Antonio Freitas Duarte
Luís Lopes da Costa *Editors*

Advances in Animal Health, Medicine and Production

A Research Portrait of the Centre for Interdisciplinary Research in Animal Health (CIISA), University of Lisbon, Portugal

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Editors
Antonio Freitas Duarte
Faculty of Veterinary Medicine
University of Lisbon
Lisbon, Portugal

Luís Lopes da Costa
Faculty of Veterinary Medicine
University of Lisbon
Lisbon, Portugal

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Foreword

The Centre for Interdisciplinary Research in Animal Health–CIISA, the research centre of the Faculty of Veterinary Medicine of the University of Lisbon, has recently commemorated its 25-Year Jubilee. Having emerged as a R&D unit to stimulate and coordinate the research activities at the Faculty of Veterinary Medicine of the University of Lisbon (FMV-UL), its mission evolved, and presently, CIISA coordinates research at multiple institutions, within national networks. Developing fundamental and applied research in the areas of animal, veterinary and biomedical sciences, it integrates research performed at the major Portuguese institutes acting in the animal sciences and animal health fields.

To carry out this role, CIISA extended its networks to integrate R&D institutions spread throughout the country, including universities, polytechnic institutes, research institutes, national state laboratories, pharmaceutical, biotechnology and food producing industries, private enterprises, cooperatives, producer's associations and other non-profit organizations. This allowed the rational use of research infrastructures and resulted in a high level of internationalization, arising from an impressive number of collaborations with foreign laboratories.

Over the years, CIISA has shown a steadily increasing trend in scientific indicators and most importantly in the quality and impact of its outputs at the scientific, economic and societal levels. This is the result of the combined efforts of a highly motivated, committed and dynamic team, including CIISA's coordination, laboratory leaders and principal investigators, postdocs, PhD and master students, technicians and administrative staff. This book showcases the latest developments in the state-of-the-art research carried out a CIISA.

Antonio Freitas Duarte
Luís Lopes da Costa

Contents

Animal Science

Ruminants' Welfare Assessment

G. Stilwell[1(✉)], A. Vieira[2], E. Can[3], C. Krug[4], S. Saraiva[5], M. Battini[6], and S. Mattiello[6]

[1] Animal Behaviour and Welfare Research Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
stilwell@fmv.ulisboa.pt
[2] Centre for Management Studies of Instituto Superior Técnico (CEG-IST), University of Lisbon, Lisbon, Portugal
[3] Division of Agricultural and Environmental Sciences, University of Nottingham, Nottingham, UK
[4] Faculty of Epidemiology and Population Health, London School of Hygiene and Tropical Medicine, London, UK
[5] School of Agrarian and Veterinary Sciences, DCV, CECAV, Centre of Animal and Veterinary Science, University of Trás-os-Montes e Alto Douro, Vila Real, Portugal
[6] Dipartimento di Medicina Veterinaria, Università degli Studi di Milano, Milan, Italy

Abstract. Most consumers expect welfare to be part of the core of animal production and will avoid products which they view as not fulfilling minimum conditions. But even if consumers did not object to poor animal welfare, there is more than enough evidence that promoting welfare corresponds to better performance and higher quality products. Thus, there are plenty of reasons vindicating welfare assessment of farm animals. Welfare is a multidimensional concept enclosing both physical and mental components, so assessment has to address its full complexity to be credible and fair. To achieve this, assessment protocols should include valid and reliable indicators pertaining the real welfare of all the animals in a group. The protocols should also include easily measured indicators to guarantee its feasibility in farm conditions. CIISA's Animal Behaviour and Welfare Research Lab has been involved in both the developing and the application of welfare assessment protocols in ruminants. As part of the AWIN project, CIISA built, together with the University of Milan research team, an assessment protocol for dairy goats. It was also the first to apply the Welfare Quality® protocol to Portuguese intensive dairy farms and to introduce changes to these protocols so that they could be used in ruminants at pasture.

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1 Animal Welfare – Definition(s)

Social concern with animal welfare and the moral status of animals has been present in different civilizations and cultures, evolving over time alongside science, namely

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through well-established disciplines, such as ethics, farming, medicine and policy (Stilwell 2014). However, for centuries the main purpose of animal breeding was directed at increasing product quantity, without caring much about quality and, above all, ignoring animal welfare. The interest towards animal welfare arose after the last Great War, and coincided with a higher level of economic well-being, significant advances in science and technology, the decrease in rural population and the consequent intensification of animal production. This interest was initially stimulated by the release of the book "Animal machine", written by Ruth Harrison and published in the United Kingdom in 1964, that received great attention from consumers and from the public, who became aware of the conditions in which animals were bred in intensive systems. These opinion movements prompted the British government to set up a commission, presided over by Professor Brambell, with the aim of investigating the welfare of intensively bred animals. The Brambell Committee (1965) proposed the famous Five Freedoms (later adopted and developed by FAWC 2009) which should be ensured so as to allow animals to live "a life worth living": 1) freedom from thirst, hunger and malnutrition; 2) freedom from physical and thermal discomfort; 3) freedom from illness and pain; 4) freedom to exhibit "normal" behaviour; 5) freedom from fear and distress. This was one of the first steps towards the design of a scientifically based framework to evaluate farm animal welfare.

Along the process of establishing "animal welfare" as a new branch of science, three different views developed (Fraser 2008): one emphasizes the affective state, one the biological function, and one selects natural living as the more fundamental measure. The first view highlights the feelings, emotions, or affective states of the animal. The second focuses on health, growth, and productivity of animals. Finally, the third view is centred on the idea that animals should be able to express their normal behaviour in a natural environment, and is referred to as the "naturalness" approach. Although these three views comprise quite different areas when assessing welfare, they constitute complementary starting points for identifying and solving animal welfare problems, often leading to similar conclusions (Fraser *et al.* 1997; Fraser 2008). However, notwithstanding all the (admirable) scientific work done within the last five decades, no universally established definition of animal welfare has emerged (Green and Mellor 2011; Keeling *et al.* 2011). As highlighted by Broom (2007), an agreement should be reached on the definition of the concept of animal welfare for standardization in scientific papers, in legal documents, and in public statements or discussions. Lack of precision in the definition opens space for distortion, misrepresentation and exaggeration, which are of no value to animals, farmers or even consumers.

In short, we can say that animal welfare is a multidimensional concept, defined as a state of complete mental and physical health through which the animal lives in harmony with its environment. It is important to point out that multidimensional means we cannot forget, devalue or replace components, at least without a very valid reason. Accordingly, welfare reflects and is a reflection of how an animal functions, how it feels and how it performs. It seems logical to integrate all that composes an animal and that differentiates it from non-living things, to reach the definition of welfare. Thus, the idea of an holistic welfare is elegantly conveyed in one of its most accepted definitions: welfare is the state of the individual as regards its attempts to cope with its environment (Broom 1986).

However, assessing how rich or how poor is the welfare of an animal, and especially of those living in large groups, is not an easy task because: there are specific needs; large populations conceal individual difficulties; farm animals very frequently adapt or disguise signs; subclinical problems take time to arise; animals may not be familiarized to artificial settings, etc. Moreover, certain dimensions will understandably be prioritized by some according to their view of what reflects good quality of life. For example, producers will say that performance is the key sign, veterinarians will perhaps choose absence of disease and pain, while ethologists will select the expression of natural behaviour.

So the important questions remain. Are domestic animals in farms always subjected to conditions which invariably will lead to poor welfare? Is ensuring natural surroundings and the exhibition of normal behaviours sufficient? Are certain needs more important than others? How can we measure the welfare of each individual (or at least of the vast majority) living in a farm? How can we avoid anthropomorphic vision to distort our observations? How can we make welfare evaluation reliable and credible to stakeholders and society in general?

Because there are no clear answers, it is crucial to be able to assess farm animal welfare in a scientific, multidimensional, objective and unbiased way. This should be the aim of a welfare assessment protocol and has been the drive of some of the investigation carried out by the Animal Behaviour and Welfare Research Lab at CIISA.

2 Why and How Should We Assess Animal Welfare?

There are four areas benefiting from methodical and meticulous animal welfare assessment: research, monitor legislation compliance (non-voluntary), voluntary certification schemes and as advisory/management tool aimed at improving health, welfare and performance (Johnsen *et al.* 2001; Main *et al.* 2001; Sørensen *et al.* 2001; Main 2009).

Welfare certification and welfare legislation are now unstoppable because sustainability, animal welfare and environmental concerns have increased consumers' interest in knowing how, where and by whom food is produced and handled from "farm to fork". Even if farmers do not always agree, food animal production will probably be unmaintainable in the future if these demands are not addressed. The market will make sure of that. So, in response to society request, mandatory or voluntary assurance schemes have multiplied all over the world in order to guarantee the marketing of high quality animal products, in terms of health, safety and respect for animal welfare (Barnett *et al.* 2009). Assessment protocols have also shown to be superlative management tools for veterinarians and producers, being used to identify subclinical problems or causes for subperformance.

Hence, welfare assessment protocols should be considered one of the pillars of modern, profitable, efficient and sustainable farming.

3 On-Farm Animal Welfare Assessment

At the end of the XX Century many researchers all around Europe started to concentrate their efforts on setting up methods for on-farm welfare assessment. Several animal welfare monitoring systems were developed, such as an ethical account of farming in

Denmark (Sørensen *et al.* 2001). However, the first real assessment schemes were the TGI 35L and TGI 200 (Animal Need Index) for the certification of organic farms in Austria (Bartussek *et al.* 2001) and Germany (Sundrum *et al.* 1994), respectively. These schemes were based exclusively on the assessment of housing structures and management (e.g., space, ventilation, stocking density, feeding regime, milking procedures). We then assisted to the proposition in different European countries of several other assessment schemes, using a wide range of approaches, essentially based on resources and management, but sometimes combined with animal-based measures, such as disease prevalence and mortality.

The high number of different attempts carried out in several countries to measure animal welfare lead to the need of harmonising the research in this field at European level. This gave origin to the European Action 846 of the COST Framework "Measuring and monitoring farm animal welfare" (Blokhuis *et al.* 2003). From this action derived the EU project "Welfare Quality® (WQ®): science and society improving animal welfare in the food quality chain" (2004–2010), the largest yet European project on animal welfare. The aim of the project was to set up welfare assessment schemes and specific practical strategies to improve the welfare of farmed animals, meeting the needs of society and market demands. The WQ® project updated the Five Freedoms, subdividing them into 12 criteria grouped into four distinct but complementary principles, allowing to describe in a comprehensive way all the aspects necessary to guarantee high animal welfare standards. These criteria and principles were expanded, leading to the development of protocols for the evaluation of dairy and meat cattle, pigs, broilers and hens' welfare. All these protocols were mainly focused on animal-based indicators.

Similarly, the "Animal Welfare Indicators" (AWIN) EU project (from 2011 to 2015) aimed at developing, integrating and disseminating animal-based welfare indicators, including pain, in goats, sheep, horses, donkeys and turkeys, addressing the same principles and criteria proposed by the WQ®. The project generated five welfare assessment protocols, with CIISA being most closely involved in the developing of the dairy goat protocol (AWIN 2015; Battini *et al.* 2015a; Battini *et al.* 2016; Can *et al.* 2016), in collaboration with the University of Milan (Italy), and also in the validation of pain indicators, such as in lameness in goats (see paper on pain assessment in this book).

A strongly characterizing aspect of both WQ® and AWIN projects lies on the close involvement of stakeholders: representatives of agricultural associations, animal rights activists and consumers, breeders, processors, technicians and operators of large-scale retail trade were systematically invited to meetings of experts, namely for the selection and development of the indicators to be used. Their contribution was essential for the development of the final protocols and contributed to the wide acceptance of these protocols.

4 Which Indicators Should We Use?

Because approaches centred only on particular aspects of welfare (e.g. behaviour, emotional state, health or performance) may fail to give a full overview of a farm (Webster *et al.* 2004), the diverse measures should be combined and integrated into a comprehensive assessment protocol (Blokhuis *et al.* 2003; Botreau *et al.* 2007a; De Vries

et al. 2014). Various studies and welfare assessment schemes have adopted different approaches throughout the last decades.

It is not surprising that the first assessment tools included only resource-based measures, because they are often easier and quicker to record, and their intra- and inter-observer reliability is usually very high. For example, if we assume that a certain environmental temperature is adequate for the welfare of a certain species, it would be easy to measure it using a thermometer, and the result would probably be the same for different assessors. However, individuals of the same species can adapt differently to the situation in which they live, according to their individual coping abilities; consequently, their welfare cannot be assessed only through environmental and management factors, but will depend on how each subject will be able to adapt to that situation.

In line with these considerations, the measures selected for WQ® and AWIN protocols were focused almost exclusively on animal-based indicators (e.g. behaviour measures, productivity, health issues). This is in agreement with the "Statement on the use of animal-based measures to assess the welfare of animals" published by the European Food Security Authority (EFSA). In this publication the use of animal-based indicators is promoted to allow for the identification of real situations of poor welfare, whereas resource-based indicators such as management, structures or practices, are considered as "risk factors" that may reduce welfare if animals cannot adapt to them (EFSA 2012).

Each indicator can provide information on a specific criteria, or sometimes even on more than one criterion. For example, hair coat condition in goats can be indicative of both appropriate nutrition and absence of disease (Battini *et al.* 2015), and can be useful for a quick general screening. However, if an overall and detailed assessment has to be carried out, then a range of animal-based indicators that cover all principles and criteria should be selected. Obviously, if we need to assess specific issues or answer specific questions, we should select the most appropriate indicator(s) for our context (Botreau *et al.* 2007a; Main 2009). Several indicators have been established for almost all farmed species, but they have only been validated under specific situations. This means that they may not be valid, reliable and feasible if applied in different contexts. Unfortunately, the majority of the existing indicators have been tested only for the most common breeds, often in intensive rearing conditions. Many of them may also be relevant to other breeds and farming (especially extensive and free-ranging) conditions; however, they should be used with care, and the development and testing of specific indicators for each breed, animal category, farming system, season, etc., should be encouraged, in order to ensure valid results.

5 Building a Welfare Assessment Protocol Step by Step

5.1 Selecting Indicators – Validity, Reliability, Feasibility

Animal welfare is a science constantly growing and everyday new papers containing potentially promising indicators are published. However, are these indicators valid in all circumstances? Or reliable? Or feasible? Building a welfare assessment protocol is a complex work requiring that all the indicators follow these specific attributes.

Validity tells us the extent to which an indicator measures what it is supposed to measure. This attribute is particularly relevant because it defines the usefulness of an

indicator. However, valid indicators are not useful if not reliable. Reliability is the extent to which a measurement is repeatable and consistent and it can be further divided into (Martin and Bateson 2007):

– Intra observer reliability, the agreement between successive observations of the same individual or group by a single observer, based on statistical significance of correlations ($P < 0.05$) or to Kendall's coefficient of concordance (>0.7). According to time between measurements, reliability may be classified as short-(1–7 days), medium-(1 week to 1 month), or long-term reliability (>1 month); Test-retest reliability, the agreement between observations performed on the same individual on at least two different occasions (Scott *et al.* 2007). A case of test–retest reliability is the consistency over time, where results represent a long-term farm condition.
– Inter observer reliability, the agreement between different observers during a simultaneous observation, based on statistical significance of correlations ($P < 0.05$) or to Kendall's coefficient of concordance (>0.7).

It is paramount that indicators are valid and reliable, but there is yet another essential attribute that allows the protocol to be successfully applied on farm. This attribute is feasibility. Feasibility is the practical chance of using the indicator during on-farm assessment and its' grade should be previously defined because it depends on the objective, the farm conditions and on other potential limitations (Knierim and Winckler 2009). For example, attention should be paid to time constraints as it has been pointed out that any on-farm welfare protocol should take less than 2 h/farm and less than 5 min/animal (EFSA 2012). Furthermore, it was previously defined that indicators should not require further processing after collection (e.g., laboratory analysis). Collection of indicators should also be straightforward and inexpensive, as cost may be one other important constraint. Last, but not least, the whole protocol should be accepted by stakeholders and farmers, therefore it should not: require more than one evaluator; alter the farm routine (e.g., affecting feeding or milking time); over-run biosecurity rules; require a specific location to be recorded (e.g., moving animals to a test arena); cause stress to the animals (e.g., isolation, fear); or, require individual identification of the animals. Also, it should be easily applied to all animals or to a representative sample of the herd (Battini *et al.* 2015c).

5.2 Testing Prototypes and Training Assessors

As a starting point for the development of a welfare assessment protocol for farm animals, experts should review the relevant scientific literature to select promising animal-based indicators. A screening period involving experts and researchers should follow in order to address potential lack of data regarding validity, reliability, and feasibility of some indicators (Battini *et al.* 2015a; Fig. 1). When validity is questionable, specific validation studies should be designed and performed.

For each of the indicators finally elected, specific interactive learning material should be prepared and used to provide a common training to all future assessors. From our experience, the learning material should consist of a PowerPoint file starting with a brief description of the indicator, followed by a detailed explanation of the assessing and

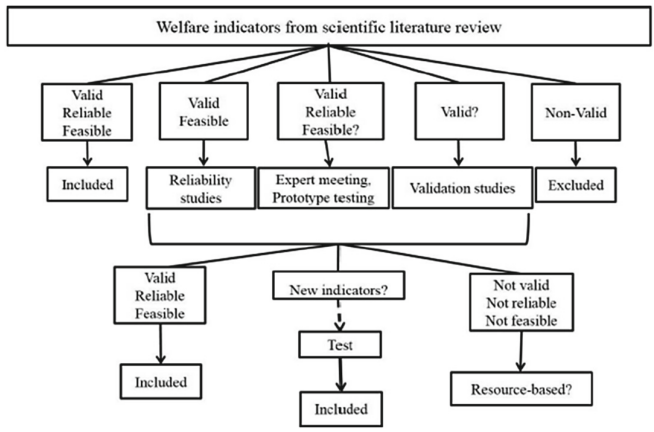

Fig. 1. Characteristics and process to identify promising animal-based indicators (Battini et al. 2015).

scoring procedures. Pictures and video-clips should be given as examples in order to simulate on-farm conditions and therefore improve the understanding and assessment of the indicator. At the end of the learning process, the level of knowledge should be tested to determine if the assessors are ready to perform the farm visits. In the case of the AWIN protocol, this preliminary phase was followed by a one-week training period at the FMV-UL, which included both theoretical and practical sessions on farm, to ensure similar level of training among assessors.

In order to facilitate and speed gathering and analysis of data collected during on-farm inspections, a digitalized data collection system can be used, adapting an Open Data Kit (ODK), a free and open-source set of tools that manages mobile data collection solutions, developed by the University of Washington's Department of Computer Science and Engineering. This system also automatically records the time needed to assess each indicator, providing important information about the feasibility of the prototype (Battini *et al.* 2015d). The use of ODK system was a milestone for the development of the AWINGoat app, freely available on Google Play Store. This app was specifically designed to guide the user step-by-step when collecting data during the farm visit and to provide an immediate visual output with the results of the assessment. AWINGoat can be used by veterinarians and technicians in their everyday practice. The real-time output can facilitate dialogue with farmers, suggesting actions to improve the welfare of animals. Farmers can use the app to compare the welfare status of their animals to that of those on farms with similar husbandry or management systems, and furthermore can easily identify causes for sub-performance.

5.3 Common Problems and Constraints

Herd size is one of the main constraints when assessing production animals that are group-housed. The high number of animals may demand a lot of time for the evaluation, or data collection may become too complicated due to problems in separating

and restraining a large number of animals. This is particularly true when performing individual-level assessment. Whenever possible, locking the animals (e.g. at the feeding rack or in a race) is the preferable way to restrain animals for examination, but if farmers agree, an alternative location can be the milking parlour to where dairy animals can be moved to outside milking hours. In the case of the AWIN prototype protocol testing, it was clear that manually restraining goats in the pen was unfeasible because it caused too much stress to the animals, put the assessors safety at risk and frequently required the help of the farmer. This is also an issue when working with cattle and especially beef cattle not used to humans' proximity. That is the reason restraining and handling are not included in the large ruminants' protocols.

The herd size and species behaviour can also influence the collection of some group-level indicators: an example is the decision to use the latency to the first contact test to evaluate the human-dairy goat relationship rather than choosing the avoidance distance test in the pen, that is commonly used in other species (e.g., dairy cows). In the avoidance distance test the assessor enters the pen and, slowly walking, approaches each animal in order to test its reaction to human. Even if accidents have never been reported, goats may start running around making it difficult to conduct the test on individual animals (especially in large groups), and to individually mark animals (ear tags in goats often are not big enough to be read at a distance as in cows).

Irrespective of the indicators selected, assessing large herds may pose some flowing related issues, namely the time needed to finish the assessment. For this reason, an effective sampling strategy is usually required. Both the WQ and the AWIN protocols were developed taking into account this aspect and both include a table that will advise on the size of the sample taking into account the dimension of the population (Welfare Quality 2009; AWIN 2015).

The protocol success implies that observers are well trained and know the normal and abnormal behaviour of the particular species. Most of the indicators are quantitative, but some problems can be found when qualitative indicators are used. Qualitative Behaviour Assessment (QBA) is a scientific method conceived to evaluate the emotional state of farm animals using the judgement of observers of animals' behaviour and body language. QBA requires experienced assessors, as they must be able to properly interpret and recognize, e.g. aggressive vs. play behaviour or relaxed vs. bored animals. An exhaustive training can overcome the limits due to lack of experience with a particular species, however this is usually very time consuming, affecting the practicability of a protocol.

6 CIISA's Work with Farm Animal Welfare Assessment Protocols

As mentioned before, CIISA has been deeply involved in building and applying cattle and goat welfare assessment protocols. The CIISA's Behaviour and Welfare research teams not only applied validated protocols to Portuguese dairy cow farms, in this way helping to understand the welfare status of production animals in our country, but also developed and tested adaptations of the protocol to other production systems. As will be shown in the few examples below, CIISA has acquired strong expertise in farm animal welfare assessment.

6.1 Cattle – Applying the WQ® Protocol in Portuguese Intensive Dairy Systems

Not much was known about the level of welfare in Portuguese dairies, with only a few studies having been conducted on the subject. Two studies were about farmers perception of the level of welfare of their cows (Jacinto 2011; Silva *et al.* 2013) and in another study several ways of evaluating dairy cows' welfare were investigated (Cerqueira 2013). As a result, the aim of this CIISA study was to perform the first methodical welfare assessment of dairies from the centre and north of Portugal using the WQ® protocol (Krug 2013; Krug *et al.* 2015). This was a cross-sectional study conducted in 24 dairy herds selected by convenience during the period between January and March 2013. Of these, thirteen farms were operating in the centre of Portugal, having been selected based on contacts from another study (Barros 2013); and 11 farms were from the north of the country, having been selected through a veterinary practitioner working in that region. Two herds had 400–680 milking cows, seven had 200–399, nine had 100–199 and six had 20–99 milking cows. All farms used free-stalls except for one, which was based on an open bedded system. Holstein–Friesian was the predominant cow breed. Each farm was visited once by one of the authors (CK).

Five farms did not meet the minimal welfare requirements and were therefore 'Not Classified', according to the WQ® guidelines. The majority of farms was scored as 'Acceptable' (18/24 farms), one was scored as 'Enhanced' and there were no farms scored as 'Excellent'. To help understanding the reasons for these final scores, collected data is summarized in Table 1. In general, the four welfare principles (i.e., Good Feeding, Good Housing, Good Health, and Appropriate Behaviour) used for the final welfare classification yielded low average scores relative to the maximum score of 100.

The principle 'Good Feeding' was scored low because 75% of farms had either an insufficient number of functioning drinkers (9/24 farms), or only partly sufficient or dirty drinkers (9/24 farms); on the other hand, cows were generally in good body condition.

The principle 'Good Housing' had a relatively better average score, as no animals were tethered, therefore leading to the maximum score on one out of two criteria used for this principle's classification. Nevertheless, on average, dairy farms had a low score on the criterion 'Comfort around resting', with 29% (7/24) of farms presenting more than one third of their cows colliding against the cubicles. Number of cows lying outside the stall (partially or completely) was a severe problem (>5%) in half of farms (11/24) and a problem (>3%) in three farms. Results for cleanliness were also undesirable, with most of farms overpassing the threshold for serious problem in all three body regions: lower legs (>50%), udders (>19%) and hindquarter (>19%).

Regarding the principle 'Good Health', most farms (19/24) yielded the lowest classification on the criterion 'Absence of injuries' due to high percentage of animals with at least one lesion or showing moderate lameness. Specifically, an average of 80% and 40% of cows' lower back legs presented hairless patches and lesions, respectively. Nasal discharges and coughing prevalence were generally higher than the threshold defined by the WQ® protocol. For instance, 88% of farms (21/24) had too much coughing, or ≥0.06 mean coughing per cow per 15 min. Average on-farm mortality was 10%, which is much higher than the threshold of 2.25% defined by the WQ® protocol. The 'Absence of pain induced by management procedures' criterion was also low, as only one out of all 23 farms did not perform disbudding, being scored with the maximum score in

Table 1. Results from WQ® protocol application to 24 Portuguese intensive dairy farms (Krug 2013; Krug et al. 2015).

	Mean	SE
Overall good feeding score	37.5	29.6
Criterion-score 'Absence of prolonged hunger'	86.7	14.3
Lean cows (%)	1.9	2.3
Criterion-score 'Absence of prolonged thirst'	32.1	35.2
Water points	–	–
Overall good housing score	54.9	18.6
Criterion-score 'Comfort around resting'	28.4	29.5
Cows with dirty hindquarter (%)	45.1	29.1
Cows with dirty lower legs (%)	29.1	23.5
Cows with dirty udder (%)	54.4	27.5
Cows that collide against cubicle while lying (%)	22.7	22.5
Time to lie down (secs)	5.2	2.0
Cows lying outside (%)	9.8	11.5
Criterion-score 'Ease of movement' (Tethering)	100.0	0
Overall good health score	18.7	6.8
Criterion-score 'Absence of injuries'	15.6	6.8
Moderately lame cows (%)	39.0	14.7
Severely lame cows (%)	11.5	8.8
Cows with no lesions (%)	2.5	4.0
Cows with hairless patch but no lesion (%)	26.8	12.9
Cows with at least one lesion (%)	70.7	14.2
Criterion-score 'Absence of diseases'	32.0	17.2
Mortality (%)	10.1	37.7
Subclinical mastitis (%)	15.0	10.9
Vulvar discharge (%)	1.6	2.0
Dystocia (%)	3.0	1.9
Downer cows (%)	4.3	3.3
Diarrhoea (%)	2.4	2.5
Frequency of coughing (/cow/15 min)	0.1	0.1
Cows with increased respiratory rate (%)	0.1	0.6
Cows with nasal discharge (%)	13.3	9.5
Cows with ocular discharge (%)	0.4	0.7
Criterion-score 'Absence of pain induced by management procedures'	26.6	19.5

(*continued*)

Table 1. (*continued*)

	Mean	SE
Partial score for disbudding/dehorning	13.8	17.0
Partial score for tail-docking	87.8	33.1
Overall appropriate behaviour score	22.0	7.0
Criterion-score 'Expression of social behaviours'	67.6	17.8
Displacements (/cow/h)	0.5	0.4
Head butts (/cow/h)	0.4	0.3
Criterion-score 'Expression of other behaviours'	1.2	5.8
Criterion-score 'Good human-animal relationship'	45.2	13.4
Cows that can be touched (%)	35.9	15.5
Avoidance distance 0–50 cm (%)	37.2	7.4
Avoidance distance 50–100 cm (%)	9.1	4.9
Avoidance distance >100 cm (%)	17.8	10.2
Criterion-score 'Positive emotional state'	40.5	22.1

this criterion (100). On the other farms, disbudding was performed in animals with an average (min-max) age of 6 (2–12) weeks typically through the use of thermocautery (11/23 of farms with only 2/11 applying local anaesthetic) or caustic paste (9/23 of farms with only 1/9 using post-procedure analgesic). Tail docking was routinely performed on two farms. Finally, the low classification for the principle 'Appropriate behaviour' was mostly due to the criterion 'Expression of other behaviours': only one out of the 23 farms that answered the questionnaire had dairy cows on pasture during the dry period.

From this assessment, it was concluded that the welfare of Portuguese dairy farms was generally acceptable, meeting minimal requirements. In order to improve it, several changes were suggest regarding number and dimension of cubicles, along with type of bedding (to decrease the number of lesions). In addition, advice on the need to improve cleanliness of both drinkers and pens, and to increase number of drinkers per pen, was given to some farms. Finally, it was recommended to introduce pain management measures when disbudding calves.

Making sure that cows are comfortable and healthy is a crucial factor towards good farming, as poor animal welfare is very clearly associated with lower productivity (Charfeddine and Pérez-Cabal 2017), and thus reduced profitability. It is, therefore, at the farmers' best interest to have their farms in excellent levels of welfare. At the end of this study, reports about the welfare problems were sent by e-mail and by post to each farmer, so that effective improvements could be made. Additionally, the results could be used for certification purpose, as we will see in the next example (dairy cow in pasture). Consumers are increasingly concerned about animal welfare (Herrwagen *et al.* 2013; Thorslund *et al.* 2017), therefore improving it in Portuguese dairies is anticipating consumers' expectations and future regulatory changes.

With this study it was possible to confirm the main disadvantage of the WQ® protocol – the long duration, which makes the task of assessing the welfare of all farms within a country, a difficult and unrealistic mission. Fortunately, national cattle databases hold a variety of potential welfare indicators that could be used to screen farms with good or poor animal welfare (Sandgren *et al.* 2009; Nyman *et al.* 2011; de Vries *et al.* 2014; Krug *et al.* 2015; Thomsen and Houe 2018). By using these easily attained indicators (e.g. on farm mortality; or calving intervals) it is possible to identify those herds with higher risk of having poor welfare and in need of urgent intervention, and in this way decrease the number of farms to go through the field assessment. However, further research is needed to document the associations between the different potential welfare indicators and real animal welfare (Thomsen and Houe 2018).

6.2 Cattle – Welfare Assessment of Cows at Pasture

Recently, EFSA (2015) presented a scientific opinion on the assessment of animal welfare in small-scale dairy cow farming systems in Europe. A CIISA researcher was a member of the working group writing this report. The pilot project took place in four countries, with 32 farms in both Austria and Spain, 37 farms in Italy, and 23 farms in France being tested. To do so, the WQ® cattle protocol (Welfare Quality 2009) was revised and modified, resulting in the removal of some indicators (*e.g.* duration of lying down movement, collision with housing equipment when lying down, coughing), and the addition of others more adapted to the production conditions (*e.g.* age at culling reflecting longevity, claw condition, clinical mastitis, use of pasture). This study showed that the modified WQ® protocol allowed for an efficient collection of data on a great number of animal-based measures although some had to be replaced by ones that are more appropriate.

With the experience attained from developing the AWIN protocols and from applying the WQ® protocol to Portuguese intensive dairy farms, CIISA was prepared to cooperate with parties interested in promoting animal welfare. This knowledge and experience was sought by the company BEL-PORTUGAL that wished to assess the welfare of dairy cows in farms to be included in their "Milk from Happy Cows" programme. This programme was established in the Azores Island of S. Miguel (Portugal) and aimed at providing certification of good practices and high animal welfare in dairy farms. This programme was also seen as a way to help milk farmers survive in regions where competitiveness through low prices is virtually impossible.

As was above mentioned, the WQ® protocol was intended for intensive production systems, so adaptations had to be tested for conditions in the Azores, such as permanent pasture. A final innovative welfare assessment protocol was developed by CIISA by removing some of the WQ measures while adding some other validated and more suitable ones (Stilwell *et al.* 2018). In summary, cows were first assessed at pasture (indicators include flight distance, isolation, thermal comfort and ease of movements), then on the path to the milking parlour (e.g. lameness, human-animal relationship), at the waiting pen (e.g. BCS, agonistic behaviour, lesions) and finally when inside the milking parlour (udder dirtiness, skin injuries, teat lesions, hoof condition) (Fig. 2). At the end of the assessment a survey was applied to farmers seeking to collect further indicators such as Somatic Cell Count, mortality and culling prevalence, pain management at disbudding and many more.

Fig. 2. Indicators' flow in CIISA welfare assessment protocol for cows at pasture.

The protocol was first presented to farmers and then applied by assessors trained by CIISA, to the 33 dairy farms seeking to enter the programme. Thresholds for the different indicators were established based on the Azores average farms' prevalence or on previously defined rules for the "Milk from Happy Cows" programme. Hence, each indicator could be graded as GREEN (threshold fully attained), YELLOW (close to threshold), RED (distant from threshold). The overall assessment resulted from the number of indicators in each grade and certification was awarded to farms with no RED indicator or no more than two YELLOW indicators. Farms with one or two YELLOWS were given a period of time to resolve these welfare issues. Some constrains were encountered when applying the protocol, namely on how to achieve threshold values through benchmarking and how to discriminate critical indicators (e.g. mortality) from more prevalent but less significant ones (e.g. overgrown claws). It is also imperative to work on ways to distinguish farms that have one RED from those having several REDS or many YELLOWS. Although all these farms may fail to reach acceptance, some will have more serious welfare issues and will need a much closer and immediate follow up.

The first assessment in 2016 of the 33 farms of the programme resulted in 79 YELLOWS and nine REDS in total. The main problems identified in adult population were related to management of downer cows, teat hyperkeratosis, broken tails and tethering bulls with chains. This was a way of identifying the major welfare problems and to quickly address the most urgent. Most farms at a second audit (2017) had already resolved most of the cows' issues.

Meanwhile a pioneer protocol was also developed by CIISA for calves and heifers belonging to farms from the Milk from Happy Cow programme, assessing mostly animal-based measures such as BCS, dirtiness, prevalence of coughing, diarrhoea or injuries (Fig. 3). Most farms had severe shortcomings such as high incidence of dirtiness, coughing, diarrhoea, poor hair coat that resulted from tethering, small pens, lack of bedding

material, low light and poor ventilation. By applying the protocol it was possible to recognise the most prevalent and serious issues and to suggest solutions. Most farms followed the advices and invested in improving the housing, colostrum feeding and management of their young stock. The following audits did show improvements with the welfare of calves being greatly improved (Fig. 4), as not complying with recommendations could mean exclusion from the programme.

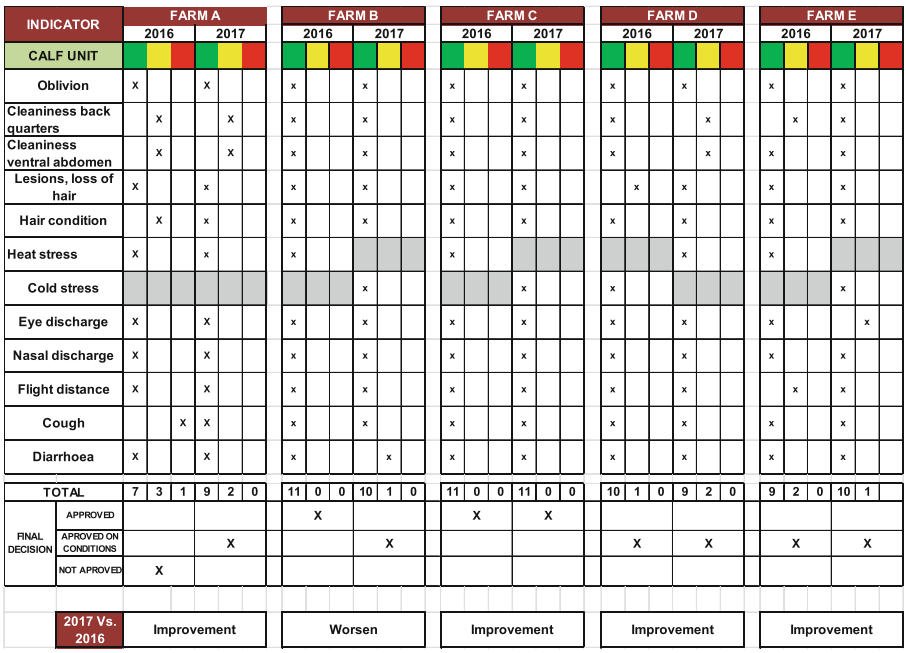

Fig. 3. Example of results from application of CIISA Protocol to dairy calves in the Azores.

Although the progress was slow at first, because most changes had to be structural, the most rewarding result from this CIISA cooperation was the perception that farmers were proud of the corrections they had made for the benefit of their animals.

6.3 *Dairy Goats – Intensive System*

The AWIN protocol for dairy goats was conceived to allow for a feasible and reliable animal-based welfare assessment, providing an accurate and consistent overall picture of the animals' welfare state, regardless of farm size, therefore preventing some welfare problems from remaining undetected.

Indicators, Assessment Flow: A Two-Level Approach

The development of the AWIN protocol for dairy goats was initiated by the selection of approximately 50 potential animal-based indicators from the literature (Battini et al.

2014). Of these, 25 were identified as promising and classified according to the '4 principles and 12 criteria' theoretical framework developed by WQ® (Botreau et al. 2007). Some indicators demanded further studies to increase validity (hair coat condition: Battini *et al*. 2015; thermal stress: Battini *et al*. 2015c; Qualitative Behaviour Assessment: Grosso *et al*. 2016) or were modified/adapted in order to increase on-farm feasibility (Body Condition Score in dairy goats: Vieira *et al*. 2015a; lameness in dairy goats: Vieira *et al*. 2015b). The acceptability and feasibility of these 25 indicators were conferred through stakeholders' consultation, via meetings or surveys (Vieira *et al*. 2015b).

The prototype protocol was then tested in 60 intensive dairy goat farms (30 in Italy and 30 in Portugal) with different sizes (mean ± SE = 192.25 ± 29.22; min 14; max 1147 lactating goats) and characteristics (e.g., presence/absence of feeding rack, stocking density, feeding space, etc.), in order to test the feasibility of the selected indicators in different farming conditions. To check indicators' inter-observer reliability and their consistency over time, 20 farms out of the 60 in assessment were selected by convenience to be visited by two assessors at the same time, and 20 farms out of the total number were visited twice (in winter and in summer) by the same observer. Most of the indicators included in the final AWIN welfare assessment protocol for dairy goats proved to be consistent over time and presented high levels of agreement and reliability between observers, either collected at group level (Spearman's correlations; $p < 0.001$) or at individual level (index of concordance ranging from 80.27% to 100%), therefore justifying their inclusion in the final protocol (Can *et al.* 2017; Vieira *et al.* 2018).

After this period of testing it was decided that a two-level approach would increase feasibility and acceptability among stakeholders, without losing scientific validity. This strategy allows for the assessment of many farms, selecting those that may have welfare issues that should be more deeply evaluated.

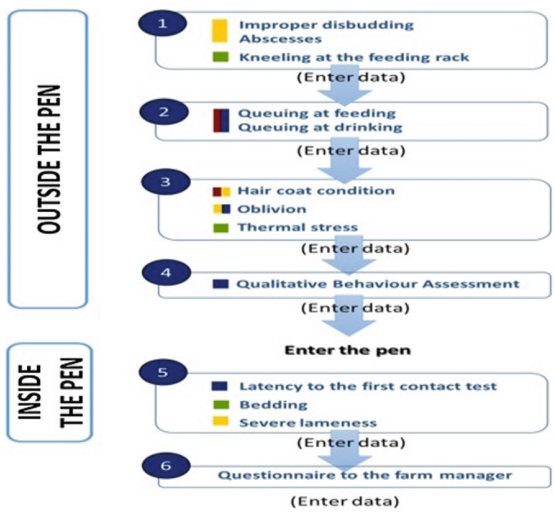

Fig. 4. First level welfare assessment collection flowchart (AWIN 2015).

At a first level, the protocol delivers a quick-screening of the farm based on a selection of robust and feasible indicators that can be promptly applied, not requiring individual animal restrain. Indicators are recorded at group level by a single assessor in only one pen. The assessment begins at feed distribution (main meal) from outside the pen and continues inside the pen, following a pre-established fixed order (Fig. 4) so biased results on behavioural indicators can be avoided. All indicators are animal-based, except for 'Bedding'[1].

A second level assessment consisting of a more comprehensive and in depth evaluation may be recommended if welfare problems are detected on farm, i.e. if there is a noncompliance with certain basic conditions (e.g. legislation, farms in the worst 5% of the reference population for one indicator). In this level, more pens are evaluated and a detailed individual evaluation (e.g. BCS, overgrown claws), supported by an effective sampling strategy, is carried out by two assessors. As in the first level, indicators should be collected following a pre-established fixed order (Fig. 5). Even though animals often need to be handled, the assessment is still feasible and can be conducted in a reasonable amount of time (AWIN 2015).

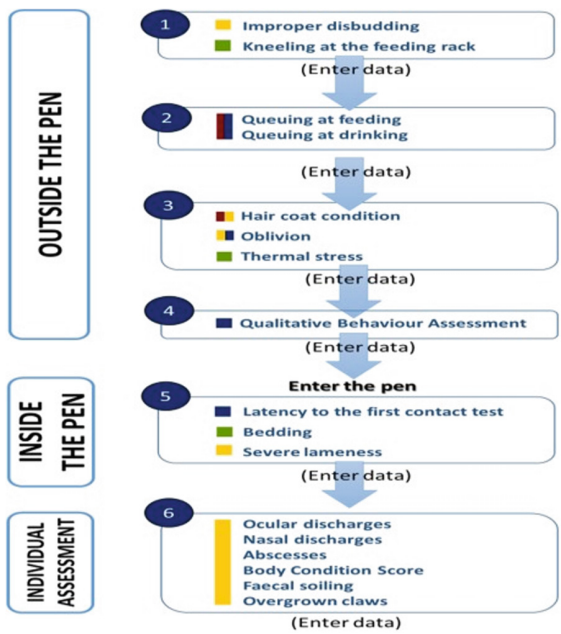

Fig. 5. Second level welfare assessment collection flowchart (AWIN 2015).

[1] Detailed information on description, assessment, and method of scoring of each indicator can be found in the AWIN welfare assessment protocol for goats (AWIN 2015), Battini *et al.* (2016) and Can *et al*. (2016).

Results from Application in Portugal and Italy

The application of the AWIN protocol both in Portugal and Italy revealed similar welfare areas of concern (see Battini *et al.* 2016 and Can *et al.* 2016 for welfare assessment results). These areas consisted broadly of claw overgrowth, poor hair coat condition and queuing animals at the feeding rack, with larger farms having higher concerns. Interestingly, the prevalence of most health indicators was affected by farm size, being higher in larger farms. This is most likely explained by larger farms being more difficult to manage, which in turn may lead to a reduced care of individual goats as a result of higher stocking densities and workload. Furthermore, most of the assessed indicators presented similar prevalence to those considered in previous studies in northern European countries (Anzuino *et al.* 2010 in the UK; Muri *et al.* 2013 in Norway), suggesting common problems.

Potential Limitations of the Protocol

A prevailing limitation of the protocol is its rather small reference population, as this is currently based only on the assessment of the aforementioned 60 farms (30 Portuguese and 30 Italian commercial dairy goat farms). Knowing the general prevalence of welfare indicators in the dairy goat industry is crucial not only to set thresholds of acceptability, but also to provide data for benchmarking purposes. Determining such thresholds will enable farmers to identify main welfare issues in their own farms, and thus set plans of action to improve the general welfare/health condition of the animals (Can *et al.* 2016). Furthermore, the application of the protocol to a larger number of farms in different countries will also help updating the reference population delivering a more reliable benchmark for comparison (Battini *et al.* 2015a).

Future and Potential

The work developed towards the AWIN welfare assessment protocol for lactating adult dairy goats has raised significant attention from multiple research teams across different areas of interest, particularly, claw overgrowth (Canotilho 2018), dehorning and disbudding (Acharya 2017), transportation (Alcalde *et al.* 2017) and udder asymmetry. The assessment protocol has also been referred in several studies as an example of a tested tool providing a comprehensive welfare assessment rationale for developing welfare protocols for other farmed species (e.g. Dai *et al.* 2016; Dalla Costa *et al.* 2017; Richmond *et al.* 2017; DuBois *et al.* 2018). All these examples provide additional scientific evidence to establish the AWIN protocol for goats as an official on-farm welfare assessment protocol for lactating adult dairy goats. However, it is important to acknowledge that the protocol in its current version does not cover all the questions and every aspect of this particular species, but rather raises new questions, discussion points and demands. Considering the increasing societal request for farm animal welfare, the next important step would be to develop and implement specific protocols for other categories (e.g. goat kids, bucks), different purposes (e.g. meat, fibre production) and production systems (e.g. extensive management systems).

6.4 Dairy Goats – Extensive System

An adapted version of the AWIN protocol was applied to 41 farms in the centre of Portugal (Canavarro 2016). Farms included had flocks of goat and sheep running together, being housed at night in sheds or stables underneath farmers' houses and taken to pasture almost every day by the owners, reflecting the reality of many Portuguese rural areas. The milk produced at these farms is generally used for homemade cheese or sold to local cheese producing companies. This type of production comprises perhaps 70% of the goat industry in Portugal so validating welfare indicators and testing potential protocols is essential. These farms are most times cheese producing units, and consumers would probably pay more to guarantee that health and welfare conditions are suitable. Several changes were introduced to the original AWIN protocol – e.g. queuing at the feed trough was removed – but in general it was concluded that a modified protocol was acceptable and useful. For instance, the results showed that goat flight distance from strange people (usually the assessor) was very high but, in contrast, was extremely low when the owner/shepherd performed the test. This is probably the result of small ruminants being more used to be handled by their owner rather than other people, when compared to intensive systems. In conclusion, it was shown that a modified AWIN protocol is of fast, simple and inexpensive application and meets the criteria of validity, reliability and feasibility. Its feasibility and applicability suggest it may be used as a basis for regulating the implementation of minimum welfare standards and certification, in a farming system that although often overlooked, is still very prevalent in Portugal and many other European countries.

6.5 Broilers and Laying Hens – Assessing Welfare at Farms and at Slaughterhouses

Several welfare indicators have been developed to be assessed at various stages of fowl production, including transport and slaughter. For example, the WQ® protocols for layers and broilers (Welfare Quality 2009) includes clinical scoring measures to be collected on farms but also animal based measures to be assessed at the slaughterhouse. Validating some of the indicators collected at the slaughterhouse as a way of assessing the welfare at farm level, was the aim of studies carried out by CIISA in cooperation with Universidade de Trás-os-Montes (UTAD).

One study evaluated the welfare conditions of 64 broiler farms on the basis of feather conditions and clinical score measures collected at the slaughterhouse (Saraiva *et al.* 2016). A 3-point scale was used to classify dirty feathers, footpad dermatitis and hock burns measures, while a 2-point scale (present or absent) was used to classify breast burns, breast blisters and breast ulcer measures. Flocks were also allocated into three body weight (BW) classes (A, B, C): class A (light) $\geq$1.43 and $\leq$1.68 kg; class B (medium) $\geq$1.69 and $\leq$1.93 kg; class C (heavy) $\geq$1.94 and $\leq$2.41 kg. The absence of hock burns was more common in class A, while mild hock burns was more common in class B flocks. Breast ulcer was observed in class C flocks. A positive association between mild hock burns, breast burns and severe footpad dermatitis was demonstrated, indicating a simultaneous occurrence of these painful lesions. Very dirty feathers and severe footpad dermatitis relationship suggest litter humidity to be the common underlying cause. In

conclusion, a significant overview of broiler farms' welfare can be obtained by applying simple scoring scales to slaughtered birds. Thus, a systematic welfare evaluation at the slaughterhouse may work as an early warning leading to the adoption of measures to improve on-farm birds' welfare. In the studied flocks, footpad dermatitis, feather conditions and hock burns were the main restrictions for good welfare and should be considered reliable welfare indicators of the rearing conditions.

Transportation represents a brief period in the total lifespan of layers and broilers, but there are indications that both mental and physical suffering can be high (Knezacek *et al.* 2010; Siegel and Honaker 2014). In another study conducted by our team, transport conditions were evaluated by assessing welfare indicators (e.g. dead on arrival (DoA), presence and locations of bruises and dehydrated carcasses) in broilers unloaded at the slaughterhouse. DoA rate increased with the increase in transport distance, the catching of birds after midnight and longer lairage duration. It was concluded that close attention to and control of all of these factors are essential to ensure high standards of animal welfare in the transportation of broiler chickens to slaughter.

Feather pecking, feather damage, keel bone lesions and flock mortality may be considered signs of stress and fear that remain common problems in most systems for laying hens (Gunnarsson *et al.* 1999; Odén *et al.* 2002; Ramadan and Von Borell, 2008; Lay *et al.* 2011). A meaningful overview of the mental welfare of laying hens in barn systems can be obtained by tests (Tonic Immobility Test) evaluating fear level (Hansen *et al.* 1993; Wang *et al.* 2013). The relation between BW, age and presence of feather damage with fear indicators, was also studied in laying hens. It was shown that the increase in BW, presence of skin injuries and high feather-damage scores was positively correlated with fear response.

Finally, correlations between causes of carcass rejection at the slaughterhouse (Salines *et al.* 2017) and characteristics of birds and types of housing systems, were considered. Ascites and peritonitis lesions increased with hens' age, while emaciation and septicaemia were observed more frequently in younger hens. Regarding BW, it was shown that DoA, emaciation, and septicaemia were more frequent in lighter hens, which can be related to the presence of infectious agents or poor management procedures that may lead to a low growth rate. The type of housing systems influenced the percentage of ascites and total condemnation rates, with hens from cages showing higher prevalence than those from organic systems. Monitoring condemnation causes of end-of-lay hens at slaughter can help to support farm managers and veterinarians to initiate regular check-ups, to define the age at slaughter and to improve health and welfare in their housing systems.

An overview of keel bone integrity for laying hens can be obtained by using simple scoring scales in slaughtered birds, highlighting the importance of applying simple welfare indicators at the slaughterhouse. In CIISA/UTAD studies the type of housing system had a great effect on the prevalence of keel bone deformations/fractures. However, even in the more confined conditions the prevalence of keel bone deformations/fractures was high, raising other welfare concerns. Moreover, the high prevalence of keel bone protrusion found in all housing systems indicate that the size of breast muscle mass may be diminished in modern laying hens, leaving the keel bone more exposed (Fleming *et al.* 2004; Sherwin *et al.* 2010).

In summary, these studies assessing the welfare of domestic fowl allowed for:

i) strategy proposals designed to minimize the effects of some pre-slaughter factors impacting upon the welfare of broilers in transit;
ii) establishing a relationship between different welfare indicators collected at the slaughterhouse, and also select the most adequate welfare indicators according to body weight (BW);
iii) determine the relationship between fear and welfare indicators in laying hens from different barn systems;
iv) investigate the effect of housing system (furnished cages, barns and free range) on the prevalence, severity and morphology of keel bone deformations/fractures and on the prevalence and severity of keel bone protrusion;
v) determine the prevalence of DoA birds and of carcass condemnation causes in end-of-lay hens flocks and investigate the effects of age, BW and housing system.

7 Final Comments

Welfare assessment should be seen as a fundamental component of animal production and husbandry. It not only answers to society concerns but also identifies problems and subclinical causes of sub-performances and ensures that good farming practices are being followed. Because animal welfare concept is multidimensional, comprising physical and mental features, protocols using animal welfare indicators are the best way to comprehensively evaluate the welfare of most, if not all, individuals within a group. The suitability of a welfare assessment protocol is related to how well the combination of indicators reflects the global welfare of (all) animals and how practical the protocol is in the field. Currently available protocols were applied and successfully adapted to different systems by CIISA's Animal Behaviour and Welfare Research Lab. Additionally, data obtained at central level was investigated as a way of screening large farms.

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Pain Management for Ruminants During Common Farm Husbandry Procedures

G. Stilwell[1,2(✉)], P. Windsor[3], and D. M. Broom[4]

[1] Animal Behaviour and Welfare Research Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
stilwell@fmv.ulisboa.pt
[2] Institute of Veterinary, Animal, Biomedical Sciences, Massey University, Palmerston North 4442, New Zealand
[3] Faculty of Veterinary Science, University of Sydney, Camden, NSW 2570, Australia
[4] St Catharine's College, Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, UK

Abstract. Pain in farm animals usually results from injury or disease and is sometimes caused by humans, particularly during husbandry procedures. Chronic pain, and to a lesser extent acute pain, are major causes of poor welfare in production animals. Ethical, economical and marketing reasons demand the best practices in pain management and for this scientific evidence is crucial. Alleviation of pain during and after common farm operations is challenging due to several constraints, including time, cost, safety and public health issues. For example, the most efficient drugs may not be allowed in food producing animals (e.g. opioids) and reduced practicality may exclude others (e.g. general anaesthesia).

Studies on disbudding, dehorning, castration, calving and lameness, have shown that topical or local anaesthesia, preferably accompanied by use of NSAID analgesia, will reduce pain during and after many of these procedures. CIISA and its research partners from many countries have conducted studies on these painful situations, contributing to the improvement of farm animal welfare. In order to ensure that they are fully observed, pain management protocols should be effective, practicable, affordable and safe. If they do not fulfil all of these criteria, the result is likely to be that farmers will undertake little pain management. On the other hand, animal producers need to recognize that consumer demands for improved animal welfare includes real pain control.

Keywords: Ruminant · Welfare assessment · Welfare indicators · Behaviour · Protocols

1 Causing Pain in Farm Animals and Its Impact on Animal Welfare

Welfare is a term which means exactly the same for humans and ruminants. That is: the state of the individual as regards its attempts to cope with its environment (Broom 1986). Similarly, the terms health, stress and pain are the same concepts whether we are

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considering a person, a calf or a trout. This point has been emphasised by many animal welfare scientists (e.g. Broom 2001c, Fraser 2008) and is one of the messages of "one welfare" (García-Pinillos 2018).

Important aspects of the welfare of an individual are its health and its feelings. Health is the state of the individual as regards its attempts to cope with pathology. Positive and negative feelings are adaptive mechanisms that help individuals to learn about their environment and pain is one of the negative feelings. Pain is an aversive sensation and feeling associated with actual or potential tissue damage, while sentience is the capacity to have feelings, including pain (Broom 2001b, 2014, 2015). The occurrence of pain in animals is widely recognised and discussed (Sneddon et al. 2014; Windsor et al. 2016).

Whilst welfare ranges from very good to very poor, most people focus on the various forms of suffering when considering our obligations towards the animals that we keep. The problem often expressed in relation to pain in species other than man is that the animals cannot tell you when they are in pain or how bad it is. The major method used in human pain studies is self-reporting, for example, on a scale from no pain to very severe pain. This method can be unreliable because people can lie or deceive themselves and there are cultural and ethnic variations in relation to expression of pain. Perhaps measures of observed behaviour or physiological changes in people, like those used commonly in non-human studies, will in future be considered more accurate than human reporting (Broom 2001c).

Some people think that pain is a feeling limited to humans or to mammals, but many studies of anatomy, physiology and behaviour show clearly that pain systems are very similar in all vertebrates, cephalopod molluscs and decapod crustaceans (Broom 2014). There are variations in the area of the brain that does the pain analysis but little variation in the function. A further misconception is that animals such as cattle do not feel pain because they have thick skin. The simple observation that cattle react to individual mosquito bites, demonstrates that the skin does not prevent responses to painful stimuli. Thicker skin can reduce the likelihood or extent of abrasions following some contacts but the nociceptive cells within and under the epidermis function fully.

Sophisticated behavioural measures are being used more frequently in studies of pain. However, there are problems in pain recognition which make comparisons between species difficult. Severe pain can exist without readily detectable signs. For example, a possible response of calves after caustic paste disbudding was a state of inactivity and almost lethargy (Stilwell et al. 2009). Although species vary greatly in the kinds of behavioural responses displayed in response to pain, individuals within a species also vary in the thresholds for the elicitation of pain responses (Morton and Griffiths 1985; Rutherford 2002). Hence, it is important to consider which behavioural pain responses are likely to be adaptive for any species that is being considered. Humans, like other large primates, dogs and pigs, live socially and can help one another when attacked by a predator. Parents may assist offspring and other group members may help individuals when attacked or otherwise in pain. Hence, distress signals, including loud vocalizations are adaptive when pain resulting from an injury is experienced. Those species which can very seldom collaborate in defence, like the smaller ruminants, do not have obvious responses to pain as these are maladaptive. However, subtle changes in facial expression can be useful indicators of pain (McLennan et al. 2016). The sheep

pain facial expression scale recently developed, involves scoring five facial areas: orbital tightness; cheek tightness; ear position; lip and jaw profile; and nostril and philtrum position. When farm ruminants are in pain, increased cortisol production as an indicator of a stress response, and increased occurrence of pain-related behaviours (Stafford and Mellor 2005; Stilwell et al. 2008b, 2009, 2010; Lomax et al. 2010), can be quantified. Measures of brain activity can also be used (e.g. Gibson et al. 2007) and, as explained below, this pain can be prevented using anaesthetics and analgesics (Broom and Fraser 2015).

The idea that animals used by people should not be treated like inanimate possessions but should be protected from actions that might cause suffering, is very old and widespread in human society (Grandin 2014). The term 'sentient' is now used in legislation about animals. The European Union Treaty of Lisbon, (European Union 2007), says in the course of a statement about animal protection and welfare (Article 6b), "since animals are sentient beings…". This wording had the intention to protect the animals commonly used by man, for example on farm, in the laboratory, or as companions. It came about because public concern about animal welfare has increased in many countries during the last thirty years and especially in the last ten years.

The attitudes of consumers to sustainability in general and animal welfare in particular is changing methods of housing and managing farm animals (Bennett et al. 2002; Broom 2010, 2012, 2017). One powerful effect of consumers is when they refuse to buy a particular product or when they say to a supermarket that they will not visit the supermarket if a particular housing system or painful farm procedure is used in the production of anything sold in the supermarket. Such actions result in food and fibre processing and retail companies establishing animal welfare standards for production systems. These standards have commenced inclusion of requirements for the avoidance of pain in farm operations. Hence, farmers have to comply if they wish to sell to the product retail companies. For farm ruminants, the standard may prohibit unavoidably painful operations such as hot-iron branding or require anaesthetic and analgesic during castration or disbudding.

A multimodal approach with topical or local anaesthesia plus nonsteroidal anti-inflammatory drugs (NSAIDs), is a new paradigm addressing the welfare concerns of aversive procedures used routinely in livestock husbandry, particularly if international consumers of animal products are made aware of these improved welfare attitudes and practices in livestock agriculture. This 'pain management revolution' has empowered farmers to reduce suffering in animals, enhance animal welfare practices, and importantly, addressed concerns raised in activist-led campaigns advocating that livestock production is inherently cruel. Pain relief in animals is now an important risk management intervention for most global livestock industries.

2 What Kind of Pain Are We Talking About?

As already stated, pain is an aversive feeling or sensation associated with actual or potential tissue damage resulting in physiologic, neuroendocrine, and behavioural changes that indicate a stress response (Molony and Kent 1997). Pain responses have two main objectives: (i) to elicit behavioural changes to prevent further damage (e.g. escape) or to

promote healing (e.g. through rest); (ii) allowing the animal to identify, remember and, if possible, avoid additional contact with the source of the noxious stimulus. Although it has a protective function, it is obvious that freedom from pain is an extremely important component of welfare because pain, especially continuous or chronic pain, negatively impacts both physical and mental health. This is easily demonstrated by showing that animals will trade almost anything, such as feed or rest, in order to avoid pain.

Knowledge on the mechanisms of nociception has increased greatly in recent years. Transduction, transmission, perception and modulation of pain are now much better understood, both in humans and in other animals. It is, therefore, important to recall the main features of pain physiology before discussing the ways to manage it.

Pain can be classified into three types: acute; inflammatory; and neuropathic pain (Adcock and Tucker 2018). When a noxious stimulus is induced it causes acute or first pain, also termed "physiologic pain", that serves a protective biological function by acting as a warning of on-going or potential tissue damage (Ringkamp et al. 2013). This is a very rapidly transmitted sensation that travels through thinly myelinated Aδ-fibres. It instigates defensive activity, including the "withdrawal reflex" or "fight or flight behaviour". However, even short repeated bursts of acute pain can induce long-term neuronal sensitisation (Carr and Goudas 1999). Second pain or deep pain, resulting from stimuli travelling through thin, unmyelinated, slow conducting C-fibres, is interpreted in the brain as a dull, diffuse, aching or throbbing sensation, that is sometimes called clinic, maladaptive or pathologic pain. Pathologic pain is generally associated with tissue or nerve damage and frequently involves the development of peripheral sensitization (Purves et al. 2001). It causes discomfort, mental distress and may have the role of prompting the animal to rest in order to recover from injury.

If tissue is damaged by trauma, surgery, stretching, or infection, there is usually an inflammatory reaction, involving activation of both vascular and cellular components. Blood vessels carry circulating precursors that are released into the area of injury and are activated by enzymes; mast cells release histamines and other substances; macrophages activate fibroblasts, which in turn release interleukin and Tumor Necrosis Factor (TNF); cyclooxygenase activates prostaglandin, leukotrienes, etc. Additional nociceptors termed "silent fibres" are stimulated and the C-fibre triggering threshold is reduced at the site of injury and in adjacent tissues, when exposed to the products of tissue damage and inflammation, referred to collectively as the "inflammatory soup". Continuous stimulation of peripheral nociceptors also results in a use-dependent neuronal plasticity in the spinal cord that modifies the subsequent performance of the nociceptive pathway by exaggerating or prolonging the response to noxious inputs (hyperalgesia) or enabling normally innocuous inputs to activate it (allodynia). Hyperalgesia can occur at the site of injury as primary hyperalgesia, and in the surrounding and distant uninjured tissues as secondary hyperalgesia. This inflammatory pain is a fundamental issue in farm animal welfare because it can be prevalent, intense, resilient and prolonged (e.g. chronic lameness in dairy cows) and its management is usually complex, costly and frustrating. Because of the long-lasting negative behavioural, autonomic, neuroendocrine, and immunologic effects, it is called non-protective pain and is an important predisposing factor for disease and lowered productivity. Severe pain can result in immune depression and poor quality of life, potentially leading to gradual deterioration and death.

Modulation is an important component of pain mechanisms, whereby transmission of pain impulses through the spinal cord are modified (modulated) by local and descending facilitatory and inhibitory neurons that originate in the central nervous system. These inhibitory neurotransmitters hyperpolarize spinal neurons, making them less sensitive to nociceptive stimuli. Additionally, central and peripheral terminals of nociceptive afferent fibres contain receptors through which endogenous opioids can act to modulate the ability to transmit nociceptive information. Damage to this inhibitory system is called disinhibition, and will result in hypersensitivity, hyperalgesia, and allodynia (Moore et al. 2002).

Pain inhibition can also be activated by peripheral nerve stimulation, mental distraction, environmental enrichment, or even by stress (Keogh and Cochrane 2002; Benaroya-Milshtein et al. 2004). Several studies have also shown that stressors, including novel surroundings, social stress, getting into a chute, or restraints such as having the head fixed, can led to hypoalgesia in cattle (Rushen et al. 1999; Herskin et al. 2004). This concept is important because it may influence the response of animals in studies of pain management, especially if stressful actions including animal restraining, are occurring.

3 How to Recognize Pain During and After Routine Procedures?

Most biological, animal and veterinary scientists would now say that pain mechanisms do not differ significantly amongst mammalian species, including humans. The "Analogy Principle" holds that the similarities in anatomy (pain system), physiology (pain perception), and behaviour (expression of pain) between humans and other vertebrates, suggests it is reasonable to assume that the sensation and effects of pain are analogous (Allen et al. 2005). Although it is indisputable that there are differences in the brain structure and function (for example, neocortex size) between different mammals, this seems to be irrelevant to the existence of perceptual consciousness (Griffin and Speck 2004). Although excessive anthropomorphic associations are to be avoided – 'we must avoid the anthropomorphic projection of our own conception of suffering onto other species" (Webster 1995) – empathy is surely an important factor when attributing pain to other animals. Behavioural and physiological studies have allowed us to identify painful procedures and even grade them, meaning that we can now opt for those that are more acceptable, and scientifically justify the decision. Importantly, we are now aware of the difference between "pain detection threshold" and "pain tolerance threshold". Contrary to traditional thinking, there are no animals "immune" to pain but merely animals that show less behavioural reaction and therefore appear more "resilient" to pain. Spontaneous pain behaviours may be rare in ruminants (stoic species), probably because it would be an evolutionary disadvantage for prey species. This means that they will endure pain if a superior interest is at stake. Hence it is nowadays clear for scientists, vets and farmers that not recognizing signs in ruminants does not mean that pain is not present. However, it should be said that some scientists show signs of being reluctant to advance further in the area of pain perception and emotions in animals. It has been suggested that "the reluctance of scientists to attribute complex abilities and feelings to non-humans has slowed the development of our knowledge of sophisticated brain function in non-humans" (Broom 2014).

The appraisal and measure of pain intensity during and immediately after certain procedures have been well studied because experiments are easy to devise and replicate and pain indicators easy to identify. The most common measures used in these studies are: direct measures of pain, including behaviours intended to relieve or to impede new painful stimuli (*e.g.* sensory testing of noxious stimuli, flight or fight reactions, vocalization, changes in movements, etc.); and indirect measures of pain, including assessment of physiological changes (*e.g.* catecholamines, ACTH, cortisol). Measuring just one indicator can lead to incorrect conclusions because animals react differently to stress and pain (Broom and Johnson 2000). These differences are linked to age, sex, breed, previous experiences, temperament, body condition, nutritional status, other concurrent diseases or disorders and type and duration of stressors (von Borell 1995). The genetic role in establishment of these variances is now being investigated in both humans and non-humans (Lacroix-Fralish and Mogil 2009; Young et al. 2011; Mogil 2012).

However, these direct and indirect measures of pain rarely continue for more than a few hours or days. Since ruminants conceal signs of debility, the development of robust and more sensitive pain assays is needed for evaluating long-term pain and the efficacy of analgesic treatment regimens after routine animal husbandry procedures such as castration and dehorning. Although measures such as hair cortisol, acute-phase protein, opioids, vasopressin, oxytocin and other hormones have been used (Fisher et al. 1996; Graf and Senn 1999; Earley and Crowe 2002; Ting et al. 2003a; Coetzee et al. 2008), results have been inconsistent. Other biomarkers such as glutamate, nerve growth factor, calcitonin gene-related peptide, brain derived neurotropic factor, and substance P seem to play an important role in hyperalgesia and their concentrations appear to increase during inflammation. Some are being tested in humans and may have some future in studies on animal pain (Nam et al. 2007; Greco et al. 2008; Jang et al. 2011; Shimada et al. 2016).

Finally, it is important to address the widespread misconception that young animals feel less pain (Adcock and Tucker 2018), that has led to current legislation, policies and guidelines allowing aversive husbandry procedures to be conducted at an early age without pain management. Some of CIISA studies have proved that young animals feel pain, although they may show different signs (Stilwell 2008b). This fallacy likely derives from: firstly the ease of restraint that can be applied in younger animals and secondly, considerations that an animal with a less developed central nervous system (CNS) may experience less pain than an older animal. This may true at an early developmental stage in altricial species but is likely to be incorrect in precocial species including ruminants, where the CNS is sufficiently advanced at birth to enable neonates to be ambulatory and respond to noxious stimuli in the early neonatal period. Thus, the approach should probably be the opposite: because young animals show fewer signs and because restraint is easier, there should be more care in assessing pain and analgesia provision should be encouraged.

4 How to Manage Pain in Farm Animals – Constraints and Opportunities

Acute pain management performed routinely by veterinarians generally involves the use of sedatives to provide restraint, often with compounds that have some analgesic properties (*e.g.* xylazine), plus the prior injection of a local anaesthetic into the surgical site or vicinity of a local peripheral nerve to achieve blockage of nociception. However, heavy sedation with muscle relaxation may lead to poorer pain management because farmers, or even vets, may confuse immobility with anaesthesia. For example, CIISA work with disbudding has shown that sedated calves have very high levels of cortisol when handled even if no stress behaviour is apparent (Stilwell et al. 2012).

Recently, NSAIDs have been recruited to ameliorate sensitisation. NSAIDs act by inhibiting prostaglandin production through blockade of two cyclooxygenases, COX-1 and COX-2. These drugs, in general, have threefold effects: anti-inflammatory, analgesic and antipyretic. The multimodal approach of sedative plus local anaesthetic plus NSAID aims to capture the effectiveness of individual agents in optimal dosages, offering theoretical synergies in acute pain prevention, potentially minimizing the side effects from each drug (Young and Buvanendran 2012). The beneficial effects of NSAIDs for pain alleviation after disbudding/dehorning (Stilwell et al. 2008b, 2010, 2012), castration (Stilwell et al. 2008a) and abomasum left-displacement surgery (Godinho 2011), calving (Stilwell et al. 2014) and lameness (Stilwell, unpublished data) have been demonstrated by many of CIISA studies. However, farmer adoption of multimodal approaches is difficult due to restrictions on the availability of drugs for widespread use, particularly of sedatives and especially centrally-acting drugs.

Anticipatory control of pain or 'preemptive analgesia' has also been shown to be extremely beneficial (Stilwell et al. 2008b, 2012) because it limits the subsequent pain experience by preventing central sensitization and peripheral hyperalgesia (Woolf and Chong 1993).

The main problems in achieving optimal pain management after painful procedures in farm animals are the following:

- Some procedures are not considered to be surgery and are frequently performed by non-veterinarians, particularly in extensive livestock systems where the need for veterinary supervision when using injectable sedatives, anaesthetics and analgesics (*e.g.* opioids) is impractical.
- Difficulty in restraining farm animals for consecutive drug administration means that only acute pain is usually addressed.
- Potential food safety issues will preclude the use of some of the most powerful analgesic drugs.
- Cost is always a limiting factor when dealing with production animals.

To address some of these limitations, new approaches have arisen in recent years. Some of these have been the target of investigation by CIISA's Animal Behaviour and Welfare Research Laboratory, that continuously seeks to associate pain management efficiency with practicality. Here is a summary of some recent work on pain management:

- Post-operative analgesia is usually limited to one injection after a procedure. However, there is evidence that the analgesic effects of new longer-acting NSAIDs given preoperatively, may persist for several days after hot-iron disbudding (Theurer et al. 2012; Stilwell et al. 2008c, 2010; Allen et al. 2013) or castration (Stilwell et al. 2008a).
- The use of systemic opioids to enhance the activity of inhibitory descending pathways is still very uncommon in ruminants due to efficacy, economic and food safety concerns. However, there are signs of good analgesia being achieved when used locally (Stein and Lang 2009) and a wide range of routes that minimize the side effects of systemically administered opioids merits further investigation (Adcock and Tucker 2018).
- Wound nociception by spray-on topical anaesthetic formulation has been shown to be safe, practicable and affordable. The development of a farmer-applied topical anaesthesia formulation for routine use in livestock husbandry is a new paradigm for addressing the welfare concerns of aversive procedures. This approach empowers farmers to reduce the suffering experienced by their animals and, when applied in a multimodal approach with oral or injectable NSAIDs, has the potential to substantially address the pain caused by some routine procedures for which speed, cost and practicality is fundamental. The 'spray-on' topical anaesthesia formulation (Tri-Solfen®) was introduced in Australia in 2005 and registered for widespread commercial use in 2012, to manage the pain and hasten the healing of the open wounds incurred during the 'mulesing operation' in sheep (Windsor et al. 2016). It was quickly recognized that this topical anaesthetic formulation delivered peri-operatively or post-injury, had broader applications in livestock husbandry, with efficacy demonstrated in managing pain and improving healing of wounds incurred during surgical castration and tail docking of lambs (Lomax et al. 2010), surgical castration, disbudding and dehorning of calves (Espinoza and Windsor 2013; Lomax and Windsor 2013), and surgical castration wounds of piglets (Lomax et al. 2017). It also has potential for treating other wounds and numerous other species, including shearing cuts in sheep, lameness caused by hoof injuries, foot abscess in sheep, and open wounds in goats, pigs, horses, alpacas, dogs and wildlife (Windsor, unpublished data). A relevant example is also the use during trimming of hoof lesions in lame dairy cows, as will be further explained below (Stilwell, unpublished data).
- Oral and transdermal NSAIDs are two relatively recent approaches to pain management in cattle (Coetzee et al. 2012; Small et al. 2014; Van der Saag et al. 2018a, b, c). Although some doubts may arise in the beginning because injection is easy, quick and perhaps more effective, there is a potential place for alternative means of administration because by reducing the need for the presence of a veterinarian, the more extensive production systems can more readily adopt an affordable means of delivering pain relief. However, keeping out veterinarians will mean that more and more painful procedures will be done by non-trained workers in countries where this is legal.

5 Research on the Most Important Sources of Pain in Farm Ruminants

5.1 Cattle

The pain associated with disbudding and castration of cattle has been thoroughly studied, probably because of the number of animals subjected each year to these procedures worldwide. The results of studies on pain done by CIISA or by its partners, are summarized here.

Disbudding
Calf disbudding is a common husbandry procedure on dairy farms which is justified by human and animal safety/welfare reasons.

CIISA conducted many studies on calf disbudding along the past 10 to 15 years. Because disbudding is a painful procedure that affects many millions of calves each year, it was thought crucial to establish efficient but also practical pain management protocols, despite the limited knowledge on how long the pain associated with disbudding or dehorning persists.

Disbudding involves destroying the horn-producing cells and in dairy farms this is usually done by one of two methods: (1) thermocautery also known as hot-iron disbudding; and (2) caustic paste disbudding. Horn buds can also be physically removed, using knives, scoop or other equipment, but this method is very rarely used in dairy calves. In a survey done in 2007, it was shown that almost 70% of farms in Portugal used "exclusively" or "some-times" caustic paste disbudding. It is our conviction that this number has decreased in the last years also because our research showed that pain is less likely to be controlled when this method is used (Stilwell et al. 2007).

Our studies compared pain-related behaviours (head rubbing, head shaking, ear flicking, increased numbers of transitions between lying and rising, inert lying, etc.) and plasma cortisol levels when using thermocautery, caustic paste or amputation and when applying different anaesthesia/analgesia protocols: use of nerve block with lidocaine; use of NSAID at the time of disbudding and pre-emptively; sedation with xylazine; use of IV or trans-rectal opioid (tramadol); use of topical gel with local anaesthetics.

The most significant results of our studies are as follows:

- Hot-iron disbudding causes extreme pain during the procedure but this pain is easily eliminated by cornual nerve block (Stilwell et al. 2007, 2010, 2012).
- Caustic paste disbudding, even if done in very young calves, causes pain that is not easily controlled (Stilwell et al. 2007, 2008b, 2009). In addition, potential longer-term consequences (e.g. deep inflammatory pain, scurs, lesions from chemical run-off) are possible but have not been sufficiently evaluated.
- Amputation or scoop dehorning causes severe and prolonged pain and should not be used in dairy calves (Stilwell 2008).
- Nerve block with lidocaine is an efficient way of eliminating pain during hot-iron disbudding but is less effective in the case of caustic paste or amputation (Stilwell et al. 2007, 2008a, 2012).
- Pain signs increase after cornual nerve block wears off (2 to 3 h). However, pain is still inhibited if an NSAID is given (Stilwell et al. 2008b, 2009, 2010, 2012).

- Preemptive analgesia with NSAID (flunixin-meglumine or carprofen) should be given in advance of disbudding thereby reducing sensitization to subsequent stimuli that could amplify pain (Stilwell et al. 2009, 2012).
- Xylazine alone does not effectively control pain from hot-iron disbudding (Stilwell et al. 2010). Our research shows higher cortisol levels in sedated calves even when no painful procedure is applied, probably due to distress induced by being unable to avoid human contact. Although sedation with xylazine followed by nerve blocking with lidocaine and parenteral NSAID, reduces pain related behaviours (Stilwell et al. 2010), further studies are needed to determine whether sedation improves welfare during painful procedures (Adcock and Tucker 2018).
- Trans-rectal tramadol is not effective in reducing acute pain from dehorning, but intravenous tramadol does provide some late post-operative analgesia (Braz et al. 2012) similar to a NSAID.

Meanwhile, other research teams have looked at alternatives for preoperative cornual nerve block such as the use of bupivacaine or 100% ethanol (Boandl et al. 1989; Tapper et al. 2011) and to parenteral NSAID (Allen et al. 2013; Stock et al. 2015). CIISA, in association with Massey University, is also currently looking at the effect of Trisolfen® on the healing of hot-iron burns, compared with the traditional spray containing oxytetracycline. Preliminary data analysis show no differences between groups in the incidence of granulation tissue, necrotic tissue, crust or infection.

In summary, our research that has been confirmed by other studies, shows that hot-iron disbudding after cornual nerve block and parenteral NSAID, is an efficient and practical pain management protocol that should be used by all intending to disbud young calves.

Castration

Because of the number of animals involved and the pain the procedure causes, castration should be seen as a very important welfare issue. Castration is usually done to reduce aggressiveness and sexual activity and to modify carcass characteristics by increasing marbling and reducing the incidence of dark-cutting meat. Chemical or immuno-castration is a potential alternative that stirs controversy but in future may replace physical castration if consumer concerns can be ameliorated.

Castration methods described for cattle are those that involve surgical removal of the testicles, application of a constricting elastic band (rubber ring) at the base of the scrotum and bloodless castration by external clamping (Stafford et al. 2002). Independently of the method used, castration will produce physiological, neuroendocrine and behavioural changes indicative of pain and distress when done without analgesia (Earley and Crowe 2002; Ting et al. 2003b; Ting et al. 2004; Bretschneider 2005; Stafford and Mellor 2005a; Boesch et al. 2008; Pang et al. 2008; Stilwell et al. 2008; Gonzalez et al. 2010; Marti et al. 2010; Pang et al. 2011; Webster et al. 2013; Meléndez et al. 2017; Park et al. 2018; Roberts et al. 2018).

Many studies have shown good results in reducing pain-related behaviours, hair or serum cortisol and weight loss when utilizing anaesthetics/analgesics at the time of castration (Pang et al. 2008; Stilwell et al. 2008a; Marti et al. 2010; Lomax and Windsor 2013b; Webster et al. 2013; Meléndez et al. 2017; Roberts et al. 2018). However, there

are also studies that show small or no effect of local anaesthesia plus NSAID (Park et al. 2018) proving that it can be problematic to compare studies as they utilize different age groups, various castration techniques and different methods of regional blocking and different drugs for analgesia (Bretschneider 2005).

CIISA and some of its research partners have conducted studies on pain management during and after cattle castration. Generally, pain research in cattle has focused on the first few hours following the procedure, and few studies have looked at the progression of pain during healing. In one study (Ting et al. 2003b) the effects of repeated ketoprofen injection after surgical castration were evaluated. However, under field conditions it is unlikely that two injections of an analgesic drug would be given. Therefore, because inflammation and pain associated with the castration will probably last for more than 24 h CIISA assessed the advantage of using long acting NSAID after external clamping castration (Stilwell et al. 2008). Forty Friesian 5- to 6-month-old calves were allocated to four groups: castrated only; castrated after lidocaine epidural injection; castrated after epidural and flunixin-meglumine; castrated after epidural and carprofen. Plasma cortisol concentration was measured before and 6, 24, and 48 h after castration. Time of arrival at the feed trough at 24 and 48 h was observed. Pain-related behaviours (turning the head toward the hindquarters, alternate lifting of the hind legs, abnormal postures), were registered at 24 and 48 h after clamping. Cortisol and behaviour showed that epidural plus any of the analgesic drugs controlled pain up to 24 h but only the long-acting NSAID had some effect one day after castration. It was concluded that long lasting pain is a problem that should be seriously addressed in calves when castrated by this method.

Surgical castration is the most reliable method but is associated with severe complications such as severe oedema, infection, tetanus and haemorrhage (Turner and McIlwraith 1989; Ewoldt 2008). Surgical techniques may vary yet very few published studies have compared differences in pain (Stilwell 2012; Roberts et al. 2018). CIISA conducted a study comparing pain and healing after one (longitudinal over the scrotum midline) or two incisions (longitudinal over each testicle) (Stilwell 2012). While the one incision technique offers the advantage of speediness and less tissue damage the two incision method facilitates the access to both testicles and allows for better drainage (St Jean 1995; Ewoldt 2008). The study compared inflammation (scrotum thickness and rectal temperature), pain related behaviours (reaction to palpation) and signs of distress at 1, 2, 3 and 6 h in 6 months old calves. Eighteen calves were randomly allocated to two groups, castrated through one central scrotum incision or two longitudinal incisions over each testicle, after sedation with xylazine and regional block with lidocaine. Of these eighteen animals, eleven were submitted to a sham-procedure one week before to act as a control group. All indicators were increased in both castrated groups compared with the sham castration group. Calves with one incision showed less pain-related behaviours in the first hours but showed a thicker scrotum and more pain behaviours towards the end of the study. It was concluded that, although one incision causes less initial pain, the inflammation, probably due to reduced drainage, leads to more intense and prolonged pain than when performing two incisions.

A research team from Sydney University looked at the effect of the same topically applied gel containing lidocaine, bupivacaine, adrenaline and cetrimide that CIISA has been using in some of its studies. The objective was to allow farmers to provide some

pain control in extensive conditions where blocking pain through anaesthesia provided by veterinarians is unrealistic. One study showed a trend for treated castrated calves to display lower cortisol concentrations than control calves (Van der Saag et al. 2016). Two more recent studies by the same team showed that a combination of the same topical anaesthetic and buccal analgesic (meloxicam) did have some effect in reducing pain following castration and dehorning, with improved weight gain, increased lying activity and reduced scrotum inflammation in the first few days following the procedures (Van de Saag et al. 2018a; Van der Saag et al. 2018c).

Another innovative approach for pain management in castration may be use of transdermal NSAID. Transdermal flunixin reduced plasma cortisol concentrations and mitigated other responses for 8 h when given at the time of castration (Kleinhenz et al. 2018).

Lameness

CIISA has conducted several studies on the impact and the management of pain in lameness of dairy cows. One study on the effect of lameness on milk quality showed that somatic cell counts were affected by the occurrence of hoof lesions from 3 months to almost 4 months after taking the cow to the chute for curative paring (Stilwell et al. 2014).

It is well established that lame cows have reduced nociceptive thresholds consistent with hyperalgesia (Whay et al. 1998). To treat cows with hoof lesions, trimming is crucial. However, trimming which extends to the pododerm causes or exacerbates pain, as well as local bleeding. Cows will react violently to these procedures, this being also a safety issue for trimmers (Becker et al. 2014). Likewise, pain arising from pressure of the skeleton through the third phalanx on a damaged or destroyed sole is likely to persist after trimming (Stoddard and Cramer 2017). Very rarely will any pain management be performed when trimming (Becker et al. 2014). Therefore, the aim of our study was to test the efficiency of Tri-Solfen®, a combination of local anaesthetics in a topical gel form, containing lidocaine, bupivacaine, adrenaline, and cetrimide on pain during trimming. Sixty-two lame dairy cows were scored for lameness before entering the crush for treatment. After confirming the presence of a hoof lesion, animals were randomly distributed to two groups: C – usual trimming with no pain control; T – trimming with local anaesthetics being applied immediately after live corium was exposed. During curative trimming, behaviour observation was conducted by two observers blind to treatment. Algometry measurements were performed before and after the procedure, to assess animal reaction to pressure. Lameness scoring was again performed as the cow left the chute. The results showed that the use of these topical anaesthetics significantly reduces reaction to trimming and lameness score after trimming, when compared with non-treated animals. Algometry values showed increased pressure threshold after application of topical anaesthetics. In short, this study demonstrated that the use of topical local anaesthetics will improve welfare and trimmers' safety, and that Tri-Solfen® is well-suited for farmers' use because of its low cost, practicality and easy application (Stilwell et al. in prep).

Calving

Although some degree of hypoalgesia has been demonstrated in cows during parturition (Aurich et al. 1990) calving is still considered a painful event in most mammals

(Mainau and Manteca 2011). Providing analgesia to the postpartum cow is not a common intervention following an unassisted calving, but may be used following dystocia and is commonly used following caesarean section (Huxley and Whay 2006). Laven et al. (2012) concluded that pain management after calving is probably underused and that further research on the use of NSAIDs in the post-calving cow is required.

A collaborative study between CIISA and Cambridge University looked at the effect of giving an analgesic (carprofen) soon after calving on behaviour, milk production and fertility (Stilwell et al. 2014). In a commercial dairy unit in Portugal, parturient cows were allocated alternatively to either the analgesia group (carprofen at a dose of 1.4 mg/kg i.v.) or a control group. There were no caesareans or complicated dystocia and all calves were born alive. Behaviour observations were recorded continuously up to 10 h post calving: time spent attending the calf, eating or drinking, proportion of time spent standing or lying, the amount of time the whites of their eyes were visible, ear position, teeth grinding, kicking the abdomen, and vocalizing. The time to placental expulsion was recorded as less than 6 h, between 6 and 12 h, and more than 12 h after parturition. Cows were monitored for signs of clinical illness, such as metritis, mastitis, and abomasum displacement, and their rectal temperature was recorded daily for three days. At 220 and 305 DIM, the total milk yield was recorded for each cow, as well as the number of inseminations and the pregnancy status. Data analysis showed that more animals from the analgesia group were seen eating during the observation period. If calvings were divided into unassisted and assisted, the rectal temperature at 24 h was lower in the unassisted animals that received analgesia ($P < 0.01$). No difference was observed between the groups in clinical disease incidence or delay before placental delivery. The milk yield of first-lactation animals at 220 DIM tended to be higher in the analgesia group than the control group and was significantly higher at 305 DIM. The number of animals pregnant by 220 d postpartum was higher in the control group than in the analgesia group (11 vs. 6). Five cows in the control group and two in the analgesia group were never inseminated, being listed for culling at the end of lactation. Apparent lower fertility may result from animals within the analgesia group having higher milk yield, delaying resumption of ovarian function (Walsh et al. 2007).

These results have been partially confirmed by other studies using NSAID after calving (Farney et al. 2013; Carpenter et al. 2016; Antanaitis et al. 2018). Lower cortisol levels and higher milk production (305 days) in primiparous cows treated with carprofen were demonstrated, but contrary to our results, also a reduced calving interval by 43 days (Antanaitis et al. 2018). In contrast, in a study in which meloxicam was given after calving, only activity was increased in animals treated (Mainau et al. 2014). In conclusion, behavioural, clinical, and production data suggest that analgesic use does have a positive effect on the welfare of post-parturient cows and on milk yield from primiparous animals, but that further studies are required before a recommendation can be made.

5.2 Goats

Lameness

Lameness in intensive dairy goat farms ranges from 9.1% to 24% (Hill et al. 1997; Mazurek et al. 2007; Christodoulopoulos 2009; Anzuino et al. 2010). The AWIN project

detected up to 5% of very severe lameness by using its welfare assessment protocol (see paper elsewhere in this book). It should be remembered that goat locomotion is better when they walk on soft straw surfaces, so assessments in the pens may underestimate the severity and prevalence of the problem (Vieira et al. 2015). Lame dairy goats will reduce milk yield, weight, fertility, as well as being more predisposed to pregnancy toxaemia and premature culling (Christodoulopoulos 2009).

Contrary to expectations, infectious diseases are not the main causes of lameness in dairy goats in intensive systems. Although these animals are permanently kept in straw beds that are sometimes wet and hot, footscald and footrot are rarely seen in farms in Portugal (Stilwell, personal observation). In contrast, overgrown and deformed claws are very prevalent and are probably the main cause of lameness in intensively kept dairy goats. The wall horn grows at about 5 mm a month and in these farms soft bedding and little movement will reduce natural wear. This is often aggravated by poor practices and management, such as insufficient frequency of foot trimming. A UK report found that of 1,520 examined animals, 79.8% had overgrown claws and the problem was present at different levels of severity in all the farms surveyed (Anzuino et al. 2010). The percentages of overgrown claws were reported as between 83.1% and 95.5% (Hill et al. 1997) although were found to occur at 66% in Norwegian dairy goat farms (Muri et al. 2013). The AWIN studies showed that more that 62% of goats had moderately overgrown claws, up to 12% had very long and severely deformed claws, and there was a positive correlation between lameness score and the number of deformed claws.

Studies carried out by a team from CIISA working in the AWIN project showed that lameness increased with exercise on hard surfaces and that trimming solved most lameness cases, with the exception of those in which deformation was already advanced and very severe. This is the reason why early and frequent trimming is crucial, with routine foot trimming associated with a lower prevalence of lameness (Hill et al. 1997). Another solution for claw overgrowth, proposed and applied in some farms, was environment enrichment by providing large stones or rocks or wooden structures onto which goats can climb.

Although the pathophysiology of small ruminant foot diseases is generally well known, there is still a lack of knowledge of the effect of lameness on other structures such as joints and on the degree of pain involved. Although it seems reasonable to propose that claw overgrowth induces stress on joints, tendons and ligaments, few papers have been published to demonstrate this. One of the techniques that has been developed in other animals to evaluate the impact of biomechanical alterations on adjacent anatomical structures, is comparative thermography before and after exercise. CIISA conducted two studies on the effect of goat claw overgrowth and deformation on joints, bones and tendons and the role these conditions can have on the incidence of other diseases, such as pregnancy toxaemia.

1. Thermographic images of goats' limbs before and after exercise and before and after claw trimming, showed an increase in temperature of the distal inter-phalangeal joint after 5 min exercise and a significant difference in temperature between animals with overgrown claws and those for which natural weight bearing was regained by trimming.

2. Computerized Axial Tomography (CAT scan) images of the limbs of goats with different grades of claw deformation showed alterations in structural topography and relations (Canotilho 2018). This provided some evidence that overgrown/deformation is not only a mechanical problem or a temporary cause of pain but may cause long term musculoskeletal changes that reduces activity, performance and fertility, ultimately leading to premature culling.

Disbudding

Cautery disbudding is a routine practice in intensive dairy goat farms as hornless animals are easier to handle, cause less damage and have lower space requirements. In spite of being extremely painful, no anaesthesia or analgesia is generally used because the procedure is very quick and the pain management protocols are not sufficiently efficient, practical or safe. CIISA work on goat kid disbudding aimed to investigate an analgesic protocol that complies with these criteria, so as to be accepted by farmers.

Tri-Solfen® is a gel with two local anaesthetics that have proved to be effective in reducing pain after procedures such as castration and dehorning in cattle and mulesing and castration in sheep (see elsewhere in this paper). We first studied the analgesic effect of TriSolfen® in 5 to 7 days old disbudded goat kids (Boto 2015). Two independent trials were performed with a total of 72 kids split into two groups of 36 animals. Trial one compared behaviour between control (group C), treated with Tri-Solfen® (group T) or sham disbudded (group S) animals, and the second trial consisted of wound and surrounding skin sensitivity testing with Von Frey monofilaments at 12, 24 and 48 h after disbudding. Behavioural assessment indicated lower pain levels in group T. However, surprisingly, there were no differences in behaviour between the S group and the C group. This reveals inconsistency in our results probably due to difficulties in differentiate normal from pain related behaviours. As for the Von Frey monofilaments test, the results did not show significant differences in the sensitivity of the areas evaluated at any time. The results suggest that the Tri-Solfen® may have an effect in reducing pain between the second and the fourth hours after the disbudding.

A more recent study assessed the efficacy of ketamine, either when used alone or in combination with an NSAID, in reducing pain-related behaviours in disbudded kids (Santos 2018). The behavioural response of fourteen healthy goat kids to ketamine at three different dosages (10, 12.5 and 15 mg/kg) was observed and registered for one hour to assess drug safety. The 10 mg/kg dose was considered to be the safest after a higher one resulting in a very prolonged sedation period. Nine healthy goat kids were then randomly allocated to one of three treatment groups: K given 10 mg/kg ketamine IM; K + M given the same dose of ketamine and 0.5 mg/kg meloxicam SC; S sham-disbudded after receiving 10 mg/kg ketamine. In order to evaluate the treatment efficacy, vocalization, leg movements, tail-flicking, head-shaking, head scratching and body-shaking were registered during disbudding and for 3 h after the procedure, by three observers blind to treatment. K and K + M kids showed a lower frequency of leg movements during disbudding, suggesting some effect of the treatment in reducing pain related to the procedure. Following disbudding, K kids had a higher frequency ($p < 0.05$) of head shaking and head scratching than other groups in the second half-hour and of body shaking in the third hour, showing that the anaesthesia did not effectively control

post-operative pain. From the second hour onwards no head-scratching was performed either by S or K + M kids, suggesting less inflammation when meloxicam is used. The use of ketamine either alone or in combination with meloxicam when disbudding goat kids showed to be unsafe, with low feasibility in farm settings and not cost-effective. However, the use of meloxicam seems advisable as it ensures good after-procedure analgesia. A parallel study showed no difference in wound healing when using Tri-Solfen® instead of a spray with oxytetracycline (Santos 2018).

5.3 Sheep

Lameness

Lameness has a tremendous economic impact in sheep production, calculated in the UK as costing £84 M per annum in lost production and treatments. In Europe, footrot is the major cause of lameness in sheep. It is a highly contagious disease resulting from mixed bacterial infection with *Fusobacterium necrophorum* and *Dichelobacter nodosus* playing complementary roles. Disease prevalence and clinical signs can range from zero to severe lameness depending on host, bacterial, environmental and climatic conditions, resulting in benign, intermediate or virulent forms of foot rot.

Footrot should also be seen as an important threat for the welfare of sheep because it causes mild to very intensive pain. A study was developed by CIISA partners in the AWIN project in Scotland to analyse the perceptions of farmers, veterinarians and students in relation to lameness and associated pain in sheep. Participants were asked to watch video clips and complete a short questionnaire, which asked them to rate, using a 100 mm visual analogue scale, the level of: (i) lameness; (ii) pain they felt the sheep was experiencing; and (iii) their own emotional response. The main conclusions were: participants were able to distinguish between different lameness severities in sheep; lameness was considered to be a painful condition, the pain severity increasing with the severity of the lameness; although farmers were more compassionate than vets, the vets were more likely to agree that the provision of analgesia as part of lameness treatment was beneficial for sheep than were farmers.

Research has indicated that prompt treatment of individual sheep lame with footrot with parenteral and topical antibiotics without foot trimming reduces the duration of disease (Wassink et al. 2010). Furthermore, it has been shown that trimming sheep lame with foot rot or scald is detrimental, because it delays healing and increases the risk of infection recrudescing. Another study showed that those treating the first mildly lame sheep in a group are also those reported to have the lowest prevalence of lameness in their flock (Kaler et al. 2010). In summary, treatment of footrot should include the traditional approach such as footbaths and topical and systemic antibiotics, but also analgesics (Raadsma et al. 2013). If trimming is absolutely necessary it should always be carried out with great care, removing only obviously loose horn and avoidance of bleeding.

One study conducted by University of Cambridge, a CIISA-AWIN partner, looked at ways to recognize and score pain in sheep affected by footrot (McLennan et al. 2016). Evidence of pain that is available from facial expression and subsequent scoring systems, have been the subject of considerable scientific investigation in humans, rodents, rabbits and horses. The Cambridge team developed a standardised facial expression pain scale for adult sheep, that can be used reliably and accurately to detect pain associated with

naturally occurring painful diseases, such as foot rot and mastitis. Eleven commercial farms where lameness was reported, were visited during the study. Of 111 sheep over one year of age, 73 were identified as having footrot by a veterinarian through lameness and lesion scoring. These sheep were matched with 38 control sheep identified as having no sign of footrot or other disease. All sheep were assessed for lameness using the five point gait scoring method. Photographic images of sheep faces were taken on the day of disease identification after lameness and lesions were scored. All sheep were treated on the same day with antibiotic (subcutaneous tulathromycin and topical chlortetracycline) and with the non-steroidal anti-inflammatory meloxicam (subcutaneous). All sheep were revisited during their recovery period and final facial images were recorded on day 90. Animals were reassessed for lesions and lameness to establish that they were fully recovered. The sheep pain facial expression scale involved scoring five facial areas; orbital tightness, cheek tightness, ear position, lip and jaw profile, and nostril and philtrum position. These areas are scored as abnormal expression present (2), partially present (1), or not present (0). A total pain score of 1–10 was determined by adding the individual scores for each of the five areas for each set of photographs. On the first day, the total pain score was higher in the sheep with footrot than in controls ($p = 0.0005$) but at 90 days after treatment there was no difference. Trained observers scored faces similarly.

The facial expression pain scale showed good relationships with lameness and lesion scores of foot rot in sheep with good intra- and inter- observer reliability. Facial expression pain scale may be used to train animal keepers and veterinarians to recognise pain in sheep, thus facilitating better pain management and ensure better animal welfare.

CIISA team used the same facial expression scale in Portugal on two small studies, including pain in kids on disbudding, and pain in goats with pregnancy toxaemia. Results (Stilwell, unpublished data) were similar to the sheep studies and that it was possible to recognize animals in pain by scoring photographs of the head.

Reduction of this very painful condition should be a priority and the selection of resistant genetic lines is probably the best way to achieve it. Resistance to footrot in some breeds is well known, although some research has shown no difference between Dorset, 1/2 Dorper, 3/4 or greater Dorper (DO), Gulf Coast Native, Katahdin, and St. Croix breeds when exposed to a highly virulent strain (Burke et al. 2007). Evidence for within-breed genetic resistance to footrot is also clear as shown by the percentage of offspring affected by footrot according to their different sires. For example, infection can range from 1 to 24% for lambs reared in the same environment (farm) under natural challenge. The AWIN project used data from the previous foot rot research led by SRUC (Scotland) to investigate the molecular basis to resistance to foot rot. Early results suggest that there may be genes on two key chromosomes that are influencing susceptibility to footrot in some breeds, potentially allowing selection for increased resistance in the absence of infection.

Mulesing

Mulesing is a procedure mainly done in Australia because of the susceptibility of the common mainly Merino phenotypes to blowfly strike (Windsor et al. 2016). Because efficacy of Tri-Solfen® was first studied in sheep submitted to this procedure, these studies included establishing quantitative sensory testing as a measure of pain relief, and over 100 million lambs have now received this form of pain management, it was decided

to include mention of this work (Lomax et al. 2008). Mulesing is conducted routinely on some Australian sheep farms to remove the 'breech wrinkle' conformation that collects urine and faecal staining and increases the risk of blowfly strike following deposition of the eggs of the sheep blowfly *Lucilia cuprina.* Myiasis is one of the most serious causes of morbidity and mortality in Australian sheep and mulesing is a convenient procedure that successfully provides decreased life-time risk of sheep blowfly strike (Lomax et al. 2013a; Windsor et al. 2016). However, as mulesing is clearly painful, the availability of a topical anaesthetic formulation for management of mulesing pain was rapidly adopted by Australian sheep farmers, enabling the procedure accompanied by pain relief to continue during the extended period required until genetic alterations of Australian Merino sheep phenotypes can progress sufficiently to reduce the risk of myiasis sheep. Importantly, trials confirmed that the topical anaesthetic formulation hastened healing and provided prolonged wound analgesia, of at least 24 h (Lomax et al. 2008, 2010, 2013).

6 Conclusions

It appears obvious that the best way to prevent pain is simply to avoid causing it. Although it is true that some procedures are undoubtedly necessary as they benefit the animal in the production system in which they are farmed, there are others which justification should be carefully scrutinized. For these, expected pain and distress have to be weighed against benefits. Additionally, validity and feasibility of alternative procedures should be frequently investigated. Alternatives to disbudding would be to increase space allowance and to actively select for polled genetic lines, and immuno-castration could eventually replace current physical castration procedures if proven acceptable to consumers.

Whilst replacement of the aversive husbandry procedures is to be recommended, until these alternatives include all animals or until there are major change in many current production systems, we have to establish practices that guarantee that poor welfare of animals is prevented. This will only happen by investigating better and more feasible ways of conducting and encouraging uptake of pain management practices. Improved production animal welfare will be ensured when farmers agree to, accept and then adopt pain relief strategies based on the scientific evidence provided through studies herein described.

CIISA and its partners continue to research ways of reducing pain during and after common painful procedures in livestock and this has contributed to achieving the goal of improved animal welfare outcomes for livestock. Anaesthesia and analgesia when disbudding calves is now widespread and accepted by most farmers as a standard practice, and the use of topical anaesthesia will improve the welfare of millions of animals, especially those subjected to procedures performed by non-veterinarians.

So, while we rethink and change our husbandry and practices, we should continue to strive towards ensuring that sentient animals are treated as such. Like John Webster says in his book Animal Welfare: A Cool Eye Towards Eden (1995) "The most crucial limitation to the moral philosophy approach to animal welfare is the fact that what matters to the animal is not what we think or feel but what we do".

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Genetic Diversity and Structure of Iberoamerican Livestock Breeds

L. T. Gama[1(✉)], A. M. Martinez[2], C. Ginja[3], J. Cañon[4], I. Martin-Burriel[5], M. A. Revidatti[6], M. N. Ribeiro[7], J. Jordana[8], O. Cortes[4], N. Sevane[4], V. Landi[2], J. V. Delgado[2], and the BIOBOVIS, BIOPIG, BIOHORSE, BIODONKEY, BIOGOAT and BIOVIS consortia

[1] Animal Genetic Resources Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
ltgama@fmv.ulisboa.pt
[2] Departamento de Genética, Universidad de Córdoba, 14071 Córdoba, Spain
[3] Centro de Investigação em Biodiversidade e Recursos Genéticos (CIBIO-InBIO), Universidade do Porto, Campus Agrário de Vairão, 4485-661 Vairão, Portugal
[4] Departamento de Producción Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain
[5] Laboratorio de Genética Bioquímica, Facultad de Veterinaria, Universidad de Zaragoza, Instituto Agroalimentario de Aragón IA2, 50013 Zaragoza, Spain
[6] Departamento de Producción Animal, Facultad de Ciencias Veterinarias, Universidad Nacional del Nordeste, 3400 Corrientes, Argentina
[7] Departamento de Zootecnia, Universidade Federal Rural de Pernambuco, Dois Irmãos 51171900, Recife, PE, Brazil
[8] Departament de Ciència Animal i dels Aliments, Facultat de Veterinària, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain

Abstract. Creole breeds of the various livestock species mainly derive from animals imported to America from the Iberian Peninsula, starting in the early years of discovery and colonization. Creoles have undergone a long period of selective adaptation to a diverse set of environmental conditions in the Americas, and over the last two centuries some Creole populations were admixed with breeds from other European countries and from India. In spite of various threats, some Creole populations are still maintained nowadays, especially in marginal regions, but they need to be better known, in order to recognize their identity and establish conservation programs. Here, we review the results published over the last years by various Consortia established under the framework of the CONBIAND network, with the goal of studying the genetic diversity, structure and breed relationships in Creole breeds. Overall, Creole breeds reveal high levels of genetic diversity and signatures of Iberian origin, but many breeds also show signs of genetic erosion, due to inbreeding or admixture with exotic breeds. The vast majority of Creoles still maintain their own identity, and these results can be used as a basis for recognition, conservation and genetic improvement of Creoles, which result from over 500 years of selective adaptation.

See consortia membership in http://www.uco.es/conbiand/consorcios.html.

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1 The Origins of Creole Livestock

Domestic livestock species such as cattle, horses, donkeys, pigs, goats, sheep and chicken, did not exist in the American continent when Columbus arrived after his first trip in 1492. The Creole populations of the different species of domestic animals that are currently found in the American continent are the result of animals that were brought over the years, especially in the initial stages of discovery and colonization, beginning with the second voyage of Christopher Columbus, when various domestic animals were taken on board of ships. In the initial period, animals of different species were taken by the navigators, brought from the Iberian Peninsula as well as from the archipelagos of the Canaries and Cape Verde, where the caravels often made a stopover for resupplying. The initial group of animals would multiply rapidly, spreading throughout America, from the Rocky Mountains to Patagonia, adapting to extremely diverse climatic and environmental conditions. These domestic animals taken from the Iberian Peninsula and the islands were a fundamental instrument in the process of territorial expansion, because of their importance as a means of transportation and as a source of labor, food and clothing.

The saga of the arrival and diffusion of animals coming from the Iberian Peninsula to the American continent has been the subject of interest by several authors (Rodero et al. 1992; Primo et al. 2004, de Alba Martinez 2012; Beteta Ortiz et al. 2015), and it is believed that the number of animals that came to America to give rise to the different populations known as Creole was small, particularly in cattle (Rouse 1977). In the first years, the main point of arrival of animals was the island of La Hispaniola, and from there they spread to Mexico and North America, Central America and northern countries of South America (Venezuela, Colombia and Ecuador), migrating thereafter to the south. A few years later, animals from the Iberian Peninsula were brought to the coast of Brazil and to the Rio de la Plata, so that, about 40 years after the arrival of the first group of animals, Creole livestock were found scattered in nearly all the American continent.

Following an undisputable predominance of Creole animal populations in America until the 18th century, the import of animals from Great Britain and continental Europe began. This was followed by the introduction of zebu-type animals from India in the early 20th century, in these now represent a significant fraction of the bovine population in the Americas, particularly in tropical regions. It has also been suggested that the introduction of animals of African origin in the American continent may also have occurred, but there is no consistent historical evidence of this flow of animals.

After an initial phase of adaptation of the animals of Iberian origin to the local conditions of very diverse environments in the Americas, the later arrival of animals of other European origins and zebu resulted, in many cases, in the disordered crossing with the Creole populations that were already installed there. This has resulted in a marked degree of erosion of livestock Creole breeds, many of which have been extinct over the last century. However, some isolated populations of the Creole type still survive, often in marginal regions. At present there is an effort to gain knowledge, rescue and conserve these populations, which are the result of 500 years of adaptation to the diverse set of environmental conditions that were found in the American continent.

Over the last decades of the 20th century, several studies on morphological and demographic characterization of some Creole populations of different species were published, which in certain cases led to their recognition as distinct breeds and definitely contributed to their safeguarding.

At that point, doubts persisted as to whether these populations corresponded to what was historically known as Creole, to what extent they diverged from each other, whether they still had strong Iberian influences or had already been admixed with other breeds (European or Zebu), what degree of identity and differentiation they had, what was their genetic structure, etc.

The answer to these questions was only possible when the use of genetic markers became a current practice, allowing a detailed knowledge of Creole populations, namely their diversity, structure and relationships with other breeds. At the end of the 1990s, uniparental genetic markers were used, especially mitochondrial DNA, which allowed a preliminary assessment of the genetic structure of Creole breeds, despite the limitations of providing information only on genetic contributions of maternal origin.

At the beginning of the 21st century, the XII-H network of the CYTED program (which was subsequently followed by the CONBIAND network) brought together the activities of a large number of research groups working on the characterization and conservation of Animal Genetic Resources throughout Iberoamerica. With the foundations and exchange platform provided by these networks, the opportunity materialized to systematically address the study of the genetic diversity of Creole populations of different species. In the strategy defined by this group in the beginning of the 21st century, microsatellites were adopted as the genetic markers of choice to be used to assess genetic diversity in Creole populations, as these are neutral autosomal markers that were recommended by FAO for this purpose and have been widely used in all species of domestic animals.

As a result of the activities of the CYTED and CONBIAND networks, various consortia were established to study the genetic diversity of livestock species. From the activity of these consortia, a series of research projects and publications were generated, which represent a fundamental contribution for an in-depth knowledge of the diversity and structure of Creole breeds, and their relationships with other breeds.

In this chapter, we put together a brief review of the research work on genetic characterization of Creole breeds carried out by these consortia, covering the bovine, swine, caprine, equine and asinine species, and it is expected that, in the near future, similar research activities will be expanded to other species.

2 Cattle

Over the last few years, a number of studies have been carried out in the framework of the CYTED XII-H and CONBIAND networks on Creole cattle genetic diversity, and several research groups were organized within the Biobovis consortium. This broad-range research work included DNA samples from the American continent, the Iberian Peninsula, the Atlantic Islands (Azores and Canary Archipelagos), the European continent and the British Isles, covering a total of 3333 animals representing 81 breeds from 12 countries.

In an initial stage, Ginja et al. (2010) studied the genetic diversity of mitochondrial and Y chromosome DNA in Iberoamerican cattle populations. The Creole breeds revealed mt-DNA haplogroups of European, African, Iberian and Atlantic origin, while Y chromosome markers indicate an influence of the same groups of breeds, as well as a strong influence of zebu. It seems, therefore, that an unquestionable African influence existed in the development of the Creole breeds, which may, however, have also been mediated by Iberian breeds.

Martin-Burriel et al. (2011) studied 51 Iberian bovine breeds using 21 microsatellite markers. The expected heterozygosity was 0.685 and the mean number of alleles/locus was 7.6. There were signs of an ancient African influence in Iberian breeds, and a reduced number of breeds showed influence of exotic germplasm (i.e. commercial transboundary breeds). The proportion of the total genetic diversity justified by differences between breeds was 8.6%, and some breeds showed a deficit of heterozygosity, indicating the existence of inbreeding or substructure. The authors concluded that the relationship between the breeds studied depended more on their geographical proximity than on their morphotype.

Cañon et al. (2011) analyzed the establishment of conservation priorities in Iberian cattle breeds based on genetic criteria, using the same database as Martin Burriel et al. (2011). Depending on the weight given to the intra- or inter-racial components contributed by each breed to total genetic diversity, the conservation priorities were completely different. For example, when more emphasis is placed on inter-racial diversity, the more isolated and differentiated breeds (which are also breeds with higher levels of inbreeding) are given priority. On the other hand, if priority is given to intra-racial variability, breeds with the highest level of heterozygosity would be chosen, but there is a risk of prioritizing breeds whose higher variability results from a more extensive influence of exotic breeds. Some alternative methods were tested in which different weights were given to the intra- and inter-racial components of genetic diversity, and these resulted in quite different conservation priorities.

Delgado et al. (2012) studied 26 Creole cattle breeds using 19 microsatellites. The expected heterozygosity was 0.738, with an average number of 6.9 alleles/locus. Differences among breeds accounted for 8.4% of total genetic diversity, and several breeds showed heterozygosity deficit, but in some breeds there was an excess (possibly due to crossbreeding). The Creole breeds are generally well differentiated, so that a number of ancestral populations of $K = 21$ were estimated to have originated the 25 Creole breeds studied, which indicates an important degree of isolation between them. The genetic distances between breeds estimated in this study indicate the existence of five clusters, namely: breeds of Mexico and the Texas Longhorn; breeds of Panama and some Colombians; Caracú from Brazil and Creole breeds of Argentina and Uruguay; Romosinuano, Costeño con Cuernos, Sanmartinero and Casanareño breeds, all from Colombia; and a group of widely dispersed breeds, presumably with zebu influence.

Martinez et al. (2012) analyzed 27 Creole, 39 Iberian, 9 European and 6 Zebu breeds, using microsatellites to investigate the influence of different breeds on the genetic composition of Creole cattle. The Creole breeds presented greater genetic diversity than any of the other groups, with a higher expected heterozygosity and number of alleles/locus than any of the other breed groups (Table 1). Most Creole populations have a closer

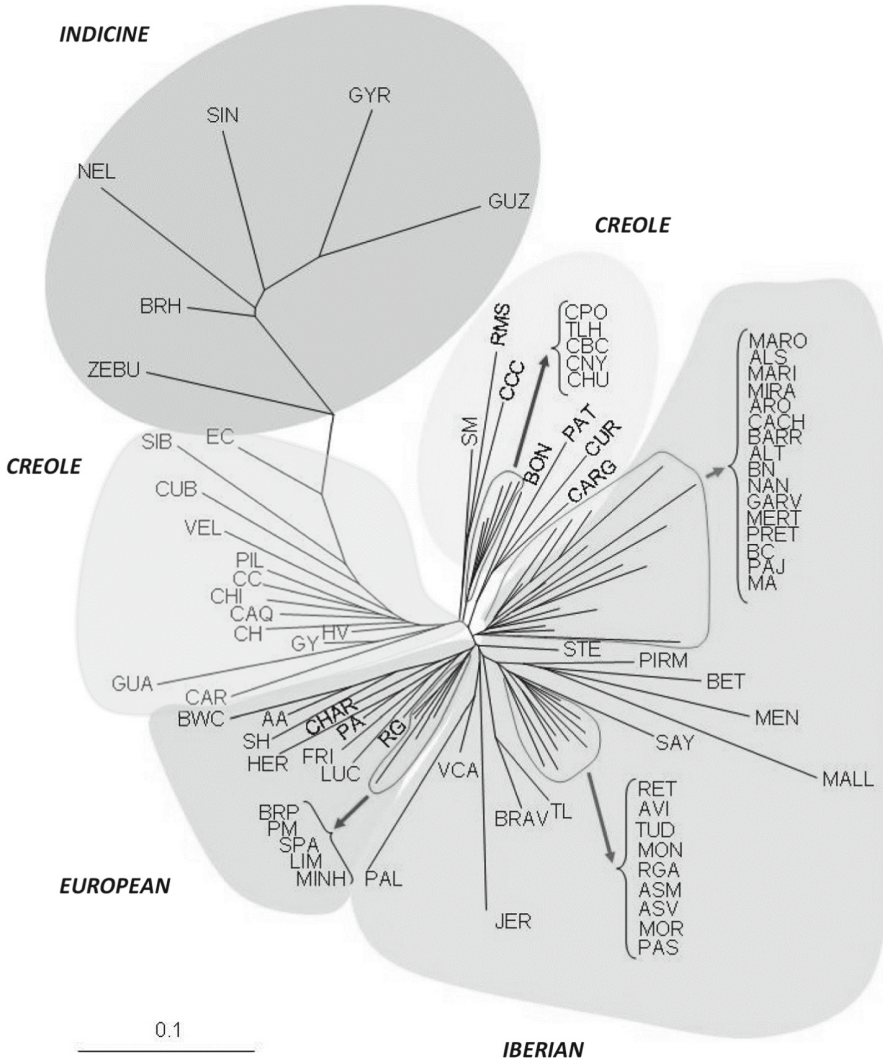

Fig. 1. Dendrogram constructed from D_A genetic distances among 81 cattle breeds from the Iberian Peninsula, Continental Europe, British islands, American Creole and Indicine cattle (adapted from Martinez et al. 2012). See Table 2 for definition of breed acronyms.

relationship with Iberian breeds (especially breeds from the south of the Iberian Peninsula), but some are influenced by British breeds and an important group reveals clear indications of crossbreeding with zebu. This could be more clearly identified in the tree summarizing genetic distances between breeds (Fig. 1), where it was clear that while some Creole breeds were close to Iberian cattle, others showed stronger influence of zebu or of British breeds. For the whole group of Creole breeds analyzed, the estimated contribution of the various ancestral populations to the Creole genetic pool was 63% from

Iberian breeds, 17% from Zebu, 10% from British and 10% from Continental European breeds. However, there were important differences between Creole groups in the relative importance of the contributions of the various ancestral groups. Overall, the results of this research indicate that the various Creole breeds have their own identity, as they differ greatly from each other, both in genetic structure and in the influences that they have received from other breeds. The erosion suffered by some Creole breeds in recent years, mainly as a result of crossbreeding with European and Zebu breeds, could compromise the results of several centuries of adaptation to a wide range of environmental conditions.

Table 1. Indicators of genetic diversity in Creole breeds of various livestock species, in comparison with Iberian and commercial breeds.

	Group	Sample used		Genetic diversity indicators[a]		
		No. breeds	No. animals	Na	Ae	He
Cattle[b]	*Creole*	*27*	*907*	*14.2*	*4.1*	*0.81*
	Spanish	26	1199	12.5	3.9	0.78
	Portuguese	13	675	10.7	3.6	0.75
	British	5	200	8.9	3.3	0.75
	Cont. European	4	184	9.9	4.0	0.76
	Zebu	6	168	11.3	3.3	0.74
Pig	*Creole*[c]	*17*	*613*	*6.3*	*3.3*	*0.62*
	Iberian[d]	17	731	5.1	2.6	0.53
Goat[e]	*Creole*	*23*	*786*	*5.7*	*2.7*	*0.63*
	Iberian	24	775	6.4	2.9	0.65
	Canarian	5	200	5.6	2.8	0.64
	African	13	441	6.6	3.0	0.67
Horse[f]	*Creole*	*27*	*1414*	*7.8*	*4.1*	*0.76*
	Celtic-type	13	478	7.5	4.1	0.76
	Iberian-type	7	320	6.8	3.7	0.73
	Cosmopolitan	2	120	6.0	3.4	0.71
Donkey[g]	*Creole*	*13*	*350*	*4.4*	*2.2*	*0.54*

[a]Na = mean number of alleles; Ae = effective number of alleles; He = expected heterozygosity.
[b]Adapted from Martinez et al. (2012).
[c]Adapted from Revidatti et al. (2014).
[d]Adapted from Gama et al. (2013).
[e]Adapted from Sevane et al. (2018).
[f]Adapted from Cortés et al. (2016).
[g]Adapted from Jordana et al. (2016).

Table 2. Breed acronyms for Figs. 1 to 6.

Cattle (Fig. 1)	SPANISH. Betizu (BET), Toro de Lidia (TL), Menorquina (MEN), Alistana (ALS), Sayaguesa (SAY), Tudanca (TUD), Asturiana de los Valles (ASV), Asturiana de las Montañas (ASM), Retinta (RET), Morucha (MOR), Avileña (AVI), Pirenaica (PIRM), Rubia Gallega (RGA), Mallorquina (MALL), Monchina (MON), Serrana de Teruel (STE), Parda de Montaña (PM), Bruna de los Pirineos (BRP), Pasiega (PAS), Berrenda en Colorado (BC), Berrenda en Negro (BN), Marismeña (MAR), Pajuna (PAJ), Negra Andaluza (NAN), Vaca Canaria (VCA), Vaca Palmera (PAL); PORTUGUESE. Alentejana (ALT), Arouquesa (ARO), Barrosã (BARR), Brava de Lide (BRAV), Cachena (CACH), Garvonesa (GARV), Marinhoa (MARI), Maronesa (MARO), Mertolenga (MERT), Minhota (MINH), Mirandesa (MIRA), Preta (PRET), Ramo Grande (RG); CREOLE. Guabalá (GUA), Guaymi (GY), Texas Longhorn (TLH), Criollo Poblano (CPO), Criollo de Baja California (CBC), Criollo de Chihuahua (CHU), Criollo de Nayarit (CNY), Criollo de Chiapas (CHI), Blanco Orejinegro (BON), Caqueteño (CAQ), Sanmartinero (SM), Romosinuano (RMS), Costeño con Cuernos (CCC), Chino Santandereano (CH), Velasquez (VEL), Lucerna (LUC), Hartón del Valle (HV), Criollo Casanareño (CC), Criollo Ecuatoriano (EC), Criollo Uruguayo (CUR), Pampa Chaqueño (PA), Criollo Pilcomayo (PIL), Criollo Argentino (CARG), Criollo Patagónico (PAT), Caracú (CAR), Cubano (CUB), Siboney (SIB); ZEBU: Gyr (GYR), Brahman (BRH), Sindi (SIN), Guzerat (GUZ), Nelore (NEL), Zebu Cubano (CUZ); Other EUROPEAN. Friesian (FRI), Hereford (HER), Brown Swiss (BSW), Aberdeen Angus (AA), British White (BWC), Charolais (CHAR), Jersey (JER), Limousin (LIM), Shorthorn (SH)
Iberian Pigs (Fig. 2)	Alentejano (ALE), Bísaro (BIS), Malhado de Alcobaça (MAL), Celta (CEL), Chato Murciano (CHM), Entrepelado (ENT), Euskal Txerria (ETX), Lampiño (LAM), Manchado de Jabugo (MJA), Negro Canario (NCA), Negro de Formentera (NFO), Negro de los Pedroches (NPE), Negro Mallorquín (NMA), Retinto (RET), Torbiscal (TOR), Spanish Wild Boar (SWB), Portuguese Wild Boar (PWB)
Creole Pigs (Fig. 3)	Mulefoot (MF), Red Wattle Hog (RWG), Guinea Hog (GH), Pelón Mexicano (PEL), Baja California (BCA), Criollo El Salvador (SAL), Criollo Cubano (CUB), Crioolo Guadeloupe (GUA) Criollo Venezolano (VEN), Zungo (ZUN), San Pedreño (SPE), Criollo del Pacifico (PAC), Criollo Ecuatoriano (ECU), Criollo Boliviano (BOL), Pampa Rocha (PRO), Criollo Argentina Northeast Wet Region (ANW), Criollo Argentina Northeast Dry Region, (AND)
Goats (Fig. 4)	Iberian Peninsula - Pirenaica (PIR); Moncaína (MON); Azpi Gorri (AZ); Blanca de Rasquera (RAS); Guadarrama (GUAD); Retinta (RET); Verata (VERA); Blanca Andaluza (BLANCA); Celtibérica (CELTIB); Blanca Celtibérica (BC); Malagueña (MALAG); Murciano-Granadina (MG); Florida (FLO); Payoya (PAY); Negra Serrana (SER); Formentera (FOR); Pitiusa (IB); Mallorquina (MALL); Bravia (BR); Serpentina (SP); Algarvia (AL); Charnequeira (CH); Serrana (SR); Preta de Montesinho (PM); Canary Islands -; Ajuí (AJ); Majorera (MFV); Palmera (PAL); Tenerife Norte (TFN); Tenerife Sur (TFS); Africa - Cape Verde (CVERDE); Barki (BARKI); Baladi (BALADI); Saidi (SAIDI); Morocco Goat (MOR); Tunisian Local Goat (TU); Bushguinder (BU); Maradi (MARADI); West African Dwarf (WAD); Sahel (SAHEL); Gwembe Dwarf Goat (ZAM); Kalahari Goat (KAL); Boer (BOER); Outgroup - Rawhiti (RW); Arapawa Goat (ARAPA); Golden Guernsey (GG); Saanen (SAAN); Alpine (ALP); Anglo-Nubian (ANG); Creole - Spanish Goat (SPA); Myotonic (MYO); San Clemente Island (SCL); Criolla Cubana (CUB); Criolla Colombiana (COL); Criolla Venezolana (VEN); Criolla del Ecuador (ECU); Galapagos Goat (GAG); Criolla Peruana (PER); Azul (SAZ); Moxotó (MOX); Marota (MRT); Canindé (CND); Repartida (REP); Graúna (GRN); Sem Padrao Racial (SRD); Criolla Boliviana (BOL); Criolla Paraguaya (PGY); Criolla del Nordeste (NEA); Neuquina (NUQ); Chilluda (CHI); Pampeana Colorada (PCA); Angora (TAN)

(*continued*)

Table 2. (*continued*)

Horses (Fig. 5)	Cr. Argentino (CAR), Mangalarga (MGB), Marajoara (MJB), Pantaneiro (PNT), Puruca (PUR), Chilote (CHI), Isla de Pascua (ISP), Cr. Colombiano Vaqueria (CVC), Paso Fino Colombiano (CPF), Trocha Pura Colombiana (CTC), Trocha y Galope Colombiano (CTP), Trote y Galope Colombiano (CTG), Cr. Cubano (CUB), Cr. Ecuatoriano (CEC), Cr. Salvadore ~ no (CSA), Cr. Panameño (PAN), Cr. Paraguayo (CPA), Peruano de Paso (PEP), Cr. Uruguayo (CUR), Appaloosa (APA), Morgan (MOR), Quarter Horse (QHO), Rocky Mountain (RMU), Saddlebred (SDB), Spanish Mustang (SMU), Tennessee Walking (TWH), Mount Taylor Mustang (MTM), Arabian (ARA), Asturcon (AST), Burguete (BUR), Cabalo do Monte Galego (GAL), Hispano-Breton (BRT), Hispano-Arabe (HIA), Jaca Navarra (JNV), Losino (LOS), Mallorquin (MAL), Menorquin (MEN), Marismeño (MAR), Monchino (MON), Pirinenc Catala (PIC), Potoka (POT), Pura Raza Español (PRE), Thoroughbred (PSI), Retuertas (RET), Trotador Español (TRO), Garrano (GAR), Lusitano (LUS), Sorraia (SOR), Merens Pirineo Frances (MER), Barb (BER)
Donkeys (Fig. 6)	Creole: Argentina (ARG); Bolivia (BOL); Brazil (BRA); Chile (CHI); Colombia (COL); Cuba (CUB); Ecuador (ECU); Guatemala (GUA); Mexico (MEX); Paraguay (PAR); Peru (PER); Uruguay (URU); Venezuela (VEN); European: Mirandesa (MIR); Andaluza (AND); Balear (BAL); Catalana (CAT); Encartaciones (ENC); Majorera (MAJ); Zamorano-Leonesa (ZAM); Pantesco (PAN); Grigio Siciliano (SIC); Ragusano (RAG); Greek (GRE)

Ginja et al. (2013) studied conservation priorities in 67 Iberoamerican cattle breeds, based on genetic diversity criteria. Overall, Creole breeds have higher levels of genetic diversity, particularly those breeds that show signs of crossing with zebu. Of course, these are the breeds considered to be prioritized for conservation when the sole criterion of choice is the contribution of intra-racial variability to the total genetic diversity. However, when priority is given to inter-racial diversity, priority is given to the most differentiated breeds (Guabalá, Romosinuano, Criollo Patagonico, Siboney and Caracú). In approaches that combine the within- and between-breed components of genetic diversity, the priority depended on the weight assigned to each of them. Overall, the Creole breeds had a higher conservation priority than Iberian breeds.

At present, the research groups involved in the various consortia are undertaking additional studies, including breeds of different origins and combining various types of genetic markers, to investigate in greater detail the origin and genetic structure of the Creole bovine breeds.

3 Pigs

The BIOPIG Consortium has been studying the diversity and genetic structure of swine breeds in the Iberian Peninsula and the American continent.

In this context, Gama et al. (2013) used a panel of 24 microsatellites in a sample of 844 pigs representative of the major 17 native breeds in Portugal and Spain, and some groups of wild boars from these countries. Genetic diversity was high in the breeds studied, with an expected heterozygosity of 0.53 and an average of 5.1 alleles/locus (Table 1). The breeds with reduced census presented signs of genetic bottleneck (expressed as

lower allelic diversity), while the more numerous breeds sometimes showed substructure. Between-breed diversity represented about 20% of the total genetic diversity, and was mainly explained by differences between the Celtic, Mediterranean and Basque groups, rather than differences between domestic and wild pigs. The different varieties of the Iberian Pig had a close relationship but were genetically distinct from each other and well-structured (Fig. 2). Significant genetic proximity with wild pigs was detected in the breeds of the Iberian branch, but not in Celtic breeds, which probably reflects admixture due to accidental crossbreeding with wild relatives in the Mediterranean group. The geographical distribution of genetic diversity essentially reflected differences in herd size and production systems between the north and south of the Iberian Peninsula, such that the spreading of the Iberian genetic influence across the territory almost exactly mirrored the distribution of the "dehesa-montado" system in which these pigs are traditionally raised. Hence, an interdependence between animal and agroforest resources clearly occurred, which underscores the important role played by native pig breeds towards environmental sustainability.

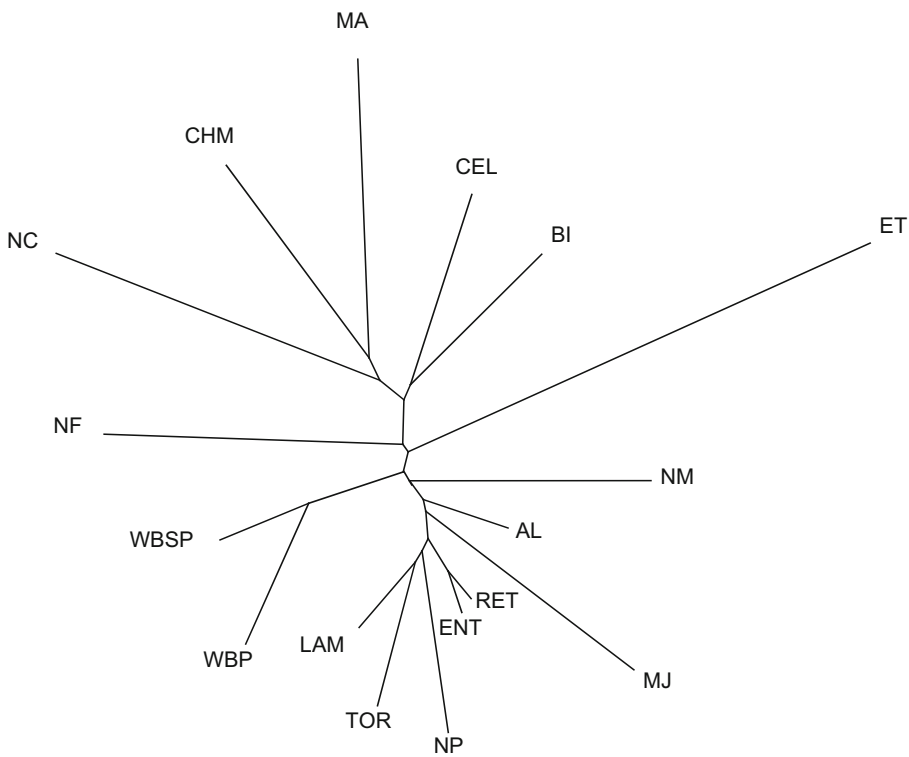

Fig. 2. Neighbour-joining tree summarizing the Nei D_A genetic distances among 17 native pig breeds from Spain and Portugal, and wild-boar pigs sampled in the two countries (Adapted from Gama et al. 2013). See Table 2 for definition of breed acronyms.

Revidatti et al. (2014) studied the diversity and genetic relationships in 17 Creole breeds from 11 countries of the American continent with 24 microsatellite markers. The expected heterozygosity was 0.62, with a mean of 6.25 alleles/locus (Table 1). The

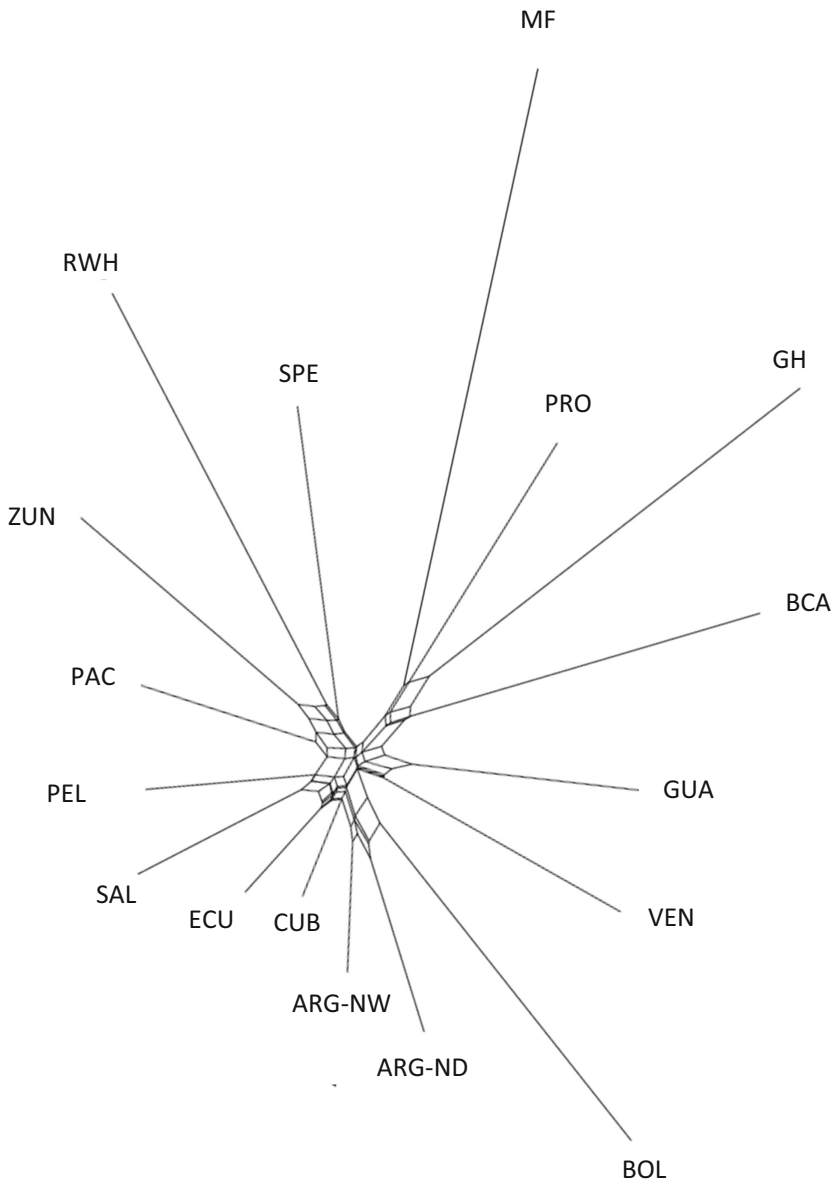

Fig. 3. Neighbournet dendrogram summarizing the Nei D_A genetic distances among 17 Creole native pig breeds (Adapted from Revidatti et al. 2014). See Table 2 for definition of breed acronyms.

proportion of genetic diversity represented by differences between breeds was 0.11 (indicating an important within-breed diversity), and nearly all breeds had a heterozygosity deficit. The Mulefoot and Guinea hog breeds from the United States formed a cluster with the Mexican from Baja California and the Uruguayan Pampa Rocha, while Colombian breeds clustered with the American Red Wattle breed (Fig. 3). The fact that some breeds with distant geographic distribution showed signs of genetic proximity between them may reflect a common origin in the initial phase of colonization.

Cortes et al. (2016) studied conservation priorities in 45 Iberoamerican and commercial swine breeds, corroborating that the priority given to each breed depends on the relative importance attributed to its contribution to inter- and intra-racial genetic diversity. When the major goal was the partial contribution to total heterozygosity, a high priority was given to Creole pig breeds, whereas Weitzman procedures prioritized breeds from the Iberian Peninsula. With the combined within- and between-breed approaches, different conservation priorities were achieved. The Core Set Kinship-based methodologies highly prioritized Creole pig breeds (in particular from Cuba, Colombia, Bolivia and Guadeloupe) while weighing the between- and within-breed components with F_{ST} and 1-F_{ST}, respectively, resulted in higher contributions of Iberian breeds. Of course, other factors in addition to the contribution to genetic diversity must be taken into account when setting conservation priorities.

4 Goats

The diversity and genetic structure of the Iberian Peninsula goat breeds were studied by Martinez et al. (2015), using 20 microsatellite markers in a sample of 975 representative animals of 25 officially recognized breeds in Portugal and Spain and 4 populations not yet recognized. The level of genetic diversity was high, with means of 0.65 for expected heterozygosity and 6.24 for the number of alleles/locus. Overall, breed differentiation accounted for about 7% of total diversity, and about half of the breeds had a heterozygosity deficit. Nevertheless, genetic differentiation between the breeds studied was generally small, with the exception of the breeds from the Canary Islands, which were quite distant from the continental breeds, possibly because of their isolation as well as the influence they received from African breeds in the past.

Ginja et al. (2017) used microsatellites to study a group of 910 animals, recruited in 24 Creole goat populations sampled in the American continent, and 112 animals from 3 cosmopolitan breeds. The genetic diversity found in Creole breeds translated into an expected heterozygosity of 0.64 and an average number of 5.82 alleles/locus, and the breeds from isolated islands (Galápagos and San Clemente) showed lower levels of genetic variability. The diversity found between the set of 27 studied breeds represented 13% of the total diversity. The breeds from Brazil were clearly differentiated relative to the others, and an additional cluster could be identified, corresponding to the Creole goat breeds from Argentina, Peru, Venezuela, Bolivia and Cuba. A small number of Creole breeds revealed the influence of commercial transboundary breeds.

A preliminary comparison of goat breeds from Portugal and Brazil was reported by Ribeiro et al. (2012), who studied in detail the 12 breeds recognized in both countries. The mean F_{ST} distances were 0.03 among Portuguese breeds, 0.07 among Brazilian

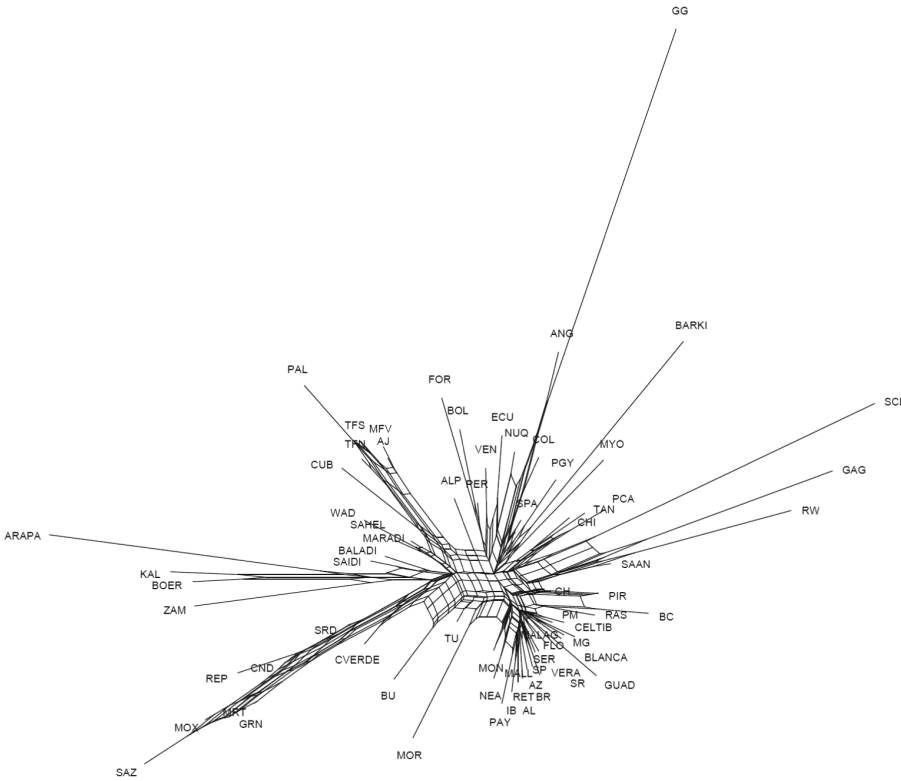

Fig. 4. Neighbournet dendrogram constructed from the Reynolds genetic distances among 71 goat populations (Adapted from Sevane et al. 2018). See Table 2 for definition of breed acronyms.

breeds and 0.15 between breeds from Portugal and Brazil. The breeds of each of the analyzed countries were in well-individualized clusters, approximately equidistant to the Alpine breed, which was used as an outgroup. However, some Brazilian breeds (Graúna and Canindé) showed signs of a possible link to the Portuguese breeds, albeit weak.

Sevane et al. (2018) studied the genetic diversity in 71 goat breeds, including 29 breeds from Spain and Portugal, 23 Creole breeds from the Americas, 13 breeds from Africa and 6 cosmopolitan plus New Zealand breeds using microsatellite markers. The major goal was to provide a comprehensive perspective on the genetic diversity in American goat populations and to assess their origins and evolutionary trajectories. The existing genetic diversity was highest in African breeds, followed by Iberian and Creole breeds (Table 1). The dendrogram representing breed genetic distances (Fig. 4) indicates the existence of three major groups, i.e., Iberian, Canary Islands and Brazilian breeds, such that most Creole populations grouped around Iberian breeds, consistent with a close genetic relationship among all these goats. The major exceptions to this pattern were the Brazilian, Colombian, and Cuban breeds, such that the Cuban breed shared their position with West African populations (Canary Islands, Bushguider from the Saharawi Camps, and Nigerian) while the Brazilian breeds grouped in one separate cluster, together with

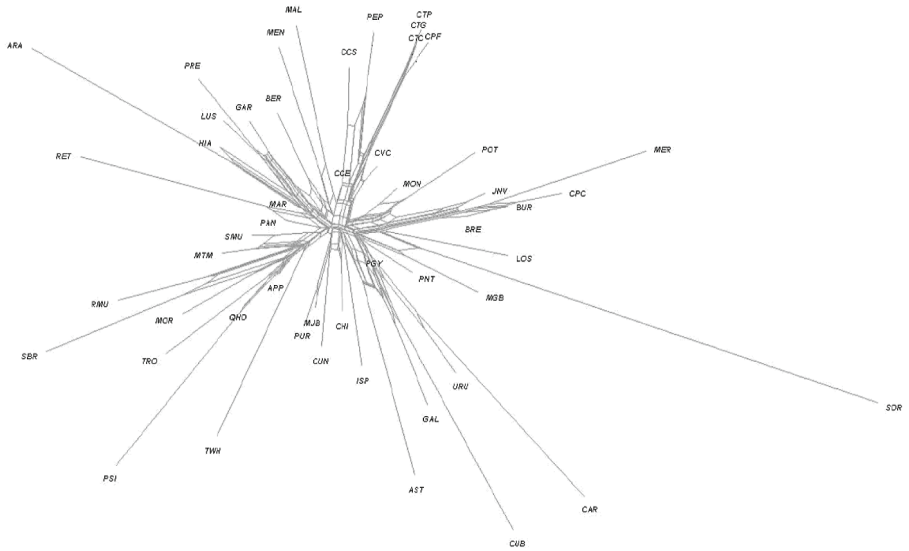

Fig. 5. Neighbournet dendrogram summarizing Reynolds genetic distances among 51 horse breeds (Adapted from Cortés et al. 2016). See Table 2 for definition of breed acronyms.

the goats from Cape Verde. Taken together, these results indicate a lack of geographical patterns in the distribution of goat genetic diversity in the Americas, with a wide differentiation among the various Creole breeds. Important Iberian signatures could be detected in nearly all Creole populations, with an important contribution of African populations to many Creole breeds. Some degree of genetic isolation was detected in Brazilian breeds, which had nevertheless some influence from Cape Verde goats.

5 Horses

The Biohorse consortium, established within the CYTED XII-H and CONBIAND Networks, gathered the efforts of various research groups working on Creole-type and related horse breeds throughout Iberoamerica.

Cortes et al. (2016) analyzed 25 microsatellite markers in 27 Creole breeds of horses sampled in the Americas, 19 breeds from the Iberian Peninsula, one breed from Morocco and one from France, and two cosmopolitan breeds (Thoroughbred and Arabian). Genetic diversity, assessed by the number of alleles/locus and expected heterozygosity, were highest in Creole horse breeds (Table 1). In the analysis of genetic relationships for the set of 50 breeds studied (Fig. 5) five clusters were identified, corresponding to: Celtic group; Iberian group; American breeds with Thoroughbred influence; majority of Colombian breeds; other Creole breeds. The last group was the one that presented greater proximity to the Iberian breeds. For the group of Creole breeds analyzed, the genetic contribution of the various founder clusters was approximately 50, 30 and 20% for the Celtic, Iberian and Arabian-Thoroughbred groups, respectively. Although they share a common origin, the Creole breeds of horses have their own identity and in most cases lack appropriate recognition and adoption of conservation programs.

6 Donkey

Donkeys played a very important role in the colonization of the American continent, both directly as working animals, but also as producers of mules, which were very important as animals used for transportation and draught. Jordana et al. (Jordana et al. 2016) used a panel of 14 microsatellites to study the genetic diversity, breed structure and relationships in a sample of 350 donkeys from 13 countries of the American continent, in comparison with samples from 476 animals from 11 asinine breeds from Italy, Greece, Portugal and Spain. The expected heterozygosity in the American breeds was 0.54, with an average of 4.4 alleles/locus (Table 1), which is much lower than the genetic diversity found in other livestock species in America. In the analysis of genetic relationships between breeds (Fig. 6), two well-identified clusters were detected in the American asinine population, including the breeds from Argentina, Bolivia, Chile, Ecuador, Paraguay, Peru and Uruguay, and in the second, the breeds from Cuba, Guatemala, Mexico and Venezuela.

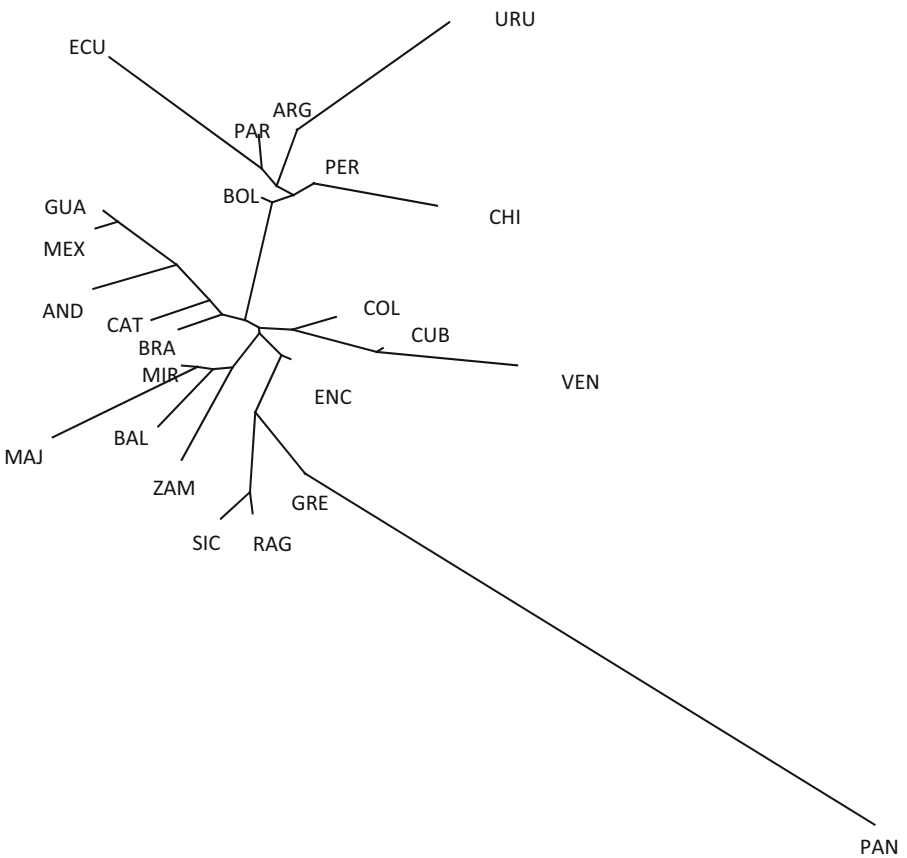

Fig. 6. Unrooted tree of Reynolds genetic distances for 24 American and European donkey populations obtained with the neighbour-joining algorithm (Adapted from Jordana et al. 2016). See Table 2 for definition of breed acronyms.

On the other hand, the breeds from Brazil and Colombia had mixed contributions from those two clusters. The breeds in the first cluster were close to each other, but away from the Iberian breeds, while the donkeys from Mexico and Guatemala showed influence of the donkeys from Andalusia and the donkeys from Brazil were close to those of Catalonia.

7 Conclusions

The Creole populations that currently exist in many countries of the American continent are the result of the introduction of Iberian breeds since the 15th century, possibly with additional influences from African breeds. More recently, Creoles have also been influenced by other breeds, especially in the last century.

In the last 10 years, a systematic research work has been carried out on the diversity and genetic structure of these Creole populations, accomplished by various research groups structured in consortia established in the framework of the CYTED XII-H and CONBIAND networks.

These studies allow us to conclude that, in all the species evaluated, the Creole breeds have high levels of genetic diversity, a unique identity and clear signatures of their origin in the breeds from the Iberian Peninsula. However, many of the Creole breeds are not adequately recognized or protected, and many show signs of genetic erosion either as a result of accumulated inbreeding due to their small census, or because of uncontrolled crossbreeding with exotic breeds.

The work carried out so far demonstrates the unique identity of the Creole breeds, their structure and genetic relationships, and therefore constitutes a fundamental basis for the adoption of measures aimed at the recognition, conservation and genetic improvement of these breeds, which result from more than 500 years of selective adaptation.

8 Future Perspectives

Research work similar to those described above is currently underway for the sheep species within the BIOVIS consortium, covering more than 70 sheep breeds, and the first results are expected to be available shortly. In recent years, other species have been incorporated into the research carried out by different CONBIAND groups working on the biodiversity of domestic animals, including chicken, turkey, guinea pigs, etc.

The development of panels of high-density genetic markers allows us to foresee new challenges, namely the possibility of investigating with non-neutral markers the diversity of Iberoamerican livestock populations. Indeed, the influence that Iberian breeds had in the development of Creole breeds, and the ability that they revealed to adapt to extremely diverse environmental conditions over the centuries, opens up excellent possibilities for investigating genetic markers associated with the unique adaptive mechanisms that these breeds have developed under very diversified environmental conditions. There is still much to be done and the synergies that have been established among the various research groups that have collaborated in the CONBIAND Network consortia are a guarantee that the fruitful cooperation that was established will continue and be strengthened,

thus raising awareness, providing better knowledge and promoting the Creole breeds of Iberoamerica.

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Genetics of Carcass and Meat Quality Traits in Iberian Pigs

M. C. Bressan[1,2], J. Almeida[3], A. Amaral[1], C. Bettencourt[4], J. Santos-Silva[3,5], O. Moreira[3], R. Bessa[5], and L. T. Gama[1(✉)]

[1] Animal Genetic Resources Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
ltgama@fmv.ulisboa.pt

[2] Faculty of Veterinary Medicine, Universidade Lusófona de Humanidades e Tecnologias, 1749-024 Lisbon, Portugal

[3] Instituto Nacional de Investigação Agrária e Veterinária (INIAV), I.P., Vale de Santarém, 2005-048 Santarém, Portugal

[4] Direção Regional de Agricultura e Pescas do Alentejo, Herdade da Abóbada, 7830-908 Serpa, Portugal

[5] Animal Production Systems Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal

Abstract. A factorial experiment was carried out with Iberian (IB, n = 60) and F1 Large White-Landrace crossbred (F1, n = 58) pigs, finished intensively or on pasture and acorns. IB pigs were genotyped with PorcineSNP60 v2 Beadchip to identify markers associated with the traits analyzed. Pigs were slaughtered in an experimental abattoir at 160 ± 5 kg live weight. Carcass properties and meat quality, including sensorial attributes, were investigated. For all traits, there was an overwhelming influence of genetic group, while finishing system had a less pronounced effect. IB pigs consistently produced fatter carcasses, with mean fat depots in IB higher than in F1 by about 25% in total abdominal fat, 94% in dorsal fat depth, 72% in intermuscular and subcutaneous ham fat, and 300% in intramuscular fat. IB pigs produced meat with higher marbling score, more appealing color coordinates and lower shear force. The sensorial properties of IB meat were more desirable in all items (tenderness, juiciness, flavor and global acceptability). This better meat quality was largely explained by the higher fat deposition in IB meat. We identified candidate genes associated with heart weight, meat moisture, index of desaturase and elongase activity, and contents of *cis*-vaccenic acid in intramuscular fat and of lauric acid in subcutaneous fat.

Keywords: Carcass · Genomic association · Pork · Sensory attributes

1 Introduction

Over the last years, selection to improve lean growth in pigs has resulted in a reduction of meat quality, with a decline in pork tenderness, juiciness and flavor (Merks 2000;

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Lonergan et al. 2001). Yet, meat quality in local pig breeds is often considered better, especially when raised under extensive conditions (Pugliese and Sirtori 2012). Among these, Iberian pigs are recognized for the excellent quality of their dry-cured products, which reach premium prices, particularly when they are obtained from animals raised on acorn and pasture (Ruiz et al. 2002; Čandek-Potokar and Škrlep 2012), produced in line with consumer concerns regarding ethics of production, animal welfare and environmental impact (Rodriguez-Estevez et al. 2012), and with a healthier fatty acid profile (Campo and Sierra 2011).

Carcass characteristics as well as physicochemical properties and sensory attributes of fresh meat from Iberian pigs are an important way to add value to this product. Traditionally, Iberian pigs are raised in free-range conditions with acorn and pasture, but in recent years in-door finishing has also coexisted (Isabel 2003; Robina et al. 2013). It is known that the sensory properties of Iberian dry-cured products is better when they are obtained from free-range pigs (Muriel et al. 2004; Ventanas et al. 2007), but the impact of finishing system on the palatability of fresh meat is not well demonstrated. Still, preliminary results suggest that consumers do not detect differences between pork derived from pigs finished extensively or intensively (Martínez et al. 2012).

When compared with commercial breeds, IB pigs usually display a higher content of intramuscular fat (IMF), consequently allowing the production of high quality pork products (Čandek-Potokar and Škrlep 2012; Pugliese and Sirtori 2012). This IMF has a higher content of monounsaturated fatty acids (MUFA) in comparison with commercial pig breeds, with about 50% of oleic acid (Serra et al. 1998; Ramayo-Caldas et al. 2012).

The various strains of IB pigs (Gama et al. 2013) represent a source of genetic variability that has not yet been fully exploited, thus representing an opportunity to identify the genetic basis of traits with high economic relevance. However, the obstacles represented by high costs of genotyping and phenotyping, as well as small size farming, have hampered the genome-wide characterization of these strains. In recent years, the availability of panels of genetic markers of variable density has made it possible to carry out in-depth research to identify genetic markers affecting production traits in Genome-Wide Association studies (GWAS). This strategy can further be combined with network construction approaches (Warde-Farley et al. 2010), enabling the study of causal inference from genotype to phenotype (Melé et al. 2015) and allowing the identification of the most probable group of candidate genes affecting the studied traits.

In the last few years, a thorough experiment was carried out to investigate how genetic group (Iberian and F1 Large White × Landrace pigs) and finishing system (outdoor-raising in oak-tree forests or conventional intensive systems) affect carcass characteristics and the physicochemical properties and sensory attributes of pork. In addition, genetic markers and candidate genes associated with the traits above were investigated in IB pigs with a medium-density SNP-Beadchip and gene interaction networks.

In this chapter we provide an integrated overview of the major results obtained in this experiment which have already been published (Bressan et al. 2016; Almeida et al. 2018; Amaral et al. 2019; Almeida et al. 2019).

2 Experimental Procedures

A total of 118 barrows belonging to the Iberian (IB, n = 60) and F1 Large White × Landrace (F1, n = 58) genetic groups were used in this study. When pigs reached a live weight of 85 kg, a sample of 30 IB and 30 F1 pigs was randomly assigned to be finished under standard intensive conditions (group IN) with a commercial diet. One other group of 30 IB and 28 F1 pigs with the same mean live weight was randomly assigned to be finished in free range (group EX), in an oak- and cork-tree forest where they had access to the fruit of those trees (acorn) and grass. At the beginning, middle and end of the finishing period, pigs were weighed and backfat measured. Pigs were slaughtered in an experimental abattoir when they reached the target weight of 160 ± 5 kg. At this time, in-depth carcass data were collected, including weight of organs, carcass cuts and fat depots, subcutaneous fat depth in the midline and in P2, and ham composition in lean, fat and bone. Samples of the longissimus thoracis were collected for determination of pH, meat color, chemical composition, drip loss, marbling score and sensory properties. After 9 d of ageing, one set of meat samples was boiled, and cooking loss and Warner-Bretzler shear force were measured. Another set of aged samples was roasted and used for sensory analysis, where meat attributes were scored by a panel of trained judges in a scale 1–8 (higher scores desirable).

Data were considered to have originated from a 2 × 2 factorial, with two genetic groups (Iberian and F1 Landrace × Large White) and two finishing systems (intensive and extensive). Data were analyzed by general linear models, using a model considering the effects of breed, finishing system and their interaction, fitting carcass weight as a covariate in the linear model. The GLM procedure of SAS (SAS Institute Inc.) was used in analyses of variance of the various response variables, and least squares means were obtained for main effects and their combination.

Experimental animals of the IB group were genotyped with the Porcine SNP60 v2 Beadchip of Illumina, which allowed interrogating the genotypes for 61,565 SNPs across the whole genome. After application of several quality control criteria, 31,434 SNPs remained for further analysis. The GWAS analyses of the various phenotypic traits assessed was conducted with the GenABEL toolset with thresholds of 5^{E-6} and 5^{E-5}, where the first threshold corresponds to 10,000 independent *Bonferroni* corrected tests and the second was proposed as the threshold allowing the detection of moderate associations (Teyssedre et al. 2012). The model used in GWAS included the effects of finishing system and carcass weight.

3 Carcass Traits

The detailed results of the analyses considering the effects of genetic group and finishing system on carcass traits have been published (Bressan et al. 2016) and are briefly summarized here.

For many traits analyzed, the interaction between genetic group and finishing system was not significant ($P > 0.05$), and genetic group had a much stronger effect than finishing system. Nevertheless, for completeness, we report here the major results for the combinations of genetic group × finishing system for the key traits in our study.

The least squares means for some of the major carcass traits analyzed are shown in Fig. 1. Carcass yield was higher in F1-IN pigs by about 0.8% relative to the two IB finishing groups, and by nearly 2.3% when compared with F1-EX. The mean heart weight was higher in EX animals of both genetic groups, and F1 pigs had heavier hearts by nearly 10% when compared with IB pigs in the same system. The prime cuts of the carcass (trimmed loin + tenderloin) represented only 4% of the cold carcass weight in IB, but they represented about 7% in F1 pigs. Abdominal fat (sum of omental, mesenteric and kidney fat depots) was slightly higher in IN-finished animals, and represented about 5.7% and 4.6% of the carcass weight in IB and F1 pigs, respectively. Backfat depth in the midline was not affected by finishing system and it was about 73 mm in IB and 43 mm in F1 pigs. In both genetic groups, backfat depth in P2 was higher in IN-finished pigs, and it was nearly twice as high in IB pigs when compared with F1. The area of the *l. thoracis* was slightly higher in EX than in IN-finished pigs, but the major differences were observed between genetic groups, such that the area was nearly 80% higher in F1 pigs when compared with IB. The percentage of fat in the ham showed minor differences between finishing systems, while genetic groups differed markedly, with a mean fat content of about 55% in IB and 30% in F1 pigs.

Our results indicate that, when compared with commercial pigs that have been selected mostly for lean growth (Tribout et al. 2010), IB pigs present lower ability for lean accretion and increased propensity for deposition of adipose tissue in different fat depots, as described by Zudaire and Alfonso (2013). For the slaughter and carcass traits considered in our study, there was an overwhelming influence of genetic group, while finishing system had a less pronounced effect, even though the interaction between the two factors was significant in some cases. Nevertheless, in most variables, particularly those related with fat deposition, the interaction essentially reflected fluctuations in mean differences rather than changes in breed ranking for the traits analyzed, such that IB pigs consistently produced fatter carcasses, regardless of the finishing system used.

The analyses of weight of organs revealed important differences among genetic groups, in some cases with relevant interactions with finishing system. The heart was larger in F1 when compared with IB pigs, and in pigs raised extensively relative to those raised indoors. These differences possibly reflect the lower physical activity of the obese IB pigs and of pigs raised indoors, as there is a well-known relationship between physical activity and heart weight (Laughlin et al. 2001).

In commercial breeds of pigs with live weight of 105 kg, the subcutaneous fat represents about 50 to 60% of total fat, while internal depots and intra-plus inter-muscular fat represent about 20 to 25% each (Kolstad 2001). The results of our study indicate that the deposition of adipose tissue is much larger in IB when compared with F1 pigs, with means for fat depots in IB which were higher by about 25% in total abdominal fat, 94% in dorsal fat depth and 72% in intermuscular and subcutaneous fat in the ham. These results confirm that, regardless of the finishing system used, IB pigs produce fatter carcasses, but the difference relative to F1 pigs is much smaller in abdominal fat when compared with other fat depots, even though the difference was observed in the various individual abdominal fat depots studied, especially mesenteric fat. The smaller difference among genetic groups in abdominal fat could be anticipated, because this depot is proportionally more important in fast-growing breeds (Kolstad 2001).

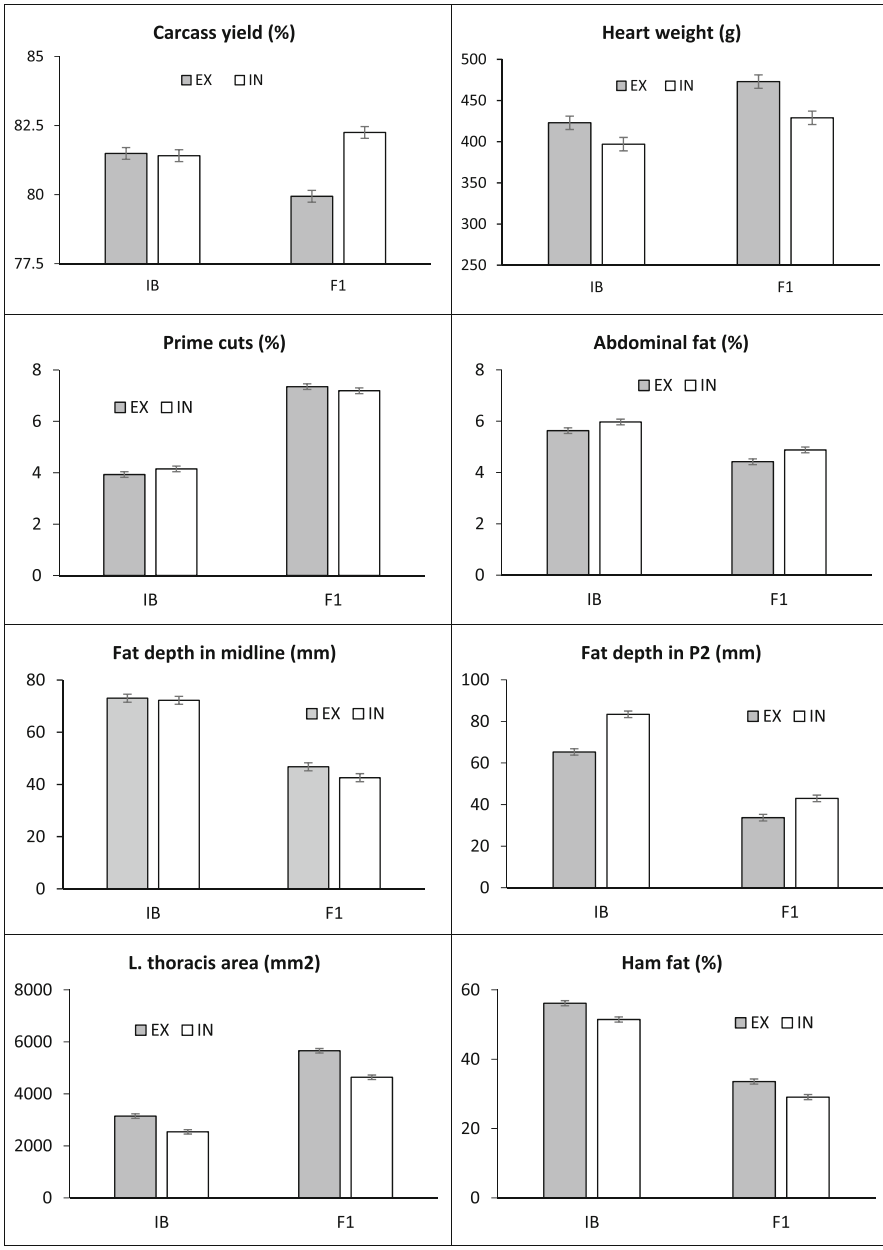

Fig. 1. Least squares means for carcass traits in Iberian (IB) and F1 pigs finished extensively (EX) or intensively (IN), with corresponding standard errors.

Differences between genetic groups in backfat depth were largely due to differences in the thickness of the inner backfat layer, which in our study represented about 77% of total backfat thickness in IB pigs and 66% in F1 pigs (results not shown). In contrast to

adipose tissue, the deposition of lean tissue was much lower in IB, such that the area of the *l. thoracis* in P2 was nearly one-half of that observed in F1 pigs in the same finishing system. These results are in general agreement with those reported by Nieto et al. (2012), and demonstrate the ability of IB pigs to deposit adipose tissue, and their poor ability for lean accretion. This pattern is due to a much higher allometric growth rate of fat relative to lean in IB pigs (Nieto et al. 2012), such that the proportion of adipose tissue increases and the proportion of lean tissue declines as slaughter weight increases. In our study, mean slaughter weight was about 160 kg, which is considered by the industry to be the appropriate weight to obtain high levels of intramuscular fat (IMF), which are desirable in high-value processed products from IB pigs (García et al. 1996). However, it has been shown (Serrano et al. 2008) that slaughter can be anticipated to 145 kg with no compromise of meat quality, and that the manipulation of energy and protein in the diet could also lead to a more desirable fat:lean ratio in IB pigs (Nieto et al. 2012).

Until the mid-20th century, subcutaneous and abdominal fat depots were considered advantageous, because they represented important sources of energy for use and consumption by humans (Kovarik 2013), but they are no longer desirable nowadays, because excessive fat is not useful in most cases and it involves a considerable expense of energy to deposit and maintain by growing pigs. Therefore, reducing subcutaneous and abdominal fat depots, either by manipulation of the feeding regime, genetic selection or reduction of weight at slaughter, could contribute to a more efficient production system. However, this could also result in a simultaneous decrease in IMF (De Pedro et al. 2007), which would compromise the organoleptic properties of IB meat, including its juiciness, tenderness and flavor (de Vries et al. 2009).

The impact of finishing system on the variables analyzed was, in general, less pronounced than the influence of genetic group. Still, some effect of finishing intensively or on pasture could be identified, mostly in slaughter traits and weight of various organs, even though interactions with genetic group were also significant. For example, F1 pigs raised intensively had higher carcass yield than those raised on pasture, but no effect of finishing system was observed in IB pigs. On the other hand, some increase in backfat thickness and an important reduction in *l. thoracis* area were observed in pigs of both genetic groups when finishing was intensive, possibly as a consequence of the higher energy level in the diet (Nieto et al. 2012).

Selection strategies aimed at changing carcass composition and meat properties, that have been adopted by various breeding programs (Knap et al. 2001), and the use of genetic markers in selection decisions could further enhance selection response (Dekkers et al. 2010). Genetic markers associated with the deposition of subcutaneous and intramuscular fat (Ciobanu et al. 2011), as well as with fatty acid profiles (Ramayo-Caldas et al. 2012), have been described for several pig breeds, but there are indications that some of these markers are muscle-specific (Quintanilla et al. 2011).

Taken together, our results indicate that IB is a slow-growing obese breed, and imply that the quality of IB meat and the marginal difference that consumers are willing to pay for its products must compensate for the much lower yield of commercial cuts obtained from IB, in addition to its lower productivity per sow (Daza et al. 2006).

4 Meat Characteristics

The detailed results of the analyses of the effects of genetic group and finishing system on the physicochemical characteristics and sensory properties of meat have been published (Almeida et al. 2018) and are briefly summarized here.

The least squares means for the various physicochemical characteristics of meat are shown in Fig. 2 for combinations of genetic group and finishing system. Meat chemical composition was similar between finishing systems, but differences between genetic groups were large, with IB pigs showing a mean IMF higher by about 8 percentage points relative to F1 pigs. The mean marbling score was higher by nearly 2.2 points in IB pigs, in line with the differences observed in IMF. Marbling also differed among finishing systems, with mean levels higher by about 0.8 points in intensively finished pigs.

Drip loss at 9 d post mortem was lower in IB pigs, with mean differences relative to F1 of about 1.3%, while between finishing systems a higher drip loss was observed in EX relative to IN pigs, with a difference of about 3.3%. The cooking loss was about 20%, with minor differences between genetic groups and finishing systems. After 9 d of ageing, the mean shear force was lower by about 15 N in IB when compared with F1 pigs, and lower by nearly 12 N in EX relative to IN pigs, but no interaction was observed between the two factors. Overall, the lowest mean shear force was observed in IB-EX pigs and the highest in F1-IN. The meat produced by F1 pigs was lighter than that of IB, while differences between finishing systems were minor but showed some interaction with genetic group. The means for redness and yellowness were higher in IB than in F1 pigs, and in EX than in IN.

Generally, an important part of IB meat produced is consumed as high-quality dry-cured products, which are highly priced, especially when they are produced under "dehesa" conditions (Andrés et al. 2000). However, in this production system it may take up to 2 years for pigs to reach the target slaughter weight of 150–160 kg (Lopez-Bote 1998; Mayoral et al. 1999) and crossbreeding and indoor rearing have been adopted as possible alternatives in the last few years (Robina et al. 2013). It is well known that the quality of dry-products is affected by both crossbreeding and finishing system (Cava et al. 2000; Ventanas et al. 2007), but it is also important to investigate how the quality of fresh meat is affected by these factors.

Drip loss reflects water-holding capacity of meat, and in the present study it was higher in meat originating from extensively finished pigs, in line with the results reported in the review by Lebret (2008). On the other hand, meat from crossbred animals had higher drip-loss than meat from IB pigs, possibly as a result of the slightly lower pH observed in meat from F1 pigs (Fischer 2007).

The mean marbling score was more than twice as high in IB when compared with F1 pigs, reflecting the much higher deposition of fat in the former group, such that the mean IMF of meat, pooled across finishing systems, was about 10% in IB and 3% in F1 pigs. The very high IMF found here in IB is a common feature of this breed, which is further enhanced when pigs are slaughtered above 150 kg (Lopez-Bote 1998; Robina et al. 2013). Still, this is a desirable goal in this breed, because IMF enhances the quality of dry-cured products (Fernandez et al. 1999). The strong adipogenic ability of genetically unimproved local breeds is widely known (Labroue et al. 2000; Franci

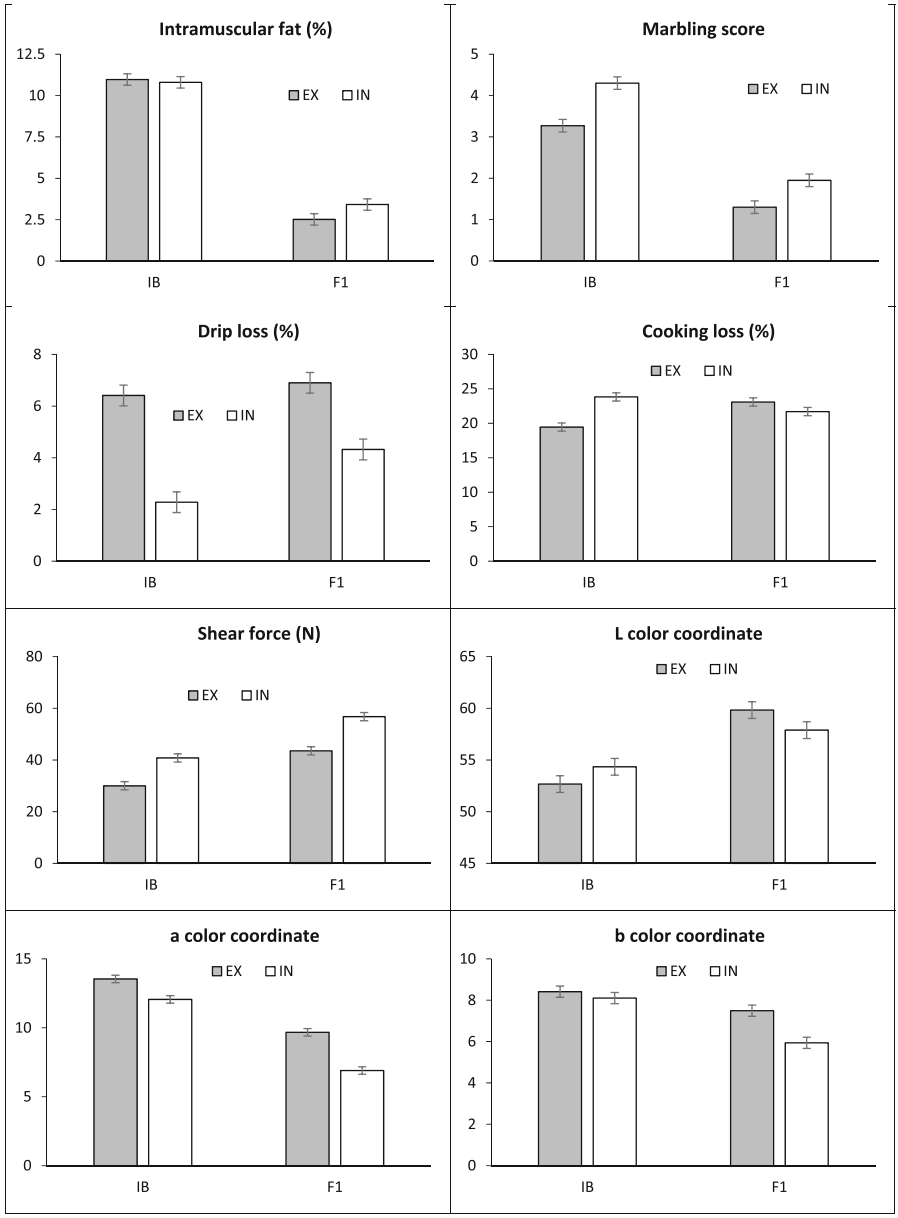

Fig. 2. Least squares means for physicochemical properties of meat in Iberian (IB) and F1 pigs finished extensively (EX) or intensively (IN), with corresponding standard errors.

et al. 2003), while commercial breeds have been strongly selected for leanness over the last decades (Sellier and Rothschild 1991), thus reducing their tendency to deposit fat. Nevertheless, breed differences in IMF strongly depend on slaughter weight, such that when IB pigs are slaughtered at a live weight near 100 kg the amount of IMF tends to be about 3% (Serra et al. 1998; Estévez et al. 2003; Cava et al. 2004), therefore much closer to other breeds, but the difference widens as slaughter weight increases, given the much higher deposition of adipose tissue in IB pigs.

The differences between genetic groups in IMF were very consistent across finishing systems, which did not differ significantly from each other. It could therefore be concluded that both the commercial and the acorn-rich diet provided enough energy for adipogenesis by the IB pig (Rodriguez-Estevez et al. 2012), even though the response in fat deposition by IB pigs may differ according to the muscle considered (Morales et al. 2003). In our study, the remaining chemical components of meat essentially reflected the higher level of IMF in IB pigs, which had lower levels of moisture and protein when compared with F1 pigs.

In the present study, the major differences in instrumental meat color were found between genetic groups, such that meat from IB pigs had a globally more intense color, showing a darker, redder and yellower color at 9 d post mortem. Juárez et al. (2009) evaluated the color of the tenderloin in various strains of IB and in crossbred pigs, and found differences for color parameters between breeds comparable to those in our study and similar findings were reported by Serra et al. (1998) in the *L. lumborum* of IB when compared with Landrace pigs. These authors also found significant differences in histochemical characteristics between breeds, which may justify color differences, with a higher content of oxidative fibers in IB than in Landrace pigs. Overall, in our study the pork obtained from IB had a more desirable color, as an intense color is preferable for consumers as well as for manufacturing dry-cured meat products (Muriel et al. 2004).

In our study, the shear force of meat at 9 d post mortem was considerably lower in IB when compared with meat from F1 pigs, and a similar pattern was observed among finishing systems, with a lower shear force when pigs are extensively finished. Surprisingly, few authors have assessed meat shear force in the *l. thoracis et lumborum* of IB in comparison with commercial pigs. Still, our results for IB are of the same magnitude as those reported by Tejerina et al. (2012) who studied IB pigs slaughtered at 90 kg after intensive or extensive finishing, and found no difference between shear force in the 2 systems. The present study confirms the lower shear force observed in meat from IB in comparison with crossbred pigs, and of meat from animals finished on acorn and pasture relative to those finished intensively. This is a very important result, that could be used to add value to fresh meat obtained from Iberian pigs raised extensively, thus contributing to support the sustainability of this production system (Lopez-Bote 1998).

5 Sensory Attributes

The mean scores for meat sensory attributes indicate that the major differences were observed between genetic groups, with higher scores in all traits consistently attributed to meat produced by IB pigs (Fig. 3). When compared with F1 pigs, meat from IB had mean juiciness higher by about 1.6 points, and meat tenderness higher by about 1 point.

On the other hand, flavor intensity and acceptability were also higher in IB pigs, by about 0.3 and 0.5 points, respectively. Overall, global acceptability was higher in IB when compared with F1 pigs, with a difference of nearly 1 point.

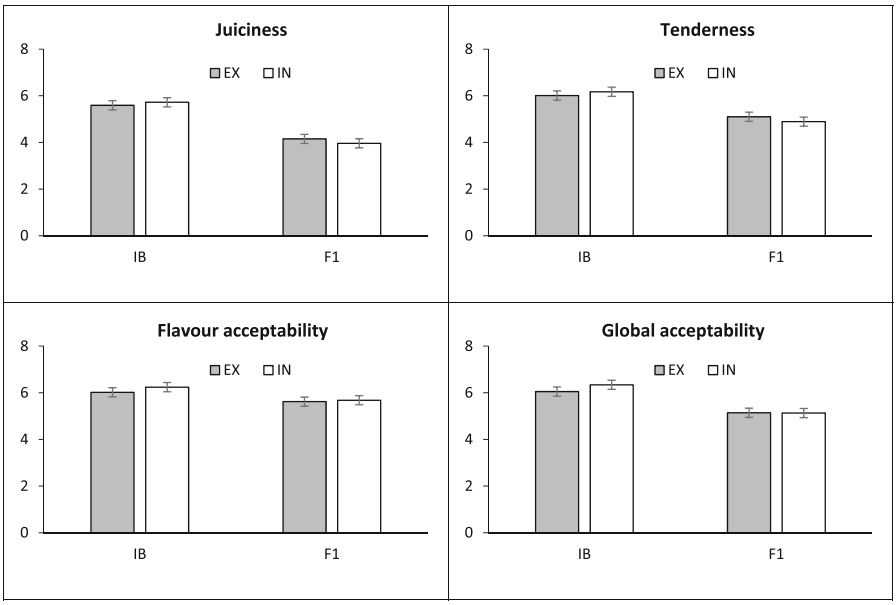

Fig. 3. Least squares means for sensory attributes of meat (score 1 to 8, higher values desirable) in Iberian (IB) and F1 pigs finished extensively (EX) or intensively (IN), with corresponding standard errors.

A stepwise regression analyses of sensory panel variables as a function of physical properties of meat was carried out, and the results indicate that, in the data set analyzed, color characteristics and pH did not have a significant influence on the various sensory attributes considered ($P > 0.10$). The standardized regression coefficients indicate that the more consistent and more influential physical variable relating to all sensory attributes was marbling ($P < 0.05$), such that there was an increase in the score of the various sensory variables analyzed when marbling score increased, as depicted for meat tenderness and global acceptability in Fig. 4. The second more influential physical variable was shear force, which had a negative relationship with juiciness, tenderness, flavor acceptability and global acceptability. Moreover, an increased cooking loss was associated with increased tenderness, flavor acceptability and global acceptability, while an increased drip loss had a minor impact on global acceptability. Overall, the coefficient of determination of the prediction model ranged between about 0.6 and 0.8, indicating that the few physical characteristics included in the linear model, especially marbling, explained relatively well the variability observed in sensory attributes. Overall, global acceptability of meat increased as marbling and cooking loss increased, and declined with increased shear force and drip loss.

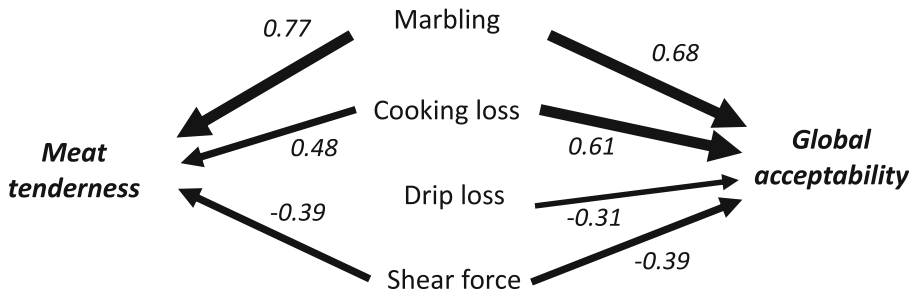

Fig. 4. Path diagram representing the relationships between sensory properties (in italic) and significant physical characteristics of meat. Numbers in italic are the standard partial regression coefficients of the sensory on the physical variables. Arrow width depicts the relative influence of each variable.

The evaluation of tenderness obtained in the taste panel was consistent with the pattern found for instrumental shear force, with a much higher tenderness score in IB pigs, regardless of the finishing system. Indeed, all the sensory traits evaluated received a higher score in IB meat, particularly for juiciness and global acceptability. Differences in flavor intensity and flavor acceptability were smaller, but still in favor of IB relative to F1 pigs, which could be a consequence of the higher IMF in IB pigs, or due to the different fatty acid profiles of IMF in the two genetic groups, with higher amounts of MUFA cis-9 and lower amounts of PUFA n-6 and n-3 in IB pigs (Bressan et al. 2017).

The results of the multiple regression analyses in our study supported that the differences observed between meats originating from the two genetic groups were largely explained by the higher fat deposition in IB pigs, such that a higher level of marbling was positively associated with all the sensory attributes evaluated. On the other hand, a higher shear force (which was observed in F1 pigs) was negatively associated with the scores attributed by the taste panel to juiciness, tenderness, flavor acceptability and global acceptability. The general conclusion was that, from the standpoint of sensory attributes, the most desirable meat should have a high amount of marbling and low shear force, and this corresponded to the profile of IB meat, regardless of the finishing system considered. This result reflects the well-known positive correlation of IMF with tenderness, juiciness and flavor of pork, confirming that marbling contributes to better meat palatability (Savell and Cross 1988). However, the high IMF level in IB pigs may have a negative impact on the nutritional value of their meat, since it contributes negatively to the fat balance in human diets (Schmid 2010).

The differences in sensory attributes between meats originating from the two finishing systems were much smaller than those due to genetic groups, but global acceptability and flavor were slightly higher in intensively finished animals. This is in contrast with dry-cured IB products, where those obtained from extensively finished animals consistently receive higher scores (Muriel et al. 2004; Ventanas et al. 2007). However, the results in the literature are somewhat inconsistent regarding the effect of indoor vs. outdoor rearing on the sensory properties of fresh meat in pigs. For example, Martínez et al. (2012) compared the sensory properties of commercially purchased meat from Iberian pigs (finished on acorns or intensively) and commercial white pigs, in a consumer panel.

In this work, panelists were able to discriminate between meats originating from Iberian and commercial pigs, mostly due to differences in texture and flavor, but the differences between fresh meats from Iberian pigs finished on acorns or intensively were minor. Overall, the results published so far regarding the effect of finishing system on pork sensorial properties are conflicting, and probably reflect the inconsistencies that can be expected from studies that use very different indoors and outdoors finishing systems, which will inevitably have different consequences in terms of meat sensory attributes.

6 Genome-Wide Association Study

The results of GWAS for carcass and meat traits using the Porcine SNP60 v2 Beadchip of Illumina have been published (Amaral et al. 2019) and are briefly summarized here. For the traits with a deflation factor lower than 1.30 and considering the genome-wide significance thresholds defined by Teyssedre et al. (2012), we have identified associations for six traits at the level of the first threshold ($P < 0.000005$) and for 17 traits at the level of the second threshold ($P < 0.00005$). Given the reduced power in detecting associations that results from the reduced sample size, these are expected to represent strong associations, which would otherwise go undetected. A summary of the identified SNPs in GWAS analysis, corresponding map locations and genes involved is presented in Table 1.

In the GWAS of meat moisture, a clear association in SSC13 (SNP rs81442486) was revealed (Table 1). This is an intron variant of gene *DOPEY2* that encodes a protein involved in vesicle mediated transport (Rachidi et al. 2005). The gene interaction network reveals that it shares a protein domain with *DOPEY1*. The pathway enrichment analysis showed enrichment of pathways related with signal transduction, lipid metabolism and muscle contraction.

Regarding heart weight, the GWAS revealed a significant association in SSC13 (Table 1, Fig. 5), where a trend of decreasing *p*-values was observed for several SNPs within the neighborhood of the significantly associated SNP (rs80826693). This SNP is located within the intron of *DSCAM*, that encodes a cell adhesion molecule (Agarwala et al. 2001). Furthermore, an orphan association with rs81389148 located within the intronic region of *CLEC3A* (cartilage-specific lectin C-type lectin domain family 3 member A) was also identified. The inference of gene interactions involving these two genes shows that they play a pivotal role connecting two hubs, one involving *DSCAM* and another one involving several genes within the same pathway, with *PAK1* and *DCC* being the strongest observed interactions, given the node size and edge weight.

Preliminary analyses of fatty acid profiles (Bressan et al. 2017), allowed the identification of genetic marker associations with the index of desaturase and elongase activity (IDA, estimated by the ratio between 20:5 n-3 and 18:3 n-3). The significant association with the IDA in IMF is among the cases of an orphan SNP which, given the magnitude of the association, should be further investigated. Regarding the IDA, we could identify four SNPs significantly associated, of which two of them are not currently mapped in the newest assembly 11.1 of the pig genome (Table 1). For the remaining two SNPs which are mapped, one is located in an intergenic region and the other is an intronic variant of gene *CRISPLD2* that encodes an immunity and defense protein (Vásárhelyi et al. 2014).

Although in lower number, we have identified well-defined SNP association signals for several traits with a pattern of *p*-value decrease in the SNP neighborhood that should be further studied. Among these, we identified a clear association of *cis*-vaccenic acid (C18:1 *cis*-11) in IMF with two SNPs in SSC2 (Table 1), while in subcutaneous fat the concentration of lauric acid (C12:0) was associated with three SNPs in SSC17 (Table 1).

Our results with IB pigs of the Alentejano strain allowed identifying new genomic regions and candidate genes associated to several traits, some of these related with the levels of fatty acids that occur in higher abundance in Iberian pigs and contribute positively to consumer health. The knowledge of these associations may allow for the inclusion of genetic markers in the selection program of IB pigs, enhancing the fatty acid profile of meat while maintaining the superior quality of its processed products.

Table 1. Summary of the identified SNPs in GWAS analysis, corresponding map locations and genes involved.

Trait	SNP	SSC	Position[a]	Type/Gene
Index of desaturase and elongase activity[a]	ASGA0016911*	NA	NA	NA
	ASGA0013633	3	14,992,005	Intergenic variant
	ASGA0083219*	NA	NA	NA
	MARC0039494	6	3,931,665	Intron variant/*CRISPLD2*
cis-vaccenic acid (C18:1*cis*-11)	ASGA0009136	2	12484742	Intergenic variant
	MARC0092956	2	24914867	Intron variant/*LDLRAD3*
Lauric acid (C12:0)	ASGA0001384	1	16,450,113	Downstream gene variant/*PPIL4*
	H3GA0053054	1	35,736,923	Intron variant/*SOGA3*
	H3GA0006318	2	23,579,817	Intergenic variant
	MARC0042230	6	124,586,073	Intron variant/*ME3*
	ALGA0110315	9	22,669,116	Intergenic variant
	ASGA0073029	16	33,947,925	Intergenic variant
	ASGA0073030	16	33,972,855	Intergenic variant
	DBMA0000204	17	22,940,818	3 prime UTR variant/*FLRT3*
	ALGA0093900	17	24,609,494	Intergenic variant
	ASGA0078243	17	58,350,463	Intergenic variant
	ALGA0104894	18	13,509,407	Intergenic variant
Moisture	ASGA0059923	13	199,927,492	Intron variant/*DOPEY2*
	ALGA0035847	6	86,804,762	Intergenic variant
	ALGA0021130	3	115,062,568	Intergenic variant
	ALGA0089092	16	10,920,705	Intergenic variant
	ASGA0093857	14	14,821,759	Intergenic variant
	ALGA0089125	16	to be included	Intergenic variant

(*continued*)

Table 1. (*continued*)

Trait	SNP	SSC	Position[a]	Type/Gene
	MARC0104064	15	31,211,129	Upstream gene variant/*U4*
	ALGA0093017	17	8,087,662	Intergenic variant
	CASI0005117	3	17,642,214	Intron variant/*PHKG2*
	ASGA0059934	13	200,078,329	Intergenic variant
Heart weight (kg)	DRGA0015908	16	17,220,511	Intergenic variant
	ALGA0034540	6	9,908,473	Intron variant/*CLEC3A*
	ALGA008072	15	42,123,213	Intergenic variant
	MARC0068484	13	203,996,202	Intergenic variant
	ALGA0073954	13	203,697,265	Intron variant/*DSCAM*

*SNPs currently not mapped in Sus scrofa Build 11.1 assembly.
[a]Position in porcine assembly 11.1.

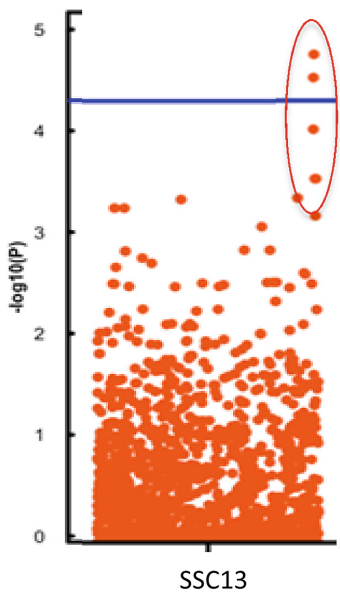

Fig. 5. Example of results of the Genome Wide Association Study for representative traits, indicating a significant association of genetic markers in Chromosome 13 with heart weight. Horizontal blue line represents the threshold of significance.

7 Conclusions

Taken together, our results indicate that IB is a slow-growing obese breed, producing carcasses that have high amounts of fat in the various fat depots studied (subcutaneous,

abdominal, intramuscular and intermuscular). For most traits analyzed, genetic group had a much stronger effect than finishing system, such that IB pigs produced meat with much higher marbling and IMF, more intense darker and redder color, lower shear force, and more desirable sensory properties. The investigation of genetic markers associated with carcass and meat quality allowed the identification of novel candidate genes associated with several traits of importance in animal physiology as well as in carcass and meat quality. Selection strategies aimed at changing carcass composition and meat properties have been adopted by various breeding programs worldwide, and the use of genetic markers in selection decisions could further enhance selection response. The availability of high-density panels of genetic markers opens the possibility of genomic selection in IB pigs, with the goal of modifying the relative importance of accretion of lean and of the various fat depots, thus improving production efficiency without compromising the outstanding quality of IB meat products.

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The Fine Structure of the Cellulosome Defines the Intricacies of Carbohydrate Deconstruction in the Mammalian Gut

P. Bule[1], S. Najmudin[1], J. Brás[1,2], V. Pires[1], V. Fernandes[1,2], A. Sequeira[1,2], K. Cameron[1], A. Leitão[1], J. A. M. Prates[1], L. M. A. Ferreira[1], V. D. Alves[1(✉)], and C. M. G. A. Fontes[1(✉)]

[1] Animal Nutrition and Biotechnology Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
{vdalves,cafontes}@fmv.ulisboa.pt

[2] NZYTech Genes and Enzymes, Estrada do Paço do Lumiar, Campus do Lumiar, Edifício E – 1º Andar, 1649-038 Lisbon, Portugal

Abstract. Protein-protein interactions play a vital role in many cellular processes. One of the most notable examples is the assembly of the cellulosome, a bacterial multi-enzyme complex that efficiently degrades cellulose and hemicellulose. Cellulosome assembly involves the high-affinity binding of type I enzyme-borne dockerins to repeated cohesin modules located on non-catalytic structural proteins termed scaffoldins. The complex is then anchored to the bacterial surface through the interaction of a C-terminal type II dockerin, present on the primary scaffoldin, to cell-bound cohesins. Initially, the architecture and organization of cellulosomes were thought to rely uniquely on these type I and type II cohesin-dockerin interactions but it was recently suggested that cellulosomes from rumen bacteria have developed divergent type III cohesin-dockerin complexes. In this review we will discuss the recent findings suggesting that a different mechanism operates to organize cellulosomes in the gut of mammals.

Keywords: Cellulosome · Protein-protein interactions · Glicoside hydrolases · Carbohydrate interaction · Rumen

1 Introduction

The challenge of replacing hydrocarbons as a primary energy source with cleaner, renewable and sustainable alternatives remains a priority in today's society. In recent years, a significant amount of resources has been applied to investigate the potential use of lignocellulosic biomass conversion to obtain fermentable sugars that could sustain the production of renewable fuels, such as ethanol. Plant cell walls, predominantly composed of cellulose and hemicellulose, are the most abundant source of biologically utilizable carbon on Earth's surface. Photosynthetically fixed carbon is recycled by numerous microbial enzymes that hydrolyse cell wall polysaccharides, therefore playing a crucial

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role in the carbon cycle, while presenting a significant biotechnological potential. The energetic constraints posed by anaerobic ecosystems led to the evolution of a remarkably highly efficient multi-enzyme complex, termed cellulosome, which is attached to the microorganism cell surface and efficiently degrades a variety of plant cell wall polysaccharides. Anaerobic organisms generally have a lower capacity for protein synthesis and thus, the improved efficiency resulting from enzyme assembly, leads to a higher performance in lignocellulosic biomass degradation. This chapter reviews the recent progress on cellulosome research based in both molecular and structural data collected from different enzymatic systems.

2 Hydrolysis of Structural Carbohydrates

Plant cell wall polysaccharides, primarily cellulose and hemicellulose, are a major reservoir of carbon and energy. Furthermore, the deconstruction of these complex macromolecules is of growing environmental and industrial significance as the demand for renewable bioenergy sources and substrates for the chemical industry increase (Himmel and Bayer 2009). However, the chemical and physical complexity of plant cell walls result in an increased resistance to enzymatic degradation and only a restricted number of microorganisms have acquired the ability to deconstruct these structural carbohydrates (Fontes and Gilbert 2010). Not surprisingly, the microbial degradation of plant cell walls is in itself a complex process in which an extensive battery of hydrolytic enzymes attacks an heterogeneous, insoluble and highly recalcitrant substrate. These enzymes are generally classified as Carbohydrate-Active enZYmes (CAZYmes). One example of this complexity is that, even though only a single type of reaction, hydrolysis of β-1,4-glycosidic bonds, is required to convert cellulose into soluble products, degradation of this carbohydrate is hindered by the low solubility of the substrate and the inaccessibility of the glycosidic bonds, especially in the crystalline regions. Thus, the microbial degradation of polysaccharides entails diverse glycoside hydrolases with different specificities and modes of action. The spectrum of enzymes involved in plant cell wall degradation also includes polysaccharide lyases and carbohydrate esterases (Bule *et al*. 2018).

It is now well documented that, at the molecular level, microorganisms can organize their plant cell wall CAZymes in two different systems. In aerobes, enzymes are secreted in copious amounts into the extracellular space and can act freely or associate into the outer membrane. Although these enzymes do not physically associate, they do display extensive biochemical synergy during plant cell wall hydrolysis. In addition, many of these biocatalysts possess a multi-modular structure composed of a catalytic module linked to one or more Carbohydrate-Binding Modules (CBMs), which improve enzyme efficiency by targeting the catalytic module to its target substrate (Fontes and Gilbert 2010). Alternatively, as mentioned above, CAZymes in most anaerobic fibrolytic bacteria and fungi associate into cellulosomes, which are usually anchored to the microorganism surface. The catalytic modules are integrated onto a non-catalytic scaffolding protein (scaffoldin) that may also contain a CBM, thus creating an intimate link between the cell and the substrate (Fontes and Gilbert 2010). This integration is possible due to a strong interaction between the dockerin (Doc) modules, appended to the enzymes, and

the cohesin (Coh) modules present in the scaffoldins. Scaffoldins also contain dockerin modules that they use to interact with other scaffoldins. It is believed that the evolution of these highly efficient nanomachines is a result of the greater selective pressure imposed by the anaerobic environment (Bayer *et al*. 2004).

3 Mammalian Gut Fibrolytic Activity

Grasslands and savannas, covering about 20% of the Earth's landscape, are a major source of nutrients for wild and domestic herbivores. In addition, annual forage crops are often the primary source of nutrients for domestic mammals. To maximize the value of these resources there is a continuing search for methods to improve the digestibility of both grasses and forage crops with the focus of these studies being the plant cell wall. Ruminants make up a significant proportion of the domesticated animal species worldwide and, among farmed livestock, they are the best adapted to utilize the energy of plant cell walls (Hungate 1966). For a long time, it has been recognized that a complex community of fibrolytic microorganisms catalyses the degradation of fibre in the rumen. The three species of ruminal bacteria considered to be primarily responsible for plant cell wall biodegradation are *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens.* These species gain selective advantage in the rumen by optimizing cellulose hydrolysis (depolymerization) and efficient utilization of the hydrolysis products (cellodextrins). Other important species include *Butyrivibrio fibrisolvens,* a highly xylanolytic Gram positive bacterium inhabiting the rumen; *Prevotella* spp. which produce a range of xylanases, and a number of less well characterized cellulolytic bacteria, such as *Eubacterium cellulosolvens.* In addition, anaerobic rumen fungi are considered critical to fibre digestion with *Neocallimastix* sp being one of the best-studied fibrolytic fungi (Hobson and Stewart 1997). Finally, there is also increasing evidence that rumen protozoa may play a role in fibre digestion (Devillard *et al*. 2003).

Rumen bacteria have been the subject of intensive studies over the past 60 years, and numerous studies have described the isolation and characterization of a variety of bacterial strains from various ruminant animals (Hobson and Stewart 1997). Out of the three main fibrolytic rumen bacteria, *F. succinogenes* is often the dominant species, although still representing a very small percentage of the total ruminal bacterial population (~0.1%). *F. succinogenes* activity owes much of its cellulolytic capacity to a strong binding to the surface of plant materials via adhesions which leads to extensive plant cell wall degradation. The fibrolytic enzymes of *F. succinogenes* are amongst the best studied within the rumen bacteria with at least 24 genes encoding endoglucanases and cellodextrinases already identified (Krause *et al*. 2003) and 23 genes presumed to encode xylanases and other enzymes that hydrolyse non-cellulosic polysaccharides. A large range of glycoside hydrolases has also been isolated from several *R. albus* strains. A number of ORFs containing type I dockerins has been identified in *R. albus* supporting recent biochemical and genetic evidence that this species produces a cellulosome-like complex (Ohara, Noguchi, *et al*. 2000; Ohara, Karita, *et al*. 2000). Despite that, only one cohesin sequence has been identified in *R. albus* genome which argues against the existence of an authentic cellulosome in this species. In contrast, *R. flavefaciens* is the only rumen bacterium known to produce a defined cellulosome. With more than 220

dockerin-containing proteins and several cohesins identified in its proteome, *R. flavefaciens* produces one of the most complex CAZyme rich multi-enzyme complexes known to date (Bule *et al*. 2018). *R. flavefaciens* is the second most abundant and probably most efficient ruminal fibrolytic bacterium. Recent genome sequencing analyses revealed that *R. flavefaciens* strain FD1 possesses at least 107 genes encoding glycoside hydrolases (Dassa *et al*. 2014) suggesting that a large majority of the proteins assembled into the cellulosome presently have an unknown function.

4 The Cellulosome

In the early 1980s, the cellulosome complex was first described in the highly cellulolytic thermophilic anaerobe *Clostridium thermocellum* (Bayer *et al*. 1983; Lamed *et al*. 1983). For many years it was known that bacteria and fungi produce many different types of cellulases that function collectively to promote an efficient degradation of cellulose. It started with biochemical studies of the cellulolytic activity of *C. thermocellum* that revealed the involvement of a large extracellular multi-component complex which is organized on the cell surface (Bayer *et al*. 1998). The presence of cellulosomes on the surface of *C. thermocellum* was first visualized by immuno-cytochemical labelling and electron microscopy. The multienzyme complexes were found to be initially located in protuberances of the outermost layer of the cell envelope and to be subsequently released into the culture medium (Bayer and Lamed 1986). After binding to cellulose, the cellulosome-containing protuberances elongate and form filamentous protractions which tether the bacterial cells to their substrate. Since all known sequences of cellulosomal polypeptides begin with a signal peptide, they are believed to be secreted individually through a general secretion pathway, therefore suggesting that cellulosome assembly takes place at the surface of the cells.

All cellulosomes that have been documented appear to have a similar molecular base for organization. They are composed of two main types of building blocks: dockerin-containing enzymes or other types of ancillary proteins, and cohesin-containing structural proteins, which are termed scaffoldins. Cohesins and dockerins are complementary modules that bind tightly to each other (Fontes and Gilbert 2010). The binding specificity of different cohesin-dockerin pairs dictate the organization of the enzymes into the complex as well as its final architecture. Scaffoldins can also contain a dockerin module for binding to other scaffoldins and a CBM for targeting the complex and its enzymes to appropriate sites on the plant cell wall substrate. Cellulosomes can be attached to the bacterial cell surface or can be released as cell-free cellulosomes (Hamberg *et al*. 2014; Xu *et al*. 2016).

C. thermocellum has the most widely studied cellulosome, which has served as the archetypal example of these nanomachines and as a blueprint for cellulosome assembly. *C. thermocellum* cellulosomes are composed of a primary scaffoldin subunit, termed ScaA, which integrates enzymes through its nine highly conserved type I cohesins. These enzymes or cellulosomal catalytic components contain type I dockerin modules, which bind specifically to the cohesin modules located in ScaA through very tight protein: protein interactions. The C-terminal region of ScaA contains a type II dockerin which interacts with a type II cohesin module located in proteins anchored to the bacterial

peptidoglycan layer through an S-layer homology (SLH) module. Thus, type II Coh-Doc interactions tether the entire cellulosome to the bacterial cell surface (Brás *et al*. 2016).

In spite of being structurally related, there is no cross-specificity between type I and type II Coh-Doc modules, ensuring an organized mechanism for cellulosome assembly and cell-surface attachment, respectively. ScaA also contains a family 3 carbohydrate-binding module (CBM3) which interacts with crystalline cellulose and, therefore, plays a key role in targeting the cellulosome to its major substrate, the plant cell wall (Bayer *et al*. 2004). The physical association of proteins in cellulosomes is believed to potentiate the biochemical synergy between enzymes, suggesting that cellulosomes are more efficient at deconstructing plant structural polysaccharides when compared to the "free" enzyme systems produced by aerobic bacteria and fungi. The synergistic effects result from the substrate targeting of the scaffoldin-born CBM, the proximity of the enzymes and also from the elimination of substrate inhibition due to the rapid uptake of released products.

C. thermocellum exhibits one of the highest known growth rates on cellulose. It has been reported that its cellulosome displays a specific activity against cellulose which is 50-fold higher than the CAZYme system secreted by the aerobic fungi *Tricoderma* sp. (Demain *et al*. 2005). The genome sequences of several cellulosome producing bacteria are known and preliminary molecular and biochemical characterisation suggest an adaptation of the various cellulosome systems to the different ecological niches (Fontes and Gilbert 2010). The composition of cellulosomes is dependent on the carbon source and other regulatory factors, and the diverse nature within given cellulosome systems has been investigated by transcriptomic and proteomic studies (Osiro *et al*. 2017; Poudel *et al*. 2017). There is still a lack of information concerning the molecular components of the cellulosomes of other known and unknown organisms (Fontes and Gilbert 2010) and the development of metagenomics will probably identify additional cellulosome-producing bacteria.

Structural biology has recently provided vital information concerning the function of various cellulosomal components and a blueprint for cellulosome assembly. As aforementioned, *C. thermocellum*'s cellulosome serves as the archetype and many three-dimensional components of this system have been determined (Fig. 1). These include several catalytic modules, the type I dockerin module from enzymatic subunits and various scaffoldin borne components, such as type I cohesins, CBMs, X-modules, C-terminal type II dockerin and the type II cohesin module from anchoring scaffoldins (Smith and Bayer 2013). This structural information provides fundamental insights into the individual components of the cellulosome. However, the analysis of individual cellulosomal components limits extrapolations concerning the molecular basis of cellulosome assembly and the arrangement of the nano-blocks within the context of the full length cellulosome.

5 The Cohesin-Dockerin Interaction

The cohesin-dockerin interaction is fundamental for the assembly of cellulosome complexes. This non-covalent protein-protein interaction has been shown to exhibit one of the strongest binding affinities known in Nature, being remarkably difficult to dissociate. Using single-molecule force spectroscopy, the force that is required to break the

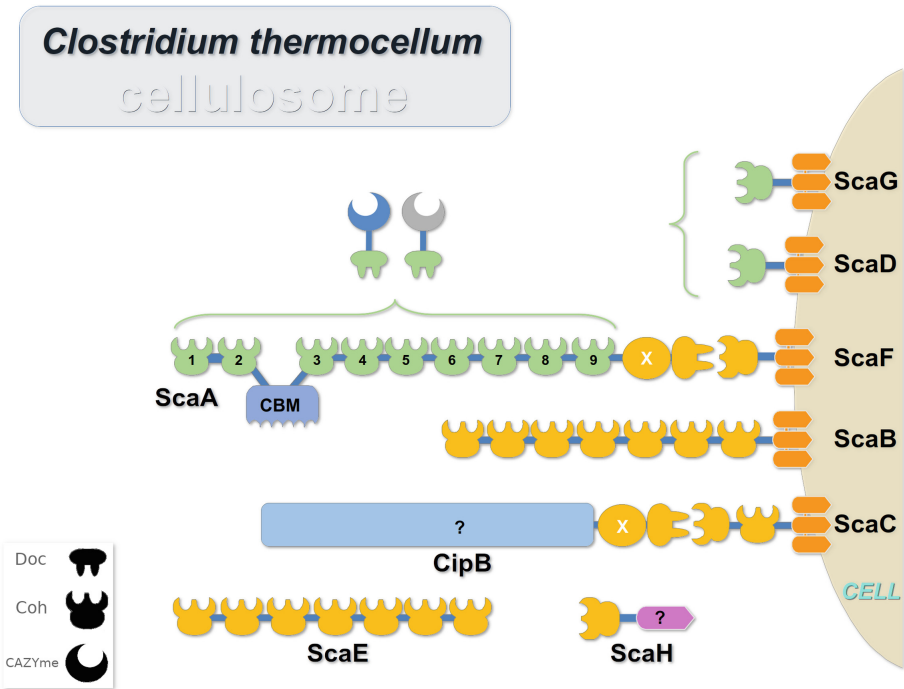

Fig. 1. ***Clostridium thermocellum*** **cellulosome.** The cellulosome architecture is organized around the non-catalytic primary scaffoldin (ScaA) that bears 9 type I cohesin (Coh) modules that congregate the catalytic enzymes via their appended dockerin (Doc) modules. ScaA can also bind the substrate through a carbohydrate-binding module (CBM) and has a C-terminal type II X-Doc that enables the anchoring of the cellulosome to the bacterial cell-wall, via type II cohesins found in several anchoring scaffoldins (ScaB, ScaC and ScaF) or to extracellular scaffoldins (ScaE and ScaH). Cellulosomal enzymes may also adhere directly to the bacterium cell surface by binding to single type I cohesin domains found in ScaD and ScaG. Coh-Doc specificities are color-coded as green for type I and yellow for type II.

interaction between a Coh-Doc pair was estimated to be half of the force required to break a covalent bond (Schoeler *et al*. 2015). Highly complex cellulosomes contain several interconnected components that articulate with each other through these strong interactions. Considering that the cellulosome is both tethered to the cell surface while also binding the substrate through its CBMs, it is likely that it will often be subjected to mechanical stress imparted by opposing forces. Hence, such a strong bond between the cohesin and dockerin modules is required to maintain the complex assembly and stability under adverse environmental conditions.

The organization and structural architecture of cellulosomes are orchestrated by the specificity of the different cohesin and dockerin modules. Based on primary structure analysis, three types of Coh-Doc interactions have been described. Type I interactions occur between dockerin-containing enzymes and cohesins of the primary scaffoldin.

Type II interactions occur between two scaffoldins (usually primary and anchoring scaffoldins). Curiously, *Pseudobacteroides cellulosolvens* is the only known bacterium to have the opposite interaction pattern, with its enzymes containing type II dockerins and the scaffoldins containing type I dockerins (Xu *et al*. 2004). Furthermore, type III interactions are observed in ruminococcal cellulosomes and are distinct from the type I and II interactions from *Clostridium* spp (Bule *et al*. 2018). Dissection of cellulosomal components requires a closer analysis of the cohesin and dockerin modules and their individual properties, as discussed below.

6 Type I Coh-Doc Interactions and Cellulosome Assembly

The first cellulosomal components whose three-dimensional structure was revealed were the type I cohesins from the scaffoldins of *C. thermocellum* and *C. cellulolyticum* (Shimon *et al*. 1997). The type I cohesin module has around 150-residues and is organized in a nine-stranded β-sandwich arranged in a jelly-roll topology with an elongated shape. The two sheets of the sandwich are composed of strands 8, 3, 6, 5 and 9, 1, 2, 7, 4, respectively, with β-strand 9 (C-terminus) and β-strand 1 (N-terminus) running parallel to each other and the remaining running anti-parallel. The nine β-strands are assembled around an extensive aromatic core (Shimon *et al*. 1997; Carvalho *et al*. 2003). All three cohesin types interact with their dockerins through β-strands 5, 6, 3 and 8 of their β-sheets (Adams *et al*. 2006; Carvalho *et al*. 2003).

Dockerins are usually present in a single copy at the C-terminus of cellulosomal enzymes. They consist of approximately 70 amino acids and contain two duplicated segments of around 22 residues, each of which comprises a distinctive Ca^{2+}-binding loop and α-helix. An NMR solution structure of the free *C. ther*mocellum type I dockerin module from cellobiohydrolase Cel48S revealed that the first 12 residues of each duplicated segment resemble the calcium-binding loop of EF-hand motifs, in which the calcium-binding residues (aspartate and asparagine) and calcium coordination patterns are highly conserved (Salamitou *et al*. 1992; Fontes and Gilbert 2010). In this context, calcium dependence for dockerin function was demonstrated experimentally (Choi and Ljungdahl 1996) and both duplicated segments were shown to be involved in cohesin recognition (Fierobe *et al*. 1999). Additionally, the presence of the duplicated segments suggested that the structure of these modules may display a two-fold symmetry. The structure of a type I dockerin from *C. thermocellum* Xyn10B in complex with the second cohesin module of ScaA scaffoldin, obtained by Carvalho *et al*. (2003), confirmed this and provided the first insights into the mechanism by which cellulosomes are assembled (Fig. 2). The dockerin module contains three α-helices, with helices 1 and 3 bearing the key conserved residues previously identified in the first and the second dockerin duplicated segments, respectively. Each duplicated segment displays remarkable structural conservation and also contributes with an F-hand calcium-binding motif. Thus, two calcium ions are present in the dockerin within the two EF-hand loops. The three α-helices present a conformation defined by a loop-helix motif followed by a helix-loop-helix motif, connected by a six-residue segment. By revisiting the Cel48S NMR structure, a long-standing enigma has been recently resolved. It was believed that the dockerin undergoes conformational changes following cohesin binding. However, new

evidence now favours an inherent cohesin-primed conformation of the dockerin without cohesin-induced alterations to its structure (Carvalho *et al*. 2003). The structure of the type I complex illustrates that the cohesin interacts with its dockerin partner primarily along one face of its flattened β-barrel (β-strands 5, 6, 3 and 8). Although the dockerin presents remarkable internal symmetry, the detailed crystal structure of the first Coh-Doc complex revealed that the dockerin prefers binding to the cohesin through its second duplicated segment (helix 3) and only the C-terminal region of the helix 1 contributes to ligand recognition (Carvalho *et al*. 2003, 2007). While hydrophobic forces dominate the Coh-Doc interface, the proteins also interact through hydrogen bonds in which a highly conserved Ser-Thr pair in helix 3 of the dockerin plays a central role in these polar interactions (Carvalho *et al*. 2003; Gilbert 2007).

In a subsequent study, Carvalho *et al*. (2007) revealed that the type I dockerin of *C. thermocellum* can bind to its cohesin partner through two distinct surfaces. By mutating the critical Ser-Thr pair located at the C-terminal helix, the binding was disrupted through this helix and reverse binding through the other helix was observed, thus revealing that the dockerin displays a dual-binding mode. Thus, substitution of the Ser-Thr pair of helix 3 with two alanine amino acids, led to a 180º rotation of the dockerin with respect to the cohesin, with helix 1 assuming the position of helix 3, and the Ser-Thr pair in the first duplicated segment dominating the hydrogen bond network (Carvalho *et al*. 2007). In essence, the equivalent residues in helix 1 of the mutant and helix 3 in the wild-type dockerin interact in the same manner with the cohesin module and so, an almost perfect overlapping of the two alternative binding interfaces was observed (Fig. 3). Karpol *et al*. (2008) performed further truncation and mutation experiments to confirm the symmetry of the Coh-Doc interaction. It was found that the first calcium-binding loop can be deleted entirely, with almost full retention of binding to the cohesin. Likewise, significant deletion of the second repeated segment can be achieved, provided that its calcium-binding loop remains intact. In addition, mutations in one of the calcium-binding loops failed to disrupt cohesin recognition and binding, whereas a single mutation in both loops significantly reduced the affinity (Karpol *et al*. 2008). These results are in accordance to the structural data previously obtained.

Interestingly, data reported by Carvalho *et al*. (2007) revealed that the mutated (C-terminal Ser-Thr replaced by alanine) and wild-type dockerins displayed equivalent affinities for the cohesin binding partner, suggesting that a dual-binding operates in solution and most possibly *in vivo*. Thus, it is believed that the dual-binding mode may be responsible for the introduction of required quaternary flexibility into the multi-enzyme complex and for the enhancement of substrate targeting and synergistic interactions between complementary enzymes, particularly the exo- and endo-acting cellulases (Carvalho *et al*. 2003; Fontes and Gilbert 2010). The stoichiometry of the binding of a variety of type I Coh-Doc complexes is consistently 1:1, which suggests that the two binding interfaces are not able to recognise their ligands simultaneously. Therefore, the dual-binding mode may be responsible for reducing steric constrains that are likely to be imposed by assembling a large number of different catalytic and non-catalytic domains into a single cellulosome.

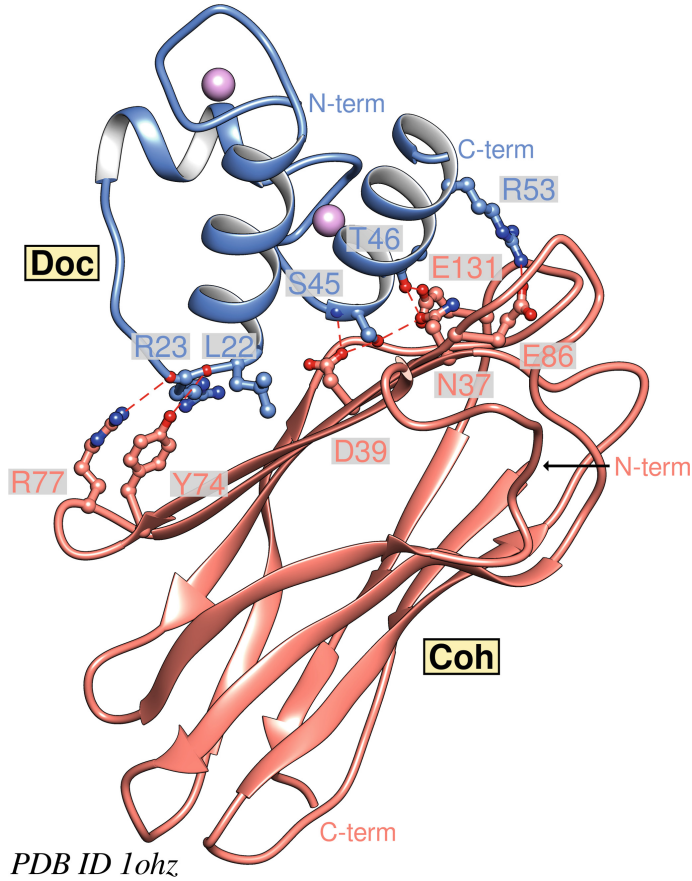

Fig. 2. The structure of the Cohesin-Dockerin complex. Structure of the type I Coh-Doc complex from *Clostridium thermocellum*. The complex is formed between the cohesin module from ScaA (salmon) and the dockerin module from the Xylanase Xyn10B (blue). The residues involved in domain contacts are labelled and shown as ball & stick models, with hydrogen-bonds shown as dashed red lines. The two Ca^{2+}-bound ions of the dockerin domain are represented as purple spheres. The N- and C-terminal ends of each module are indicated. Image prepared with UCSF Chimera (Pettersen *et al*. 2004).

Quaternary flexibility could be further provided by the proline-threonine rich linker sequences that join cohesins within scaffoldins. Indeed, probing cellulosome components by small angle X-ray scattering supports the proposal that the inter-module linkers in free enzymes are extended and flexible. The linker sequences joining the cohesin domains within the *C. thermocellum* scaffoldin are quite long, up to 35 residues, and thus the conformational freedom displayed by the scaffoldin protein may contribute to the synergy displayed by the enzymes within the cellulosomes (Fontes and Gilbert 2010). Additionally, in order to optimize the synergy between specific enzymes, the efficiency of cellulosome function may require, temporarily, the switching of the enzymatic subunits from one cellulosome position to another. Since the Coh-Doc interaction is

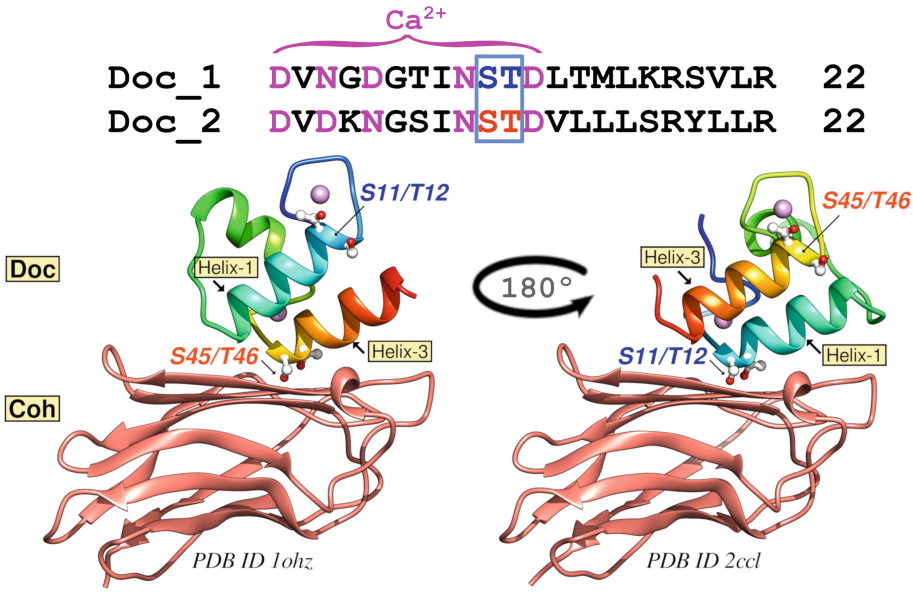

Fig. 3. Cohesin-Dockerin dual-binding mode. The archetype dual-binding mode Coh-Doc complex of CohScaA-DocXyn10B (adapted from PDB ID 1ohz/2ccl) from *Clostridium thermocellum*. A ribbon-representation of the rainbow-coloured Xyn10B dockerin module (blue, N-terminus; red, C-terminus) is bound in two orientations with ScaA cohesin (salmon). At the top, the sequence alignment of the 2 dockerin duplicated segments reveals the residue level conservation that supports the use of both dockerin interfaces. The residues involved in the Ca^{2+} coordination are shown in magenta and the critical Ser/Thr pairs, which interact with the cohesin module, are highlighted within a blue box. Ser-11/Thr-12 (blue label) and Ser-45/Thr-46 (orange label) are depicted on the structures as ball & stick models and coloured accordingly. On the left structure, helix 3 (containing S45/T46) plays a key role in ligand recognition whereas only the C-terminal region of helix 1 (in blue) is involved. On the right, a 180° rotation of the dockerin enforces a helix 1 dominated binding, consolidated around the S11/T12 pair. The Ca^{2+} ions are represented as purple spheres. Image prepared with UCSF Chimera (Pettersen *et al.* 2004).

extremely tight, the existence of a second ligand binding surface in type I dockerins may facilitate the switching of the appended enzymes onto a different cellulosomal cohesin (Fontes and Gilbert 2010).

Miras and colleagues (2002) performed site-directed mutagenesis and thermodynamic studies in different Coh-Doc complexes revealing that substitution of residues 11 and 12 (Ser-Thr pair) at one of the helices of *C. thermocellum* dockerin had no major impact on the Coh-Doc interaction (Miras *et al.* 2002). Therefore, only the substitution of both serine-threonine motifs in helix-1 and helix-3 with bulky amino acids significantly reduces the affinity of the dockerin for its ligand (Schaeffer *et al.* 2002; Carvalho *et al.* 2007; Pinheiro *et al.* 2012). These data are in accordance with the structures obtained for the Coh-Doc complexes. Similar observations in the type I interaction responsible for the binding of *A. cellulolyticus* adaptor scaffoldin ScaB to the anchoring scaffoldin

ScaC were observed: only the mutation of both DocScaB key residue pairs (Ile-Asn) were able to disrupt binding (Cameron *et al*. 2015).

Recent transcriptomic, proteomic, and complementary biochemical and structural studies have shown that type I cohesin modules are not exclusive to *C. thermocellum* cellulosome scaffoldins and the dual-binding mode is not an entirely ubiquitous feature of the type I dockerin. Four established *C. thermocellum* type I dockerin modules, two associated with cellulases (Cel124A and Cel9D-Cel44A) and two others with proteins of unknown function (termed Cthe_0258 and Cthe_0918), contain sequential substitutions that do not allow a dual-binding mode (Pinheiro *et al*. 2009). The type I dockerins from Cel124A and Cthe_0258 specifically bind the type I cohesin module of the anchoring protein ScaG, while the type I dockerin module from Cthe_918 similarly recognized the type I cohesin modules from ScaA and ScaD. The structures of CohScaG-Cel124A and CohScaD-Cthe_0918 revealed that each of these dockerins display a single mode of binding with their cognate cohesin module; each being orientated 180º with respect to the other (Brás *et al*. 2012). Thus, these data suggest that while the dual-binding mode operates in dockerins that bind to the cellulosome, dockerins used to fix the appended enzymes to the bacterial cell-surface seem to display a single-binding mode.

7 Type II Coh-Doc Interactions and Cellulosome Cell-Surface Attachment

Attachment of cellulosomes to the bacterial cell surface is a crucial mechanism for the optimal uptake of nutrients and consequently for the viability of the microbe. In *C. thermocellum*, type II dockerins tether the cellulosome to the peptidoglycan layer of the bacterial cell envelope through high-affinity interactions with type II cohesin modules located in cell-surface proteins ScaF, ScaB, ScaC. They can also bind to the cohesins from the extracellular scaffoldins ScaH and ScaE (Fontes and Gilbert 2010; Brás *et al*. 2016). The first type II cohesin crystal structure to be obtained was the type II cohesin of scaffoldin ScaA from *P. cellulosolvens*, shortly followed by the structure of the type II cohesin from *C. thermocellum's* anchoring protein ScaF (Carvalho *et al*. 2005). With the exception of a few structural elements including the presence of an α-helix, between β-strands 6 and 7, and of two "β-flaps" interrupting β-strands 4 and 8, both structures have the same jelly-roll topology observed in type I cohesins. The sequences of these three secondary elements, as well as the rest of the structural elements, are more conserved between all type II cohesins than between type I cohesins (Carvalho *et al*. 2005). The first crystal structure of the *C. thermocellum* ScaF type II cohesin in complex with the type II ScaA dockerin was obtained by Adams and colleagues (2006). The type II cohesin also displays a typical jellyroll fold. Data indicated that the cohesin does not undergo significant conformational changes upon ligand binding (Adams *et al*. 2006), a feature that is evident in type I cohesins from other microorganisms (Carvalho *et al*. 2005). It was shown that the type II dockerin displays a similar fold to its type I counterpart. However, type II dockerins closely interact with a neighbouring module of unknown function, the previously mentioned X-module, which adopts an immunoglobulin-like fold. Unlike type I dockerins, in which cohesin recognition is dominated by only one of the dockerin helices, it was found that in type II dockerins both helices contact with

the cohesin surface over their entire length. The interaction surfaces are significantly less charged, thus binding is predominantly hydrophobic. There is also an extensive hydrogen-bonding network that involves residues from the X-module, both dockerin helices and the β-strands 8-3-6-5 face of the cohesin module. Furthermore, the type II Coh-Doc complex reveals an intimate hydrophobic interface between the type II dockerin and the Ig-like X-module fold, giving the C-terminal region of the ScaA scaffoldin a rigid and elongated conformation. Besides interacting with the type II dockerin, the ScaA X-module also contributes to the different specificities displayed by the type I and the type II dockerin partners and might even contribute to structural stability and enhanced solubility of cellulosomal components.

More recently, Brás *et al.* (2016) elucidated the unique molecular mechanisms used by anaerobic bacteria for cellulosome cellular attachment. The structure and biochemical analysis of five Coh-Doc complexes revealed that cell surface type II dockerins contain two cohesin-binding interfaces, which can present different or identical specificities (Brás *et al.* 2016). In contrast to the current static model, it was proposed that dockerins utilize multivalent modes of cohesin recognition to recruit cellulosomes to the cell surface, a mechanism that maximises substrate access while facilitating complex assembly. Isothermal titration calorimetry (ITC) assays were performed in order to assess the binding affinity of the type II cohesin-X-dockerin interaction in solution. Titration of the X-dockerin into type II cohesin showed that these proteins bind with a 1:1 stoichiometry. Structural and biochemical data revealing the dual-binding mode, have dramatically affected the way that Coh-Doc interactions are perceived. Dockerins displaying a dual-binding mode contain a near perfect 22-residue repeat, unlike that of dockerins exhibiting a single-binding mode. With this in mind Noach *et al.* (2010) have hypothesised that the type II dockerin of *A. cellulolyticus* may in fact display a dual-binding mode, due to its near identical segment repeat in contrast to the type II dockerin of *C. thermocellum*. This hypothesis was recently confirmed by Brás *et al.* (2016) reporting the structure of the type II complex of *A. cellulolyticus* in both two orientations (Fig. 4).

In conclusion, although cohesin and dockerin modules have been classed by different types, it is becoming more and more apparent that this classification is only relevant in terms of phylogenetic similarities and in fact it could be argued that each of the modules from a given species should be viewed and characterised individually given its intrinsic functional properties. Despite initial evidence that the type I and type II interactions reflect dual-binding and single-binding modes, respectively, it is now clear that the mode of binding is not strictly indicative of the modular type (Nash *et al.* 2016). It is rather the conserved or divergent nature of the recognition residues in the two repeated segments that determines the binding mode of a given dockerin.

8 Ruminococcal Single-Binding Mode Type III Coh-Doc Interactions

The cohesin, dockerin and X-modules of *Ruminococcae* cellulosomal components were found to be divergent in sequence from previously known type I and type II cellulosomal modules, and their Coh-Doc interactions were therefore collectively designated

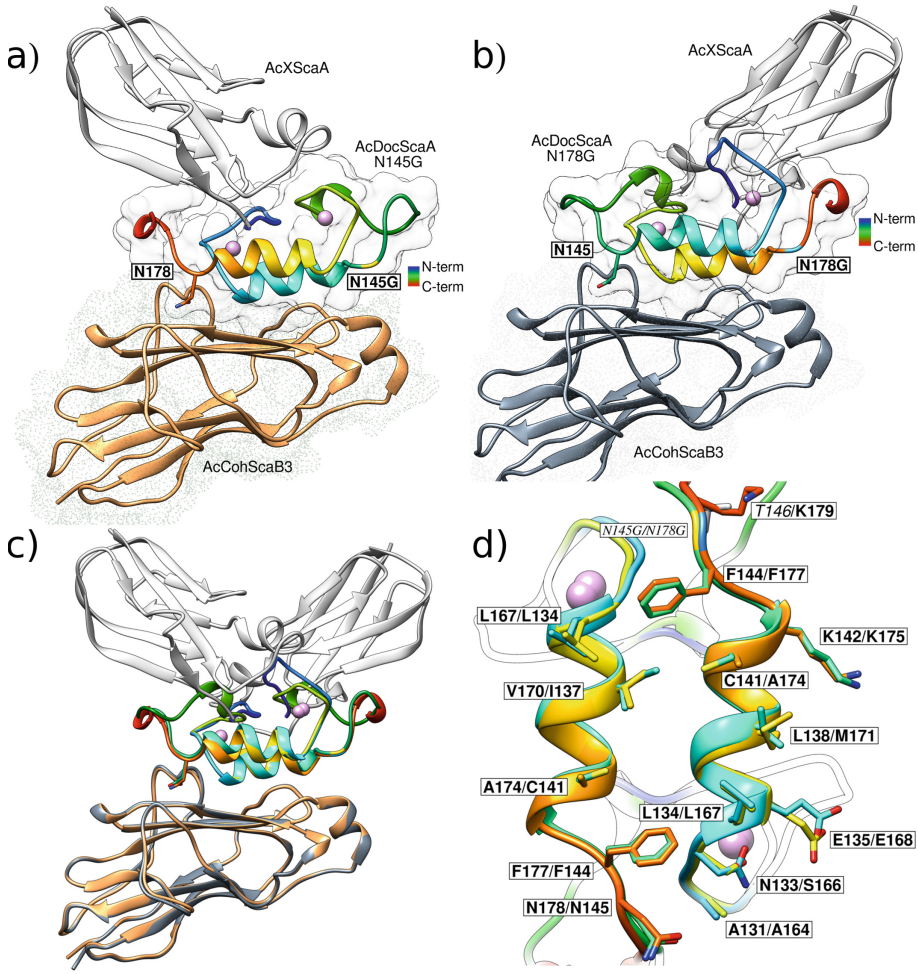

Fig. 4. The type II Cohesin-Dockerin interaction. The structures of CohScaB3-XDocSca type II Coh-Doc complexes from *Acetivibrio cellulolyticus* are shown in both orientations in Panels (a) (PDB ID 4u3s) and (b) (PDB ID 4wi0). The dockerin structure is rainbow coloured, calcium ions are modelled as purple coloured spheres and the cohesin surface is depicted as dots, whereas the dockerin surface is solid white. Relevant residues, Asn145/Asn178 and engineered mutations into Gly (N145G/N178G), are shown in stick representation or a contrasting yellow ribbon zone, respectively. The X module structure is shown as a white ribbon above the dockerin. The structures of the two complexes were overlayed in Panel (c). Panel (d) depicts the dockerin platform that interacts with ScaB3 cohesin, showing the important cohesin contact residues, displayed in stick representation, and revealing the almost perfect two-fold symmetry that supports a dual-binding mode Coh-XDoc interaction in *A. cellulolyticus.* Image prepared with UCSF Chimera (Pettersen *et al.* 2004).

type III based on their respective phylogenetic trees (Fontes and Gilbert 2010). There are currently two ruminococcal species known to produce a well-defined cellulosome: *R. flavefaciens*, found in the rumen of several herbivores, and the recently identified *Ruminococcus champanellensis*, a cellulolytic bacterium found in the human gut (Moraïs *et al*. 2016). In both species, the gene clusters that code the main cellulosomal components have a similar gene arrangement, which indeed translates into their cellulosomes having a very similar complex organization that relies on multiple different Coh-Doc specificities. Extensive structural work on these type III complexes have revealed that, although there are some structural elements that diverge between complexes, the binding interface is somewhat similar in all of them. Different specificities are the result of subtle differences in key contacting residues that allow the cellulosome to keep an organized conformation (Bule *et al*. 2016).

Besides introducing a new type of Coh-Doc complexes, these multi-enzyme complexes challenge the notion that the dual-binding mode is widespread across all cellulosomes. Evidence suggests that the vast majority of ruminococcal dockerins lack the internal two-fold symmetry, previously observed in most cellulosomal dockerins, required to support a dual-binding mode (Fig. 5). This means that both the assembly and attachment to the bacterial wall occurs via single-binding mode Coh-Doc interactions which is a rather striking observation as the dual-binding mode was believed to universally improve the flexibility of highly populated cellulosomal systems. Interestingly and in contradiction, in *R. flavefaciens*, some dockerins lacking the characteristic upstream X-module, can interact with the ScaE anchoring scaffoldin (located at the cell surface) in two different orientations. One of such dockerins belongs to the adaptor scaffoldin ScaH. Considering that the dockerin from primary scaffoldin ScaB can interact with the single cohesin of ScaH, it is likely that the dual-binding mode can be incorporated into the attachment of *R. flavefaciens* cellulosome to the cell wall via ScaH, adding flexibility to its structure.

9 Cellulosome Diversity

When cellulosomes were discovered it was initially thought that cellulosome-producing bacteria would be prevalent in Nature. However, it has become increasingly apparent that cellulosomes are specialized and rare, although essential for degradation of recalcitrant polysaccharides derived from plant cell walls in lignocellulosic ecosystems. Cellulosome-producing organisms inhabit a wide range of ecological niches, from sewage sludge and soil to the gastrointestinal compartments of herbivores and even the human gut. Since these highly elaborate cellulolytic systems were first described, only around 20 species were identified as cellulosome secretors suggesting that the cellulosome is not as prevalent in Nature as it was initially thought (Artzi *et al*. 2017). Nevertheless, with the increased availability of genomic and metagenomic information, cellulosome diversity is expanding and is likely to still increase. It is apparent that scaffoldins have the potential to be exceptionally varied in some features, such as size, structural organization and nature of their modular components (Bule *et al*. 2018).

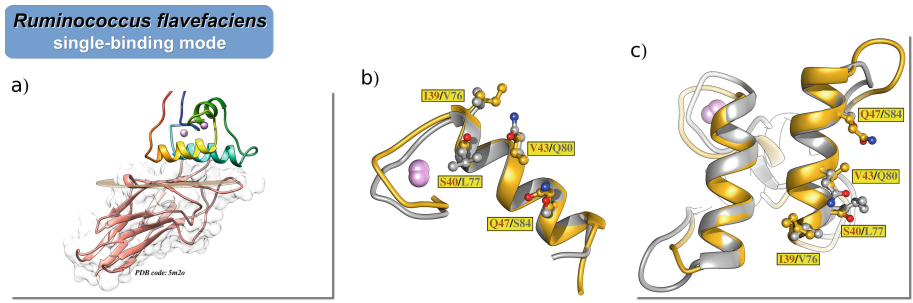

Fig. 5. Cohesin-Dockerin single-binding mode. The Coh-Doc single-binding mode exemplified by the CohScaB-Doc1a complex (PDB ID 5m2o) from *Ruminococcus flavefaciens*. In panel (a) the dockerin structure is rainbow-coloured (blue, N-terminus; red, C- terminus). The cohesin molecular surface contour is depicted in transparent white while a disk marks the plane defined by the cohesin β-strands that form the distinctive dockerin interacting plateau. Panel (b) shows an overlay of the N-terminal and C-terminal dockerin repeats. Panel (c) has a comparison of the two putative binding surfaces by overlaying the dockerin with a version of itself rotated by 180° (in grey). Important residues of the Coh-Doc interface are depicted in a ball & stick representation and labelled. Calcium ions are modelled as purple spheres. Although it is apparent that both dockerin repeats display a notable structural homology with similar main-chain tracing, *R. flavefaciens* dockerin shows a lack of conservation in key contacting residues thus forcing a single Coh-binding platform. The structure artwork was done in UCSF Chimera (Pettersen *et al*. 2004).

The recent near complete sequencing of *Pseudobacteroides cellulosolvens* genome has revealed what is now the most complex cellulosome ever described. With an impressive 212 Doc-containing proteins and 78 cohesins scattered across 31 different scaffoldins, this cellulosome can adopt numerous conformations, including one that allows up to 110 enzymes to be assembled in a single complex (Zhivin *et al*. 2017). It also revealed a new divergent type of cohesins, referred to as group R, that do not resemble any of the other 3 types previously described. Another striking feature is a reversed Coh-Doc arrangement, whereby type II assembles the enzymes into the primary scaffoldin, whereas type I modules mediate cellulosome attachment to an anchoring scaffoldin, as described above. Furthermore, data suggests a dual-binding mode for *P. cellulosolvens* cellulosomal cell anchoring, in opposition to what is observed in *C. thermocellum* (Cameron *et al*. 2015).

The remarkable complexity of cellulosomes becomes even more intriguing when the nature of its components is taken into account. Doc-bearing proteins are, in their vast majority, CAZymes whose catalytic modules can possess diverse specificities. Intriguingly, a large number of cellulosomal proteins display an activity that does not directly relate to polysaccharide breakdown, including serpins, expansins, peptidases, transglutaminases and leucine-rich repeats (Bule *et al*. 2018). The role of these biocatalysts in cellulosome function is still unclear but some studies suggest that they may exert a microbial competition protective role, a regulatory activity or even act in synergy with CAZymes to degrade highly complex substrates (Irving *et al*. 2002; Kang *et al*. 2006; Steenbakkers *et al*. 2008; O Cuív *et al*. 2013).

10 Biotechnological and Potential Applications for Cellulosomes

Structural insights into the specificity displayed by the increasing repertoire of Coh-Doc pairs coupled with the exploration of the dynamic structural features of the scaffoldin subunit are essential for the development of cellulosome based/inspired tools (Smith and Bayer 2013). Artificial multi-enzyme complexes that mimic cellulosomes were proposed over two decades ago (Bayer *et al*. 1994), and have since been produced extensively, both *in vitro* and *in vivo*. Mini-cellulosomes and designer cellulosomes have been used both as tools for the study of cellulosome action and as potential replacements for, or extensions of, native cellulosomes for nanobiotechnological applications, notably for the production of biofuels from cellulosic biomass (Fierobe *et al*. 2005; Morais *et al*. 2012). In this case, naturally evolved nanomachines could be used as a blueprint for the design, construction and exploitation of tailor-made catalytic multi-protein complexes with precise functions (Fontes and Gilbert 2010). The hydrolysis of cellulose remains a major limiting factor for the efficient utilization of lignocellulosic materials. The activities of multiple enzymes, including endoglucanases, exoglucanases, and β-glucosidases, are required to release soluble sugars from cellulose, therefore making the use of cellulosomal enzymes an ideal solution. Cellulosomes integrating fungal and bacterial enzymes from non-aggregating systems, displaying particular promise in biomass saccharification, can be generated to improve hydrolytic activities. To broaden cellulosome diversity and increase substrate degradation, these 'external' enzymes, such as β-glucosidases, lytic polysaccharide monooxygenases (LPMOs) or expansins, have been incorporated into designer cellulosomes (Gefen *et al*. 2012; Arfi *et al*. 2014; Chen *et al*. 2016). This incorporation complemented the cellulosome complex with novel enzymatic activities that generally resulted in an enhancement of overall activity. Additionally, more than a decade ago, it was estimated that the sale of industrial enzymes would reach a market value of approximately 1.6 billion dollars, of which cellulases and associated enzymes represented a significant amount. The potential of cellulosomes for incorporating a vast array of different cellulases and hemicellulases and their association with an extreme habitat variability make them an exceptional tool for the bioenergy field (Karmakar and Ray 2011). The value of the proximity effect in cellulosomes has also been proven transferable to other novel platforms. By drawing inspiration from cellulosome architecture, other structures with an increased number of enzymes in a single complex were designed. These include 12 enzyme and 18 enzyme self-assembled complexes (Mitsuzawa *et al*. 2009; Moraïs *et al*. 2010), cellulases that are covalently bound to nanospheres (Blanchette *et al*. 2012), cellulases that are bound to streptavidin and inorganic particles (Kim *et al*. 2012), and cellulases that are bound to a DNA scaffold (Mori *et al*. 2013).

11 Conclusions

A brief overview of the variety of cellulosomes identified to date demonstrates the sophistication and diversity of structural mechanisms that have evolved to organize these highly intricate multi-protein complexes. It is evident that these systems are exceptionally varied in size, structural organization and nature of their different modular components.

Continued input from structural biology initiatives will aid in the description of new and more accurate cellulosome component structures and will enable deducing functional implications.

The biological significance of the dual-binding mode remains an elusive question. There are hypothesis that range from it being a mechanism against mutation to allowing a better accommodation of the several cellulosomal components by adding some plasticity, thus avoiding steric hindrance. Interestingly, only the ruminococcal systems seem to bypass the need for this mechanism. One could justify this with the presence of monovalent adaptor scaffoldins that distance the enzymes and declutter the core of these relatively small cellulosomes. But considering that these species inhabit tightly temperature regulated gastric compartments while others endure a wide-range of physicochemical conditions, it could be that the dual-binding mode represents an adaptation to the different ecological niches. Unfortunately, there is still a lack of experimental evidence to support any of these theories.

As mentioned above, the potential contained in the Coh-Doc interaction is remarkable. Such a strong and highly specific protein-protein interaction may be explored in several ways: from the development of mini-cellulosomes that can be used in biofuel production, waste management and animal nutrition to the incorporation of cohesins and dockerins in affinity-based systems with applications in research, medical diagnosis or even pharmaceutics. However, to fully harness the Coh-Doc interaction potential, a deep understanding of the mechanisms behind such a unique system is essential, which requires a continuous effort to expand our knowledge on this subject. Fortunately, the advent of automation and high-throughput methodologies suggests a very promising future for cellulosome research.

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Exogenous Enzymes Improve the Nutritive Value of Cereal-Based Diets for Monogastric Animals Through Different Mechanisms

V. Cardoso[1,2], T. Ribeiro[3], V. Fernandes[1,2], C. Guerreiro[4], M. Centeno[1], V. Pires[1], P. Ponte[1,2], A. Goyal[5], S. Najmudin[1], V. D. Alves[1], J. A. M. Prates[1], L. M. A. Ferreira[1], and C. M. G. A. Fontes[1](✉)

[1] Animal Nutrition and Biotechnology Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, 1300-477 Lisbon, Portugal
cafontes@fmv.ulisboa.pt

[2] NZYTech Genes and Enzymes, Estrada Do Paço Do Lumiar, Campus Do Lumiar, Edifício E, 1° Andar, 1649-038 Lisbon, Portugal

[3] Cargill Nutrição Animal, SA, Rua de Adarse, Seção 26, Alverca, 2616-953 Lisbon, Portugal

[4] Poultry Health Services, Howton, Hereford HR2 0BG, UK

[5] Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati 781039, Assam, India

Abstract. It is now well established that exogenous enzymes improve the nutritive value of cereal-based diets for monogastric animals. However, the exact biological mechanisms that underpin an improvement in animal performance and health has only recently started to be clarified. Here, we revise a range of different studies that provide a clear picture for the different mechanisms used by carbohydrate-degrading enzymes to improve the nutritive value of cereal-based diets for poultry and pigs. These findings are valuable contributions to optimize the large-scale use of enzymes in animal nutrition and provide the base for a widening of applications of exogenous enzymes in different areas of the veterinary sciences.

Keywords: Exogenous enzymes · Glucanases · Xylanases · Prebiotics · Nutrition

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1 Introduction

Meat from monogastric animals has an increasing global importance to humans due to its healthier characteristics combined with a lower consumer price, when compared with other meats. Monogastric do not have the endogenous ability to digest plant cell wall polysaccharides. In addition, soluble plant cell wall polysaccharides, such as arabinoxylan and β-glucans found in wheat and barley, respectively, express anti-nutritive properties for simple-stomach animals. It is now well established that the inclusion of exogenous cellulases and hemicellulases in animal diets leads to an improvement in the nutritional process and consequently in animal performance. Today, commercial enzymes used by the feed industry contain a broad range of substrate specificities to ensure a broad spectrum of action. However, in some particular circumstances this may be unnecessary and irrational since removal of the anti-nutritive soluble non-starch polysaccharides (NSPs) may simply result from the action of individual enzymes expressing the correct specificity. Thus, it is clear that the rational use of exogenous enzymes in animal nutrition requires a clear understanding on the molecular mechanisms used by these enzymes to improve the efficiency of the digestive process and thus enhance animal performance. Here we review our recent knowledge on the different mechanisms used by exogenous carbohydrate-degrading enzymes to improve the nutritive value of cereal-based diets for monogastric animals.

2 Carbohydrate-Active Enzymes (CAZymes)

Within the plant cell wall, a diversity of polysaccharides displaying different structures are interconnected to exhibit remarkable recalcitrance and rigidity. It is not surprising that hydrolysis of plant structural carbohydrates involves a correspondent diversity of enzymes displaying different specificities and modes of action (Warren 1996). Generally, Carbohydrate-Active enZymes (CAZymes) are modular proteins containing both catalytic and non-catalytic domains connected by flexible linker sequences (Fig. 1). Non-catalytic modules are predominantly involved in protein-carbohydrate recognition and substrate targeting, and are termed carbohydrate-binding modules (CBMs, described in detail below), or in protein-protein interactions (the most predominant of these being the dockerins involved in the assembling of enzymes in high molecular mass protein complexes termed Cellulosomes). In recent years, many CAZymes have been identified, characterized and their individual modules grouped into multiple families according to their sequence and structural similarities (Henrissat 1991; Henrissat et al. 1998). These protein families are accessible at the constantly updated CAZy database (http://www.cazy.org) (Cantarel et al. 2009). CAZymes of the same family display a common fold, while the catalytic apparatus and mechanism are similarly conserved (Gilbert 2010). Significant sequence similarity (usually over 30%) is a strong sign of folding similarities. Thus, if the three-dimensional structure of one member of a family is known, it is possible to do homology modelling and deduce structural insights for other family members. Consequently, this system of cataloguing CAZymes in families is used to classify protein modules of unknown function, of which the only recognized feature is sequence similarity. When a novel family is created (sequence homology less than

30%), previously released information is reanalysed to take the additional new family into account (Henrissat 1991; Cantarel et al. 2009). However, it should be noted that classification of a protein module within a family does not directly establish a function for an enzyme, since substrate specificity is not conserved among CAZyme families.

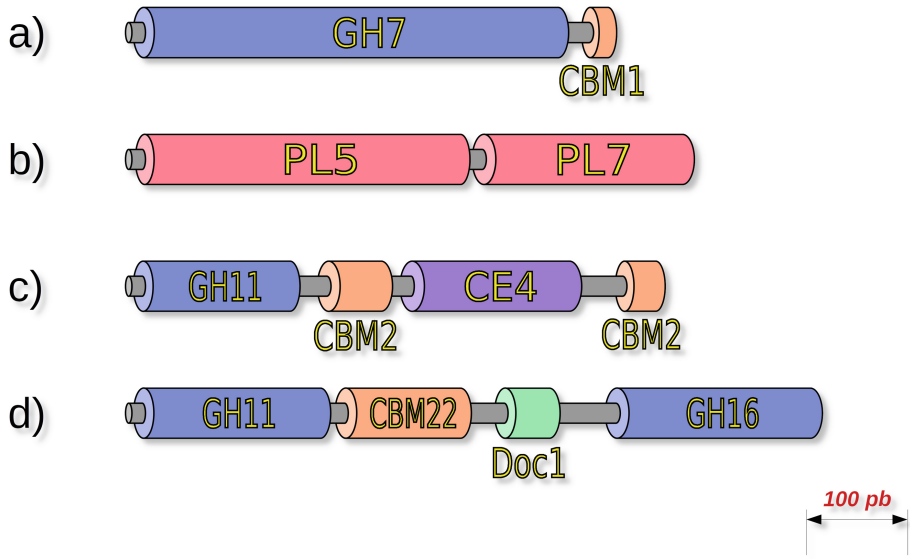

Fig. 1. Modular architecture of Carbohydrate-Active enZymes (CAZymes). Examples of modular CAZymes, composed of various glycoside hydrolase (GH), polysaccharide lyase (PL), carbohydrate esterase (CE), dockerin (Doc) and carbohydrate-binding (CBM) modules from different CAZy families. a) cellobiohydrolase I from Hypocrea jecorina (SP P00725); b) alginate lyase from Sphingomonas sp. A1 (GB BAB03312.1); c) xylanase from Cellulomonas fimi (GB CAA54145.1); d) xylanase D/licheninase from Ruminococcus flavefaciens (GB CAB51934.1). Adapted from (Cantarel et al. 2009).

CAZymes are enzymes which make, break or modify glycosidic bonds and can be classified into four main categories: glycoside hydrolases (GHs), polysaccharide lyases (PLs), carbohydrate esterases (CEs) and glycosyl transferases (GTs). Recently, the CAZy database incorporated a new category, named “auxiliary activities” (AAs), which covers redox enzymes that act in conjunction with CAZymes and groups families of lytic polysaccharide monooxygenases (LPMOs) as well as ligninolytic enzymes. (Levasseur et al. 2013). CAZyme terminology was proposed by (Henrissat et al. 1998) based on the family to which the enzyme belongs and its target substrate. Thus, the first three letters of the enzyme abbreviation identify the substrate, followed by the family number and by an uppercase letter corresponding to the order by which the catalytic domain was reported. For example, a family 5 GH will be named Cel5 or Man5, depending on its substrate (cellulose or mannose respectively) and by Cel5A or Cel5B if there were two catalytic domains with the same specificity but reported at different times. The microorganism abbreviation may also be included before the

enzyme name, in order to differentiate similar enzymes of different origins. For example, the enzyme from Clostridium thermocellum composed of a lichenase family 26 A (first to be discovered) catalytic domain fused to a cellulase of family 5 E (fifth one to be published) will be CtLic26A-Cel5E, written in the conventional sense from the amino- to the carboxyl-terminus of the protein.

Glycoside hydrolases (EC 3.2.1.-) also referred as Glycosidases or Transglycosylases attack either β- or α-glycosidic bonds in di-, oligo- and polysaccharides. The CAZy database categorizes GHs in 156 families (data collected on December 2018). They can be retaining or inverting enzymes, when catalyzing transglycosylation or hydrolysis reactions with retention of configuration at the anomeric center, or when they catalyze hydrolysis reactions with inversion of configuration at the anomeric center, respectively (McCarter and Withers 1994). Glycoside hydrolases also differ in the products they release when acting on a particular substrate. Exo-acting enzymes remove units of one or more sugars from the ends of the polysaccharide chain. Endo-acting enzymes randomly hydrolyse glycosidic bonds within the chains, thereby producing more ends for the exoenzymes to act on (Warren 1996). Exoenzymes and endoenzymes act in synergy, which increases the efficiency of polysaccharide hydrolysis. This is crucial for long linear substrates, such as cellulose, where the numbers of polysaccharide ends for exoenzyme attack are limiting factors. However, the distinction of these two types of enzymes it is not clear, since some exo-acting enzymes have some residual endo-acting activity (Ståhlberg et al. 1993). The distinction can also be reflected by the architecture of the active sites, which fall into three general classes (Fig. 2). Endoglucanases, for example, are commonly characterized by the presence of a groove or cleft into which any part of a cellulose chain can fit. In contrast, exoglucanases bear tunnel-like active sites, which can only accept a substrate chain via its terminus, thus the hydrolysis occurs in a sequential manner, classifying them as "processive enzymes" (Davies and Henrissat 1995). Nevertheless, structural changes can convert endo-acting glycoside hydrolases into exo-acting enzymes (Gilbert 2010). Finally, enzymes acting on the removal of decorations of the polysaccharide backbone contain pockets that recognize sugar side-chains. Glycoside hydrolases exhibit different degrees of substrate specificity; some enzymes have an exclusive target, while others act on different substrates (Warren 1996). Recent observations in the bacterium Clostridium thermocellum suggest an evolutionary adaptation of some GHs to function as polysaccharide binding agents (like carbohydrate-binding modules) rather than enzymatic components, thus serving as extracellular carbohydrate sensors of the microorganism (Bahari et al. 2011).

3 Carbohydrate-Binding Modules (CBMs)

CBMs, made of small sequences that contain from 30 to about 200 amino acids, constitute the majority of non-catalytic modules identified in CAZymes. They constitute auxiliary domains with autonomous folding and display a specific capacity to recognize heterogeneous and complex carbohydrates, thus promoting the association of the enzyme with their target substrates. CBMs can be located at the N- or C-terminal ends of CAZymes, between two catalytic modules, as a single unit or arranged in tandem (Guillén et al. 2010). CBMs can also be found independently from catalytic domains, such as

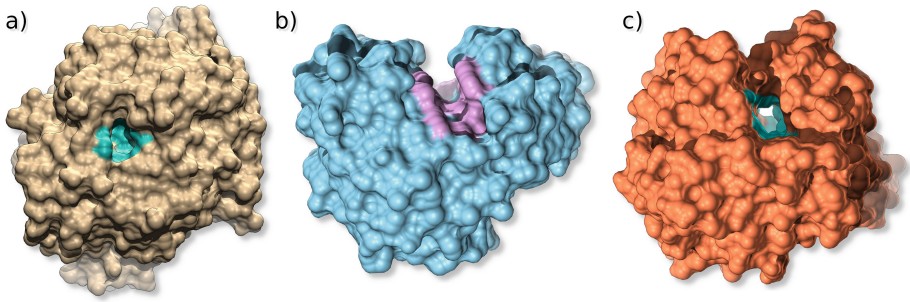

Fig. 2. The three types of active sites found in glycoside hydrolases. The active site is highlighted in a contrasting colour. a) The pocket or crater found in non-processive exo-acting enzymes (GH15 glucoamylase from Aspergillus awamori, PDB code 1DOG); b) The cleft or groove found in endo-acting enzymes (GH6 endoglucanase E2 from Thermobifida fusca, PDB code 2BOD) and c) The tunnel found in processive exo-acting enzymes (GH6 cellobiohydrolase II from Trichoderma reesei, PDB code 3CBH). Image prepared with UCSF Chimera (Pettersen et al. 2004) and adapted from (Davies and Henrissat 1995).

the CBMs located in cellulosomal scaffoldins, such as the CBM3 of C. thermocellum scaffoldin ScaA, which binds strongly to the crystalline cellulose (Bayer et al. 2004).

Initially, CBMs were defined as cellulose-binding domains (CBDs) because they were first described to have a binding capacity to crystalline cellulose (Gilkes et al. 1988). Subsequently, in order to reflect the diverse ligand specificities identified in these modules, the more inclusive term of CBM was proposed (Boraston et al. 1999). Today, the ligand specificity of CBMs has been recognized for a large variety of polysaccharides including crystalline cellulose, non-crystalline cellulose, chitin, β-1,3-glucans, β-1,3–1,4-mixed linkage glucans, xylan, mannan, galactan or starch (Boraston et al. 2004).

As described for CAZymes, CBMs are divided into families based on amino acid sequence similarity on the continuously updated CAZy database (Cantarel et al. 2009). There are currently 84 defined families of CBMs (data collected on December 2018). With respect to nomenclature, the rules employed to describe CBMs are similar to the ones described for CAZymes. At its simplest, a CBM is named by its family but one may also include the organism and even the enzyme from which it is derived. If there are many modules of CBMs belonging to the same family in tandem, a number corresponding to the position of the CBM in the enzyme relative to the N-terminus is included. For example, Clostridium stercorarium contains an enzyme with a triplet of family 6 CBMs, being the first CBM referred as CsCBM6-1, the second as CsCBM6-2 and the third as CsCBM6-3 (Boraston et al. 2004).

CBMs with similar fold are observed to present different ligand specificities (Guillén et al. 2010). The most common fold of CBMs is the β-sandwich followed by the β-trefoil. The β-sandwich fold comprises two β-sheets, each of which consisting of three to six antiparallel β-strands. An example of a β-sandwich conformation is the C. thermocellum CBM11 (Carvalho et al. 2004). In contrast, CBMs with the β-trefoil fold contain 12 β-sheets, forming six hairpin turns. An example of a β-trefoil conformation is the C. thermocellum CBM42 (Ribeiro et al. 2010).

Based on the topology of CBM-ligand binding site, CBMs have been classified into three types: A ("surface binding"); B ("glycan chain binding") and C ("small sugar binding") (Boraston et al. 2004) (Fig. 3). Type A CBMs have a flat or platform-like hydrophobic surface composed of aromatic residues. Due to complementary conformations, the flat Type A binding sites interact with the flat surfaces of crystalline polysaccharides such as cellulose or chitin. The binding-sites of Type B CBMs have a cleft arrangement in which aromatic residues decorating the concave ligand-binding surface interact with free single polysaccharide chains. Aromatic side chains are oriented in such a way that they form twisted or sandwich platforms. The binding site architecture of Type B allows binding to amorphous cellulose or xylan. These CBMs also recognize substrates like β-1,3-glucans, mixed β-1,3–1,4-glucans, β-1,4-mannan, glucomannan, and galactomannan. Type C CBMs or lectin-like CBMs only bind mono-, di-, or trisaccharides due to steric restriction in the binding site (Boraston et al. 2004; Guillén et al. 2010). Recently, some refinements to the classification in Types A, B and C were proposed by (Gilbert et al. 2013) whereby the Type B CBMs are classified as CBMs that bind internally on glycan chains (endo- type) and Type C CBMs are defined as CBMs that bind the termini of glycans chains (exo-type) (Gilbert et al. 2013). The importance of the side chains of aromatic amino acids for carbohydrate recognition, in particular tryptophan but also tyrosine, is well known. They form stacking interactions with sugar rings resulting in strong Van der Waals interactions that stabilize the structure of the protein-carbohydrate complexes (Guillén et al. 2010). Also hydrogen bonds and calcium-mediated co-ordination play a key role in ligand recognition by CBMs (Boraston et al. 2004).

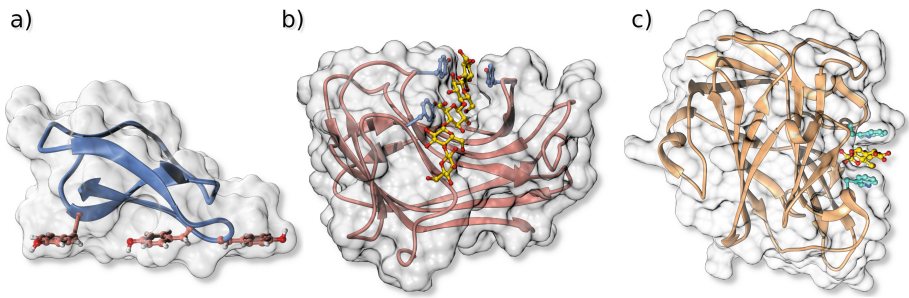

Fig. 3. Structures of the three different CBM types based on topology of carbohydrate binding site. Below the molecular surface, the relevant aromatic residues are shown in a ball & stick representation while the carbohydrate ligands are shown in a yellow ball & stick representation. a) Type A CBM (surface- or platform-like binding site) depicting the aromatic residue-defined hydrophobic flat surface - CBM1 from Trichoderma reesei cellobiohydrolase I (PDB code 1CBH); b) Type B CBM (endo-type glycan chain binding site) showing the aromatic residue-lined cleft binding site for single polysaccharide chains - CBM4 from Cellulomonas fimi endo-β-1,4-glucanase C (PDB code 1GU3) and c) Type C CBM (exo-type glycan chain termini binding site) showing the aromatic residues that sterically restrict the binding-site to mono- di- or trisaccharide "small-sugar" termini - CBM9 from Thermotoga maritime xylanase 10A (PDB code 1I82). Image prepared with UCSF Chimera (Pettersen et al. 2004) and adapted from (Guillén et al. 2010).

It is well established that the main function of CBMs is to recognize and bind specifically to carbohydrates. When appended to catalytic modules, CBMs fulfill the following three different roles to potentiate the efficiency of the associated enzymes: (1) proximity effect (2) targeting function and (3) disruptive function (Boraston et al. 2004). CBMs can increase the concentration of the enzymes on the polysaccharide surface, which improves the proximity between the enzyme and the substrate, thus enhancing catalysis (Bolam et al. 1998). Therefore, it is clear that removal of CBMs from their appended enzymes or from cellulosomal scaffoldins significantly decreases the activity of the associated catalytic modules on insoluble carbohydrates (Bolam et al. 1998; Boraston et al. 2003).

4 Anti-nutritive Properties of Non-starch Polysaccharides (NSPs) in Animal Nutrition

Non-starch polysaccharides are a large variety of structural carbohydrates, comprising some of the most representative compounds of the cell wall (Williams et al. 1997). They can be insoluble or soluble in water. From a nutritional point of view, insoluble NSPs are largely indigestible for simple stomach animals. However, they have the ability to absorb large amounts of water maintaining the normal motility of the gut. Soluble NSPs are more susceptible to biological hydrolysis especially in the last compartments of the birds' gastro-intestinal (GI) tract, such as the caecum but display an anti-nutritive effect for poultry and pigs due to the resulting increase in digesta viscosity. The alteration of digesta viscosity results, predominantly, from the solubilization of carbohydrates presenting a high degree of polymerization. An increase in digesta viscosity causes a reduction in digesta passage rate and hence a reduction in feed consumption. In addition, the lower digesta passage rate and its higher viscosity determines a modification in gut physiology that results in an enlargement of the GI tract (Choct 1997) and the proliferation of a fermentative anaerobic microflora in the upper compartments of the GI tract (Vahouny 1982). This microflora can bind some proteins and form complexes that limit protein hydrolysis (Vahouny et al. 1981). The normal and healthy microflora is composed of facultative anaerobic microorganisms in jejunum and strict anaerobic microorganisms in caecum.

An increase in digesta viscosity has a direct impact in animal performance because feed intake, as well as feed digestibility, decreases. The decrease in digestibility is due to an increase in size and stability of digesta layers without motility in mucosa surface leading to a minor contact between feed and enzymes (Chesson 2001), which leads to a decrease in nutrient availability (Bedford and Morgan 1995). In addition, there is considerable evidence suggesting that both soluble and insoluble NSPs protect nutrient digestion due to molecular encapsulation. NSPs digestion depends on the species and age (the presence of microflora able to digest NSPs increases with animal age), NSPs solubility, chemical structure of the polysaccharide (linkage between sugars determines the fermentation extension of the different carbohydrates) and the amount of NSPs in the diet (anti-nutritive effects of NSPs are related with NSPs concentration in the diet) (Choct et al. 1996).

As described above, there is a negative correlation between the NSPs content in the diet and its nutritive value. Addition of cellulases and hemicellulases into monogastric animals´ diets reduces the degree of polymerization of NSP which then have a lower capacity to affect digest viscosity. Commercially available enzymatic mixtures contain a variety of enzyme specificities which cleave different polysaccharides acting in synergy (Zyla et al., 1999). Exogenous enzymes added to monogastric diets need only to cleave the carbohydrate at a few places in the polysaccharide chain to greatly reduce the viscosity of solutions and thus enhance nutritive value (Williams et al. 1997). This supplementation improves nutrients digestibility and feed intake, increasing animal's performance. The polysaccharide disruption releases and provides nutrients, increasing feed metabolizable energy. To summarize, enzymatic supplementation leads to an improvement of the nutritive value of cereals containing high levels of soluble NSP (Williams et al. 1997).

Most cereals, such as wheat or barley, are rich in NSPs. (Choct and Annison 1990) classified different cereal grains based on their total NSP content from low to high as follows: rice, sorghum, maize, wheat, triticale, rye and barley. Barley and oats are rich in β-glucans which are responsible for the low nutritive value of the diets based on these raw materials. Feed supplementation with β-glucanases or cellulases that cleave β-1,3–1,4 linkages decreases the polymerization degree of β-glucans allowing for a better use of the nutrients released, leading to an increase in intake and a decrease in the feed conversion ratio. In contrast, arabinoxylans are the major contributors to soluble NSP released from wheat, rye and triticale. However, arabinoxylans can vary structurally, showing different branching degrees and different substituted residues. Correspondingly, xylanases can also show remarkably different patterns of activity depending on the structure of the substrate (Chesson 2001). Much of the soluble NSP content from wheat and barley grains derives from the thin walled endosperm cells and reflects the composition of the endosperm wall. Feed enzymes have proved to be most efficacious against soluble substrates for which there is no endogenous competition (Chesson 2001). Low rates of endogenous activity detectable in cereal flour (Cleemput et al. 1997) appear to be constantly present during grain storage and may slowly degrade soluble NSPs. However, thermal processing of feed can destroy endogenous activities, but, on the other hand, promote the release of soluble NSPs (Chesson 2001).

5 Are Exogenous Enzymes Really Required for All Barley-Based Diets?

As described above most cereals contain a significant proportion of soluble non-starch polysaccharides, which are known to display a variety of anti-nutritive properties for monogastric animals, particularly for poultry (Hesselman and Aman 1986). Incorporation of exogenous β-1,3–1,4-glucanases in barley-based and β-1,4-xylanases in wheat or rye-based diets, improves the efficiency of feed utilization, enhances growth and contributes to a better use of these feed ingredients (Chesson 1993; Bedford 2000). However, there are numerous examples where enzyme supplementation had no impact in animal performance, suggesting the existence of factors affecting the efficacy of feed enzymes. We have hypothesized that the levels of endogenous enzymes present in cereals might

affect the efficacy of the exogenous cellulases used to improve the nutritive value of barley-based diets for poultry.

In a study conducted by (Ribeiro et al. 2011), approximately 60 different barley lots were acquired and levels of viscosity, β-glucan and β-glucanase activity were determined to assess the variation in barley composition in relation to those factors. The data revealed that there is a considerable variation in the levels of endogenous β-glucanase activity of the different barley lots, which ranged from more than 1300 U/kg to less than 60 U/kg. Endogenous β-glucanase activity varied much wider than the levels of viscosity and β-glucans. Taken together, these observations suggest that endogenous levels of β-glucanase activity and not exclusively the content in β-glucans may affect the nutritive value of barley.

Data presented above suggest that levels of endogenous β-glucanases in barley may affect the efficacy of exogenous cellulases used to supplement cereal-based diets for poultry. To test this possibility two different barley lots, with higher or lower endogenous β-glucanase activity (designated HA and LA, respectively), were selected for a comparative study aiming at evaluating the capacity of an exogenous cellulase mixture to improve the nutritive value of barley-based diets with different levels of endogenous plant enzymes for broilers. The data presented in Table 1 revealed that the addition of exogenous β-glucanases had no impact in the weight gain of animals receiving the diet containing high levels of endogenous β-glucanase. In contrast, addition of exogenous enzymes to diet containing low levels of endogenous β-glucanase significantly improved bird final body weight, although birds did not reach the final weight of birds receiving the HA diets. There were no variations in feed intake among the four groups (including positive and negative controls) suggesting that barley source and exogenous enzymes lead to different efficiencies of nutrient utilization rather than to an increase/decrease of feed intake.

Table 1. Performance of broilers fed on two different barley-based diets, displaying high (HA) or low (LA) endogenous β-glucanase activity, supplemented (+) or not (-) with a commercial β-glucanase preparation.

	HA-	HA+	LA-	LA+	SEM	P(F)
Body Weight (g)						
0d	48.06	47.72	47.71	48.19	0.248	0.42266
28d	1323.00^{a}	1304.57^{a}	992.65^{c}	1163.93^{b}	30.847	<0.0001
Weight Gain (g)						
0-28d	1274.86^{c}	1256.85^{c}	944.95^{a}	1115.86^{b}	30.922	<0.0001
Feed Intake (g)						
0-28d	1957.88	1888.00	1952.54	1875.27	79.271	0.8307
Feed Conversion Ratio						
0-28d	1.67^{a}	1.66^{a}	2.09^{b}	1.84^{a}	0.068	0.0002

Overall these findings suggest that enzyme supplementation dosage should be modulated by levels of endogenous β-glucanase activity present in cereals, which was demonstrated to be the most important parameter affecting enzymatic supplementation in barley-based diets. Therefore, the best way to rationalize enzyme incorporation in poultry diets may be to analyse barley lots prior to enzyme supplementation, in order to regulate the enzyme cocktail and dosage levels to the presence of the anti-nutritive carbohydrates.

6 Cellulases or β-Glucanases? What Are the Best Enzymes to Improve the Nutritive Value of Barley-Based Diets for Poultry?

In some parts of the world, barley is the most common cereal used in industrial poultry diets. It contains a significant proportion of β-glucans, a linear polysaccharide constituted by mixed linkages of β-1,4- and β-1,3-glycosidic bonds and thus presenting more solubility than cellulose (Xue et al. 2003). The high content of soluble β-1,3–1,4-glucans, categorized as NSPs, limits barley incorporation in diets for monogastric animals, in particular for poultry, due to its anti-nutritive properties related with an increase in digesta viscosity. To counteract the negative effects related with the solubilization of barley β-1,3–1,4-glucans, exogenous enzymes able to cleave mixed linked glucans are used to supplement this type of diet. Due to the different nature of glycoside bonds present in β-glucans, different types of glycoside hydrolases (EC.3.2.1.) are naturally involved in the degradation of this mixed-linked polysaccharide, in particular β-1,3–1,4-glucanases (EC 3.2.1.73) and β-1,4-glucanases (EC 3.2.1.4), also referred as cellulases. The former strictly cleave β-1,3–1,4-glucans, while the latter display a broader substrate specificity as they can degrade both 1,4-β-glucans and β-1,3–1,4-glucans. In barley β-glucan, β-1,4-linkages predominate with a ratio of β-1,3- to β-1,4-linkages of approximately 1:2.5, and can be cleaved by β-1,4-glucanases (Xue et al. 2003). Usually commercial enzyme mixtures used to supplement poultry diets based on barley, display both β-1,3–1,4-glucanases and β-1,4-glucanases. Which GH contributes more to reduce the degree of polymerization of β-glucans remains, however, to be clarified.

A recent study developed by (Fernandes and colleagues 2016) was conducted to compare the capacity of a highly specific β-1,3–1,4-glucanase and a typical endo-acting β-1,4-glucanase to improve the nutritive value of a barley-based diet for broilers. In this study, two Clostridium thermocellum enzymes, the β-1,3–1,4-glucanase 16A (CtGlc16A) and β-1,4-glucanase 8A (CtCel8A), respectively, recombinantly expressed in Escherichia coli, were used to supplement a barley-based diet for broilers. The data revealed that at the end of the trial (35 d), birds fed supplemented diets containing the commercial enzyme mixture and CtGlc16A had similar body weights which were significantly higher than those observed for birds from the negative control (No supplementation, NC) and CtCel8A groups. Differences in body weight result from similar differences in weight gain during the first weeks of age (7–14 and 14–21 days of the trial). Feed intake was not affected by the addition of exogenous enzymes. At the end of the experiment, the feed conversion ratio (FCR) for the birds fed diet supplemented with CtCel8A was similar to that observed for animals from the NC group, and the lowest values were observed for animals from CtGlc16A and positive control (supplemented

with a commercial enzyme mixture, PC) groups. Taken together, the data suggest that CtGlc16A contributes to improve the nutritive value of the barley-based diet for broilers, while CtCel8A is unable to significantly improve broiler performance. Further work revealed that incapacity of a typical cellulase to improve the nutritive value of barley-based diets for broilers is related to the formation of unproductive interactions with feed cellulose rather than with the anti-nutritive β-glucans. In vitro, it was observed that the cellulase has a reduced capacity to degrade β-glucans in the presence of cellulose, while higher levels of β-glucanase activity are revealed by the CtGlc16A in the presence of cellulose (Fig. 4).

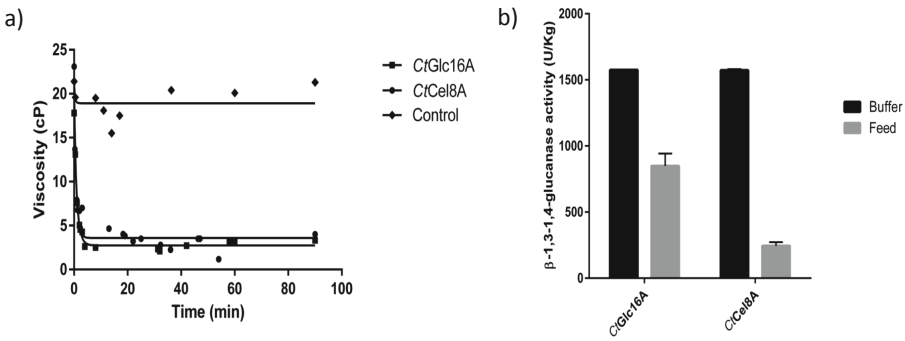

Fig. 4. Effect of CtGlc16A and CtCel8A in the viscosity of a barley β-glucan preparation in vitro (a) and effect of feed in the activity of recombinant enzymes CtGlc16A and CtCel8A (b). a) The two recombinant enzymes were incubated with a barley β-glucan solution (1.5%) and viscosity measured up to 90 min. Viscosity of the same solution was also measured when the polysaccharide was not exposed to the enzymes (Control); b) The activity of the two recombinant enzymes was measured in the absence (buffer) or the presence (feed) of the barley-based feed (Fernandes et al. 2016).

Nowadays, it is a common practice to use enzyme mixtures displaying a large range of polysaccharide specificities to supplement cereal-based diets for monogastric animals. Although further work is required, it is clear that a considerable scope to optimize enzyme mixtures used in animal nutrition may exist. In particular, levels of β-1,3–1,4-glucanases should predominate in relation to the cellulases when there is absolute requirement to degrade β-1,3–1,4-glucans in complex carbohydrate mixtures containing high levels of cellulosic polysaccharides. In addition, the observations suggest that it might be highly relevant to determine the contribution of β-1,4-glucanases to the overall β-1,3–1,4-glucanase activity expressed by current exogenous enzyme mixtures used for the supplementation of barley-based diets for poultry.

7 Role of CBMs in Exogenous Enzymes Used in Animal Nutrition

Countless different studies have widely demonstrated the efficacy of exogenous enzymes in cereal-based diets for pigs and poultry, but very few have focused on the role of carbohydrate-binding modules (CBMs) in feed CAZymes. As previously referred, plant

cell wall hydrolases usually possess a modular structure, with catalytic domains being associated with CBMs, whose main function is to anchor the enzyme to the carbohydrate. Since these CBMs promote an intimate proximity between the enzyme and the substrate, they have the potential to promote a more efficient catalysis. It has been shown that a family 6 xylan-binding domain is able to improve the efficacy of a recombinant xylanase supplementing wheat and rye-based diets for poultry (Fontes et al. 2004). The CBMs' role on the enzyme efficiency in barley-based diets for poultry has been studied by (Guerreiro et al. 2008; Ribeiro et al. 2008).

An initial assay was designed to test the effects of CBM11 from Clostridium thermocellum CtLic26A-Cel5E, a bifunctional enzyme containing β-1,3–1,4-glucanase (GH26) and β-1,4-cellulase (GH5) catalytic domains and two non-catalytic modules, one of them being CBM11, in a barley-based diet for broilers (Guerreiro et al. 2008). Birds were allocated in to four different groups, each provided with a different supplement to their basal feed: no enzyme, 30 U/kg of Lic26-Cel5E, 30 U/kg of Lic26-Cel5E-CBM11 and 15 U/kg of a commercial enzyme. The results showed that the use of enzymes improved birds' performance but that, at these levels of enzyme incorporation, the family 11 CBM of CtLic26A-Cel5E did not improve the biological effectiveness of the associated glycoside hydrolase catalytic modules.

Following these results, another study was conducted, with a lower incorporation rate of CBM11 (Ribeiro et al. 2008). The birds were fed with similar supplements to their diet, the difference being that Lic26-Cel5E and Lic26-Cel5E-CBM11 were incorporated at 10 U/kg, that is, three times less than the concentration used before. This time, the results showed that the β-glucan-binding domain improved the efficacy of the recombinant enzyme containing the CBM11 module ($P < 0.1$). Additionally, since the recombinant cellulase Lic26A-Cel5E-CBM11 functioned as effectively as the commercial enzyme cocktail, the authors suggested that accessory non-glucanase activities, such as xylanase or mannanase, become obsolete in improving the nutritive value of barley-based diets such as the one used in the study. Another interesting result from these trials is that exogenous enzymes added to barley-based diets seem to act mainly in the proximal section of the birds' gastrointestinal tract. Publications such as these are of extreme relevance for the pig and poultry industries, as they provide information about how carbohydrate hydrolases and their associated CBMs work in vivo. The use of exogenous enzymes in monogastric diets will most certainly have an increased significant place in the near future, as an alternative way to improve animals' performance, since antibiotics use as growth promoters has been already banned in several countries.

8 Temporal Restriction of Exogenous Enzyme Supplementation

It is now evident that, in some circumstances, exogenous CAZymes improve animal performance predominantly at the early stages of the production cycle while, in others, the effects are particularly notable in older animals. Presently, it remains difficult to disentangle the impact of exogenous-enzyme supplementation at different animal ages. There are logical reasons why enzyme supplementation should be beneficial to birds, both young and old. In the young chick, the production of endogenous digestive enzymes is scarce and may limit feed digestion (Nitsan et al. 1991; Dunnington and Siegel 1995). Thus, the

addition of exogenous enzymes can enhance the digestive capacity of the young bird by complementing its repertoire of intestinal enzymes. As the bird ages, its digestive ability and microbiota capacity increase, and the microflora route becomes more important in mediating the beneficial effects of exogenous enzymes (Bedford 2000). It is now well recognized that temporal response to enzymatic supplementation may vary among the sources of raw-materials used to prepare monogastric diets. In a series of three different studies our group clarified the mechanisms that coordinate different temporal responses to exogenous-enzyme supplementation in poultry (Figueiredo et al. 2012; Santos et al. 2013; Cardoso et al. 2014).

In wheat-based diets, for example, exogenous enzymes seem to be more important at later phases of the productive cycle (Fontes et al. 2004). In general, viscosity of wheat-based diets is usually not as pronounced as in barley or rye.Thus, in this type of diets, exogenous enzymes are not critical to reduce digesta viscosity, but rather to generate novel substrates, in particular xylo-oligosaccharides, which are used by the beneficial bacteria that colonize the final portions of the GI tract (Jamroz et al. 2002; Fontes et al. 2004). In wheat-based diets, exogenous enzymes influence animal performance through the caecal phase, which predominantly occurs at later stages of the productive cycle when a dynamic caecal microbial population is established. The response to xylanase supplementation tends to be greater in older than younger broilers due to an interaction between exogenous-enzyme products and bacteria (Bedford and Morgan 1996). Figueiredo et al. 2012 confirmed that in broilers fed on low-viscosity wheat-based diets and supplemented at different ages with exogenous xylanases, the response to enzyme supplementation could be restricted to the final stages of animal growth, without compromising animal performance.

In contrast to wheat, in rye-based diets, levels of soluble NSPs particularly arabinoxylans, are remarkably high, motivating high digesta viscosities that impair digestibility and absorption of dietary nutrients and lead to a depression in growth rate (Choct and Annison 1992). Mourão and Pinheiro (2009) have found that the reduction of body weight and increase in FCR with rye-based in comparison to corn-based diets were attenuated with age. In a 35-days trial, these authors found that xylanase supplementation of a rye-based diet had significant effects on FCR only until 21 days of age. Recent studies also confirm that enzyme supplementation to a rye-based diet is particularly effective in the early periods of animal growth (Santos et al. 2013). According to these authors, exogenous xylanases action must be restricted to the first 21 days of the broiler's production cycle without compromising animal performance. This is in contrast with broilers fed on wheat-based diets where exogenous xylanases action must be restricted to the final stages of the bird's growth (21–35 days) as described above (Figueiredo et al. 2012).

Broilers fed on barley-based diets also display an improved performance in response to β-glucanase supplementation, particularly at the early stages of their life (Newman and Newman 1988; Rotter et al. 1989; Nahas and Lefrancois 2001) when the young chick has a poorly developed digestive system. Hence, the production of endogenous digestive enzymes at an early stage of growth is scarce and may hinder feed digestion (Nitsan et al. 1991; Dunnigton and Siegel 1995; Kirjavainen and Gibson 1999). β-glucanases act primarily in small intestines reducing digesta viscosity (Fontes et al. 2004; Ponte

et al. 2008), thus improving digesta passage rate and nutrient digestibility at the lower parts of the intestine (Choct et al. 1999). The performance of broilers fed on barley-based diets supplemented with a β-glucanase enzyme mixture was evaluated in the first 11 and 23 days and during all the 35 days of the production cycle, by (Cardoso et al. 2014). This study suggests that exogenous enzymes act primarily in the earliest period of broilers growth and the enzyme supplementation may be restricted to the first 11 days of the production cycle, without negatively affecting animal performance. Although this activity was not sufficient to reduce digesta viscosity in the upper portions of the GI tract when compared with birds exposed to the exogenous enzymes during the entire period of the experiment, it might have contributed to attenuate the anti-nutritive effects of β-glucans at later stages of animal growth. Taken together these data indicate that understanding the mechanisms by which exogenous enzymes operate in the animal GI tract may lead to considerable optimization in the supplementation programs. In addition, these observations are of significant value to commercial feed manufacturers and poultry producers as they represent important savings to the industry.

9 Novel Insights into the Mechanism of Action of Feed Xylanases

It is now well established that the addition of β-1,4-xylanases to wheat-based diets leads to a significant depolymerization of the indigestible NSPs resulting in improved bird performance. In diets containing a high proportion of soluble NSPs, exogenous enzymes reduce the resulting high digesta viscosity, promoting feed intake and the efficacy of endogenous digestive enzymes, thus leading to an improvement in nutrient digestibility (Bedford and Morgan 1996; Bedford 2000). In low viscosity diets, the action of exogenous enzymes has been attributed to their ability to degrade cereal cell walls, thus enabling enhanced access to cell contents by digestive enzymes (Bedford 2000). However, other subtle mechanisms to explain the action of exogenous enzymes could involve the gut microbiota route (Apajalahti and Bedford 1999; Fontes et al. 2004; Figueiredo et al. 2012). Generation of prebiotic xylo-oligosaccharides (XOS) in the birds' gastro-intestinal (GI) tract could promote the proliferation of a beneficial microbiota when xylanases are added to diets. Hence, in low viscosity wheats, the beneficial effects resulting from inclusion of β-1,4-xylanases could result from the production of XOS rather from the direct reduction of digesta viscosity.

In a recent study (Ribeiro et al. 2018) show that final body weight and feed intake of birds fed the basal diet supplemented with XOS or with a commercial xylanase, was similar and significantly higher than those of birds fed on the non-supplemented diet. These results suggest that the exogenous biocatalysts may mediate their effects through mechanisms that do not involve a decrease in the concentration of the anti-nutritive arabinoxylans or the release on cell-wall trapped nutrients, but rather involves the generation of XOS that are used as prebiotics by gut microbiota. In other words, exogenous xylanases may influence animal performance through the production of XOS rather than by reducing the concentration of soluble, viscous arabinoxylans or by reducing the integrity of cereal cell walls. To confirm this hypothesis, the authors investigated how β-1,4-xylanases and XOS modulate gut microflora populations. Data, presented in Fig. 5, reveal that control samples were characterized by Lactobacillus, Akkermansia,

Clostridiales, Faecalibacterium, among other bacteria, whereas the supplemented samples were characterized by Bifidobacterium, Solirubrobacter and also members of the family Lactobacillaceae and Lachnospiraceae.

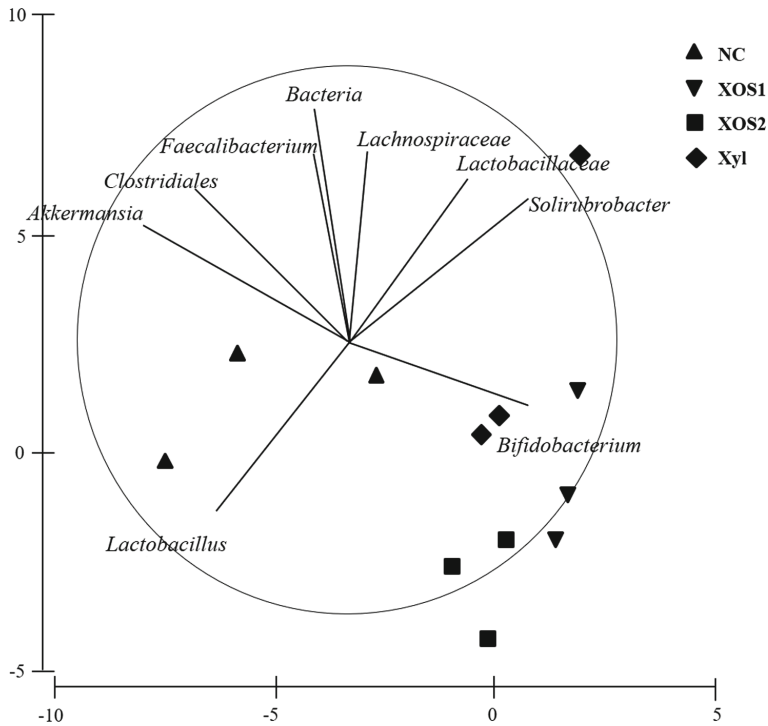

Fig. 5. Principal Component Analysis (PCA) of bacterial microbiota in the caecum of birds fed on a wheat-based diet. Caecum samples were collected in birds fed on a basal wheat-based diet with no supplement (NC), supplemented with a β-1,4-xylanase mixture (XYL), and xylo-oligosaccharides provided at two different incorporation rates, 0.1 g/kg (XOS1) or 1 g/kg (XOS2). Microbiota profiling was evaluated using NGS approach (Ribeiro et al. 2018).

Data presented above suggests that XOS resulting from exogenous xylanase activity on cereal arabinoxylans can modulate the caecal microbiota profile of chickens. To confirm that these effects could translate to other diets (Ribeiro et al. 2018) supplemented a maize-soybean meal diet with XOS. The data confirmed a prebiotic effect of XOS in non-wheat containing diets (Table 2). It was observed that optimum incorporation rates may range between 0.1 and 1 g/kg of XOS although lower doses may also be effective. All these observations suggest that XOS modulate an improvement in animal performance by optimizing feed digestion and feed intake mainly by triggering the evolution of the microbiome to a more favourable construction.

Table 2. Performance of broilers fed on the corn-based diet supplemented with a XOS preparation provided at three different incorporation rates, 0.1 g/kg (XOS1), 1 g/kg (XOS2) or 10 g/kg (XOS3). A fourth group of birds was fed on a basal non-supplemented diet (C-).

	C-	XOS1	XOS2	XOS3	SEM	P(F)
Body Weight (g)						
0d	46,5	46,6	46,1	46,3	0,368	0,777
42d	2084^{b}	2406^{a}	2262^{a}	2136^{b}	56,732	0,003
Weight Gain (g)						
0-42d	2038^{b}	2359^{a}	2216^{a}	2090^{b}	56,696	0,003
Feed Intake (g)						
0-42d	4455^{b}	4576^{ab}	4781^{a}	4299^{b}	102,386	0,023
Feed Conversion						
0-42d	2,19	1,95	2,16	2,07	0,074	0,119

10 Conclusions

In the past, the function of exogenous feed enzymes was explained by their contribution to reduce the digesta viscosity that is associated with the intake of soluble indigestible carbohydrates. In this context, exogenous CAZymes were put in a central place by creating the optimal digestive environment to improve the rate of diffusion of substrates, endogenous digestive enzymes and nutrients (Bedford et al. 1991; Bedford and Classen 1992; Fengler and Marquardt 1988; White et al. 1981). Recent data, reviewed here, provide a more comprehensive and complex role for feed xylanases and cellulases. Firstly, only now we do start to understand why in certain cases the inclusion of exogenous enzymes in diets containing a high percentage of wheat, barley or rye fails to have any effect in animal performance. As revealed above, in some of these cases the expression levels of endogenous plant CAZymes are so high that further supplementation with exogenous enzymes is redundant. In addition, it is clear that defining the most critical activity to target the anti-nutritive feed molecules is critical to maximize feed digestibility. Data presented above revealed that mixed β-1,3–1,4-glucanases, and not the classical β-1,4-glucanases, are effective in reducing the degree of polymerization of the anti-nutritive β-glucans in vivo. Furthermore, we now know that CBMs can also contribute to modulate the activity of feed enzymes leading to a higher targeting effect that could afford a reduction in enzyme loads. Knowing the mechanism of action of feed enzymes also potentiates their rational incorporation in animal diets by targeting the critical periods of animal production when the enzymes are really required, leading to a considerable savings in enzyme incorporation. Finally, we begin to understand that, in some circumstances, the role of exogenous enzymes is related with their involvement in the production of prebiotic oligosaccharides rather than with reducing the prevalence of antinutritive NSPs. All these observations open new avenues to further extend the use of exogenous enzymes in animal nutrition.

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Cistus ladanifer L. in Ruminant Diets – A Sustainable Approach to Improve the Feed Nutritional Value and the Quality of Edible Products

E. Jerónimo[1,2(✉)], M. T. Dentinho[3,4], O. Guerreiro[1,4], A. Francisco[3,4], D. Soldado[1], S. P. Alves[4], J. Santos-Silva[3,4], and R. J. B. Bessa[4]

[1] Centro de Biotecnologia Agrícola E Agroalimentar Do Alentejo (CEBAL)/ Instituto Politécnico de Beja (IPBeja), 7801-908 Beja, Portugal
eliana.jeronimo@cebal.pt
[2] MED – Mediterranean Institute for Agriculture, Environment and Development, CEBAL, 7801-908 Beja, Portugal
[3] Instituto Nacional de Investigação Agrária E Veterinária, Fonte Boa, 2005-048 Vale de Santarém, Santarém, Portugal
[4] Animal Production System Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal

Abstract. Utilization in ruminant diets of *Cistus ladanifer* (a shrub native from Mediterranean region) or its condensed tannin (CT) extracts has been intensively explored in the last years, with a wide range of studies that have provided valuable information on conditions of use, benefits and action mechanisms. Although it is considered as a feed with poor nutritional value, *C. ladanifer* can be used as component or as a source of bioactive compounds for ruminant diets in order to modulate the rumen metabolism and oxidative stability of animal products. *Cistus ladanifer* has been shown to be a good approach to improve the nutritional value of ruminant products, promoting the increase of healthy fatty acids content, which appear to be mainly due to ability of their CT to modulate ruminal biohydrogenation. On the other hand, incorporation of *C. ladanifer* in ruminant diets can also limits the lipid oxidation of meat, even in meat more susceptible to oxidation. Application of *C. ladanifer* CT extracts to silage has been shown to be effective in reducing the proteolysis, improving the silage nutritional value. Moreover, the treatment of dietary protein source with *C. ladanifer* CT extract also reduce the protein rumen degradability, allowing reduce the protein level in ruminant diets without compromise the animal performance.

Keywords: *Cistus ladanifer* · Rumen biohydrogenation · Lamb meat · Fatty acids · Peroxidation · Protein protection

1 Introduction

The improvement of feed efficiency and of quality of edible animal products associated with a sustainable use of resources is a major target in the animal nutrition research

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of the present time. The growing concern on the sustainability of livestock production systems, associated to high costs of the raw-materials, has promoted an intense search for alternative nutritional strategies focused in use of non-conventional feed resources. A great diversity of plant resources that can be used in the ruminant nutrition are available in the Mediterranean region. These include agro industrial by-products and shrubs that besides providing energy and nutrients are also an important source of bioactive compounds to diets and thus can contribute to improvement of the animal health and welfare, products quality, and to environmental sustainability of ruminant production systems. In the last decade, our research team developed an extensive work on a very abundant shrub in the Mediterranean area – *Cistus ladanifer* L. (also known as rockrose, or "Esteva" in Portuguese), from its chemical composition and nutritional value to the application of plant or its extracts in ruminant diets (list of all studies are presented in Table 1).

Table 1. Studies developed by our research team with *Cistus ladanifer* and *Cistus ladanifer* extract

References	Form of *Cistus ladanifer* presentation	Aspects evaluated
Chemical composition and nutritional value		
Dentinho *et al.* (2005)	Leaves and soft stems	proximate composition and *in vitro* digestibility of plants collected in November and January
Guerreiro *et al.* (2015); Guerreiro *et al.* (2016b)	Leaves and soft stems	Seasonal variation and plant age effect on proximate composition, *in vitro* digestibility, fatty acid profile and antioxidant activity
In vivo trials with lambs		
Jerónimo *et al.* (2010); Vasta *et al.* (2010); Jerónimo *et al.* (2012); Van Leeuwen *et al.* (2017)	Leaves and soft stems	growth performance, carcass and meat quality traits, volatile compounds and fatty profile of meat, stable carbon isotope composition of fatty acids; ruminal fermentation and biohydrogenation and lipid oxidative stability
Francisco *et al.* (2015); Francisco *et al.* (2016); Alves *et al.* (2017)	Leaves and soft stems	growth performance, carcass and meat quality, meat fatty acid profile, ruminal biohydrogenation, fatty acid desaturate gene expression and lipid oxidative stability

(*continued*)

Table 1. (*continued*)

References	Form of *Cistus ladanifer* presentation	Aspects evaluated
Francisco *et al*. (2018)	Leaves and soft stems	growth performance, carcass and meat quality traits, meat fatty acid profile, fatty acid desaturate gene expression and lipid oxidative stability
Guerreiro *et al*. (2020); Soldado *et al*. (unpublished data)	Leaves and soft stems	growth performance, carcass and meat quality traits and lipid oxidative stability
Dentinho *et al*. (2018b)	Condensed tannins extract	growth performance, carcass and meat quality traits, and blood biochemical parameters
In vitro and in situ trails		
Dentinho *et al*. (2007)	Condensed tannins extract	rumen degradability and intestinal digestibility
Dentinho *et al*. (2014)	Condensed tannins extract	rumen degradability and intestinal digestibility
Dentinho *et al*. (2018a)	Leaves and soft stems	ruminal degradability and fermentation characteristics and use of polyethylene glycol to prevent the anti-nutritional effects
Dentinho *et al*. (2018c)	Condensed tannins extract	protein degradation during ensiling and rumen degradability
Guerreiro *et al*. (2016a)	Essential oil, Dichloromethane extract, Total Phenols extract, Non-tannin phenolics extract, Condensed tannins extract	ruminal fermentation and biohydrogenation
Costa *et al*. (2017)	Condensed tannins extract	ruminal fermentation and biohydrogenation

Cistus ladanifer L. (Fig. 1), is a perennial and woody shrub native from Mediterranean area that belongs to the Cistaceae family. *Cistus ladanifer* is distributed from southern France to north of Morocco and Algeria, but is particularly abundant in the southwestern region of Iberian Peninsula (Frazão *et al.* 2018), where grows spontaneously occupying large areas in the forest and uncultivated lands. This shrub is highly resistance to drought and is known by its ability to inhibit the growth of other plants, due presence of allelochemicals (Chaves *et al.* 2001), and by its capacity to repopulate areas after forest fires (Ferrandis *et al.* 1999). Such properties, associated with the land abandonment and fire events, have contributed to the great abundance of *C. ladanifer*

in Mediterranean area. Moreover, *C. ladanifer* is highly combustible, causing problems in the prevention and control of forest fires (Dentinho *et al.* 2018c). This shrub produce an oleoresin – the labdanum, which is used in the fragrance industry as fixative and as source of specific aromas (Gomes *et al.* 2005). Moreover, in Portugal other *C. ladanifer* products as essential oil or hydrolate have been applied in the cosmetics and hygiene products. However, in addition to marginal application in the perfume and cosmetics industry, *C. ladanifer* has failed to find other uses. Hence, the development of new applications for *C. ladanifer* that lead to its regular and large-scale use constitutes a major opportunity to its valorisation, but also to its control, which is extremely relevant for the prevention and control of forest fires. Results of our research showed that *C. ladanifer* and its extracts, particularly condensed tannin (CT) extract can be applied in ruminant diets, inducing beneficial effects on feed nutritional value and quality of edible products.

Fig. 1. *Cistus ladanifer* L. morphology: A, flower buds and flower; B, young leaves; C, flower bulbs; D, open seed head with seeds. Pictures by David Soldado.

So, the main objective of this chapter is to review the works developed by our research team on the use of *C. ladanifer* and its CT extract in ruminant diets, particularly in strategies to improve the nutritional value of feeds and the quality of products, being discussed the possible action mechanisms by which them are able to induce such effects and the

impact on animal performance. Moreover, aspects related to *C. ladanifer* morphology, phenology, chemical composition and nutritional value, and biological activities will also be briefly reviewed.

2 Morphology and Phenology

Cistus ladanifer (Fig. 1) is a dicotyledonous perennial shrub, which can reach up to 2.5 m of height, with dense root and shoot systems, exhibiting branches of very rigid and lignified wood covered by a sticky and viscous bark (Frazão *et al.* 2018). *Cistus ladanifer* presents full, opposite, leathery and sessile leaves, which are welded at the base, with visible nerves. The stems and leaves are covered by labdanum gum. *Cistus ladanifer* plants present a large and terminal unique white flower, with a maroon blotch at the base of each petals (Talavera *et al.* 1993). *Cistus ladanifer* seed heads are globular and lignified, with 6–12 valves. Each seed head produces a large number of seeds (aprox. 250 per valve), and total seed production by a single adult plant may be up to 158 000 seeds each year (Demoly and Montserrat 1993; Bastida and Talavera 2002; Guzman and Vargas 2009).

In Fig. 2 is represented the established phenology of *C. ladanifer*, although the time and duration of each stage varies with the year and place of growth. Generally, it is considered that vegetative growth starts after the first autumn rains, when the new plants emerge and the new leaves regrowth in existing plants that developed in previous seasons, after being reduced during the summer dry season (Cabezudo *et al.* 1992; Talavera *et al.* 1993). The flower buds formation begins at the end of winter (lasts between January and May) and flowering extends from February to May, depending the location and the climate conditions (Talavera *et al.* 1993). During seed heads maturation, leaves and bracts fall and the pedicels elongate and lignify. On early summer, the seed heads are mature and are exposed at the ends of the wood pedicels. During summer seed heads begin to open and, by early autumn, seed dispersal occurs following shaking of branches by wind and rain, being the seeds dispersed around the parent plants. The small seed size facilitates the accumulation and penetration on soils and leads to the formation of soil seed bank, which allow the escape from unfavourable conditions and guarantees propagation, after fire, for example (Talavera *et al.* 1993; Frazão *et al.* 2018). These seeds have a crusty and impermeable coat, with significant longevity and germinate after thermal shock. Leaf drop occurs all year, being more accentuated in summer (Cabezudo *et al*. 1992). At the end of summer, when the high temperature decrease and the nocturnal dew formation recommences, the plant axes reinitiate the vegetative growth (Talavera *et al.* 1993).

3 Chemical Composition and Nutritional Value

Studies on chemical composition of *C. ladanifer* has been mainly focused on its essential oil (obtained from the volatile fraction by steam distillation of plants) (Mariotti *et al.* 1997; Gomes *et al.* Gomes et al. 2005; Vieira *et al.* 2017) and its labdanum exudate (obtained by boiling the plant material in water or by organic solvent extraction) (Chaves *et al.* 1993, 1997; Sosa *et al.* 2005; Alías *et al.* 2012). More recently, other *C. ladanifer* extracts, obtained with several solvents, have been studied (Andrade *et al.*

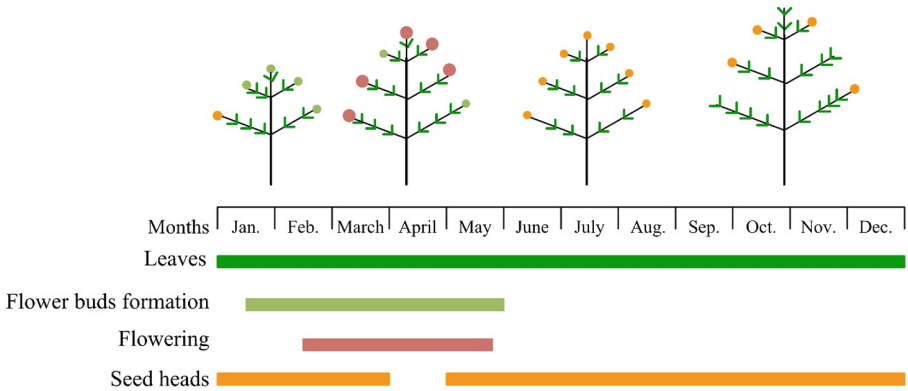

Fig. 2. Phenology of *Cistus ladanifer*, and schematic representation of the branches in successive phenophases along the year. Illustration of the leaves (dark green), flower buds formation (light green), flowering (pink) and seed heads formation and dispersal (yellow). Data from personal observation in field and adapted from Talavera et al. (1993) and Cabezudo et al. (1992).

2009; Barrajon-Catalan *et al.* 2010). The information about proximate composition and nutritional value of *C. ladanifer* plant, as far as we know, is limited to two studies. In the first study the chemical composition and digestibility were analysed in aerial part of *C. ladanifer* collected only in two consecutive seasons (Dentinho *et al.* 2005). In the other study, the seasonal variation of proximate and chemical composition of *C. ladanifer* aerial parts from plants with different ages was evaluated (Guerreiro *et al.* 2016b). Aerial parts of *C. ladanifer* of both ages, are characterized by low contents of crude protein, moderate levels of neutral detergent fibre (NFD), and low organic matter digestibility, being considered as an unbalanced food (Table 2) (Guerreiro *et al.* 2016b). As expected during phenological cycle, the proximate composition of aerial parts of *C. ladanifer* varied, with high crude protein and low NDF contents in early stages of the growth season and reduction of crude protein content and increase in the fibre fraction throughout plant maturation (Guerreiro *et al.* 2016b).

High ether extract content was found in aerial parts of *C. ladanifer* during full year (Table 2) (Guerreiro *et al.* 2016b). However, fatty acid (FA) content of aerial parts of *C. ladanifer* only represent 10% of the ether extract (Table 2) (Guerreiro *et al.* 2015). Fatty acid composition of *C. ladanifer* aerial parts throughout a full year was reported by our team (Guerreiro *et al.* 2015), and results showed that more than 70% of the total FA are saturated fatty acids (SFA), mainly 20:0 and 16:0. Only, three unsaturated FA were detected in *C. ladanifer* aerial parts, the oleic (*c*9-18:1), linoleic (18:2*n*-6) and α-linolenic (18:3*n*-3) acids. Moreover, was identified in aerial parts of *C. ladanifer* two odd mono-methyl branched chain fatty acids (BCFA), the iso-19:0 and iso-21:0, which are rarely found in the lipid fraction of higher plant and were detected for the first time in shrubs. Branched chain fatty acids, which are naturally present in ruminant fats deriving largely from ruminal bacteria (Vlaeminck *et al.* 2006), show interesting biological properties, such as antitumoral activity in human cells and reduction of the incidence of necrotizing enterocolitis (Wongtangtintharn *et al.* 2004; Ran-Ressler *et al.* 2011). So, in addition to other more known bioactive compounds, *Cistus ladanifer* may

Table 2. Chemical and nutritional characterization of *Cistus ladanifer*

Variables	Range
Dry matter (DM) (g/kg)	364–540
Crude protein (g/kg DM)	55–100
Ether extract (g/kg DM)	57–95
Total fatty acids (g/kg DM)	5.0–9.0
Neutral detergent fibre (NDF) (g/kg DM)	318–410
Acid detergent fibre (ADF) (g/kg DM)	232–339
Acid detergent lignin (ADL) (g/kg DM)	70–123
Organic matter digestibility (g/kg DM)	249–315
Total phenols (g GAE/kg DM)[1]	60–106
Condensed tannins (g/kg DM)[2]	32–161

[1]gallic acid equivalents per kg DM
[2]quantified using purified *Cistus ladanifer* CT as standard. Data from Dentinho *et al.* (2005), Guerreiro *et al.* (2015) and Guerreiro *et al.* (2016b).

also be a source of odd BCFA in ruminant diets, with potential benefits for the animal health and nutritional value of products. In our study also was found marked seasonal changes in BCFA and polyunsaturated fatty acids (PUFA), with large increase of the BCFA in summer and autumn while PUFA showed lowest concentration in summer. Such results, suggest that BCFA replaces PUFA in plant lipids in an adaptive response of plant to high environmental temperatures characteristic of the dry season of Mediterranean climate (Guerreiro *et al.* 2015).

Cistus ladanifer leaves and stems also showed high levels of CT (Dentinho *et al.* 2007; Barrajon-Catalan *et al.* 2010; Guerreiro *et al.* 2016b), but their detailed chemical profile was not yet described for *C. ladanifer* plants. Biosynthesis of polyphenolic compounds is linked to environmental conditions and plant growth stage, and throughout the seasons we assisted to variation of the total phenolic and CT contents in aerial parts of *C. ladanifer* collected in south of Portugal, with an increase of its contents during dry seasons (Fig. 3) (Guerreiro *et al.* 2016b). Such variation is probably a response to high environmental temperature and hydric stress that occur during dry seasons, conditions which are associated to high synthesis of polyphenolic compounds, including tannins (Mangan 1988). Moreover, during the growing season more carbohydrates are allocated for plant growth and reproduction than to production of the tannins, which may contribute to lower CT content during vegetative development stage (Skogsmyr and Fagerström 1992). Differences in the total phenolic and CT contents of *C. ladanifer* between plant age was also observed, with higher total phenolic content during spring, summer and winter and higher CT content in summer (more 54 g/kg DM) in aerial part of *C. ladanifer* young plants than in plants with between 2 and 6 growth seasons (Fig. 3) (Guerreiro *et al.* 2016b). Secondary metabolites of plants play an important role in its defence against herbivores, insects and micro-organisms, against other plants competing

for nutrients and light and protection against to UV light (Acamovic and Brooker 2005), and higher levels of polyphenolic compounds as CT in *C. ladanifer* young plants may constitute a defence mechanism that allows their preservation and development (Guerreiro *et al.* 2016b). Condensed tannins are considered as anti-nutritional compounds, and its high levels in *C. ladanifer* contribute to poor nutritional value of this shrub and constitute a major factor limiting of the ruminal digestive utilization of *C. ladanifer* (Dentinho *et al.* 2018a). However our results showed that treatment of *C. ladanifer* with polyethylene glycol (PEG), a polymer that binds irreversibly to tannins preventing the complexation of tannins with dietary molecules as protein, is an effective away to prevent the anti-nutritive effects of tannins, improving the utilization of this shrub by ruminants (Dentinho *et al.* 2018a).

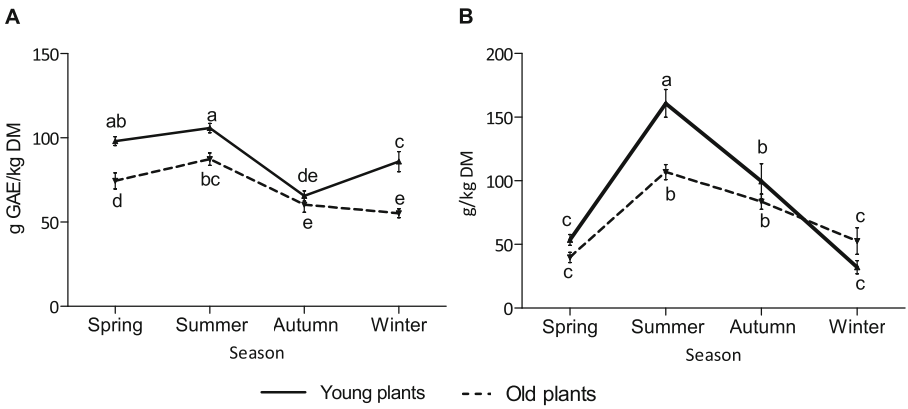

Fig. 3. Effect of season and plant age on total phenolic (A) and condensed tannins (B) contents. Total phenolic content expressed as g gallic acid equivalents/kg dry matter; and condensed tannins quantified using purified *Cistus ladanifer* condensed tannins standard. Data from Guerreiro *et al.* (2016b).

The *C. ladanifer* leaves and stems also present other phenolic compounds, as gallic and ellagic acid and as well as punicalagins isomers, that belongs to ellagitannins family, which are structurally derived from ellagic acid (Barrajon-Catalan *et al.* 2010; Barrajon-Catalan *et al.* 2011; Barros *et al.* 2013).

The most relevant volatile terpenes present on *C. ladanifer* essential oil and exudate extract, are mono-, sesquiterpenes and norisoprenes. Concerning the monoterpenes the major compounds identified are α-pinene, β-pinene (Mariotti *et al.* 1997; Rincon *et al.* 2000; Gomes *et al.* 2005; Teixeira *et al.* 2007; Vieira *et al.* 2017), and camphene (Zidane et al. 2013), and the major sesquiterpene is viridiflorol (Gomes *et al.* 2005; Greche *et al.* 2009). *Cistus ladanifer* also presents high proportions of the carotenoid derivative norisoprene called 2, 2, 6-trimethylcyclohexanone (Mariotti *et al.* 1997; Gomes *et al.* 2005; Teixeira *et al.* 2007; Greche *et al.* 2009; Zidane *et al.* 2013), which is one of the main compound responsible for *C. ladanifer* odour (Ramalho *et al.* 1999) and was not found in other Cistus species (Frazão *et al.* 2018). Diterpenes are rarely reported in *C. ladanifer* essential oil due to its low volatility. However, labdanum is rich in diterpenes

mainly labdane-type ones (Greche *et al.* 2009; Alías *et al.* 2012) as the labdanoic acid. Flavonoids aglycones are another group abundantly present on *C. ladanifer* exudate, represented by apigenin, kaempferol and its methylated derivatives (Chaves *et al.* 1997; Sosa *et al.* 2005).

4 Biological Activities

Cistus ladanifer has been recognized as having diverse biological activities, which was mostly described on *in vitro* studies with essential oil and plant extracts and only a few studies evaluate the plant activities on *in vivo* animals.

Essential oil and extracts from *C. ladanifer*, either aqueous and organic extracts, have demonstrated strong antioxidant activities in a dose-dependent manner, when several free radical scavenging methods were used (Andrade *et al.* 2009; Amensour *et al.* 2010; Guimarães *et al.* 2010; Zidane *et al.* 2013; Guerreiro *et al.* 2016b). These studies demonstrated that the *C. ladanifer* extracts can be used, at low concentrations, as natural antioxidants in the animal nutrition and food industry.

Cistus ladanifer extracts also shown antimicrobial activity against *Bacillus cereus*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Helicobacter pylori*, *Methicillin-resistant Staphylococcus aureus* (MRSA) clinical strains, and *Candida* species (Barrajon-Catalan *et al.* 2010; Ferreira *et al.* 2011; Barros *et al.* 2013; Tomas-Menor *et al.* 2013). The antimicrobial activity of *C. ladanifer* extracts can be influenced by the extraction method employed, as demonstrated by Ferreira *et al.* (2011), which tested several extraction methods and solvents against 14 bacterial strains and 3 yeasts. The results obtained suggest that the different microbial susceptibility observed, can be related with different phenolic and flavonoid compounds extracted by each extraction method (Ferreira *et al.* 2011).

Plant extracts are considered as a potential source of anticarcinogenic compounds (Kaefer and Milner 2008), and aqueous extracts from aerial part of *C. ladanifer* demonstrated to have capacity to inhibit the proliferation of some pancreatic and breast cancer cells (Barrajon-Catalan et al. 2010). The chemopreventive and tumor-inhibitory effects are associated to dietary antioxidant polyphenols which could be associated with their capacity to inhibit oxygen reactive species or free radicals (Halliwell 1996).

Anti-inflammatory and analgesic effects of *C. ladanifer* extracts were evaluated in animal models, using carrageenan-induced rats hind paw edema and hot plate method, respectively (El Youbi *et al.* 2016). These authors demonstrated a significant inhibitory effect of *C. ladanifer* aqueous extract on carrageenan-induced paw edema after the third hour of carrageenan injection, which suggest that the *C. ladanifer* is mainly involved in prostaglandin biosynthesis, that have a major role in the inflammatory mechanism (El Youbi *et al.* 2016). In the same study, *C. ladanifer* showed a dose-dependent analgesic effect in the hot plate model, and the authors suggested that *C. ladanifer* compounds are acting at central nervous system level.

Other study showed a decrease of the levels of blood glucose, total cholesterol, triglycerides, low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) cholesterol and increase of the high-density lipoprotein (HDL) cholesterol in rats treated with *C. ladanifer* extract suggesting that *C. ladanifer* extract can improve lipidemic profile, protecting against coronary heart diseases and diabetes (El Kabbaoui *et al.* 2017).

Cistus ladanifer aqueous extract also showed antihypertensive effect, inducing improvement of the vascular reactivity and the endothelium relaxation on vascular smooth muscle (Belmokhtar *et al.* 2009). Same authors also showed that aqueous extract decreases blood pressure in hypertensive rats, showing that *C. ladanifer* extract acts both in a curative and preventive way (Belmokhtar *et al.* 2009).

5 *Cistus ladanifer* in Ruminant Diets

The aerial parts of *C. ladanifer* can be incorporated in ruminant diets in association with other feeding resources that complement its nutritional imbalances. This might be particularly interesting for small-ruminants that showed lower energy requirements per animal compared to larger animals (Guerreiro *et al.* 2016b). Although, *C. ladanifer* shows better or similar characteristics to other feed resources normally used by ruminants (Guerreiro *et al.* 2016b) and represent an important source of bioactive compounds for ruminant diets, its utilization requires great care. High levels of CT in feedstuffs are of concern, because tannins are generically considered as antinutritive and/or toxic compounds. However, depending on several factors as CT chemical structure and concentration in diets, composition of basal diet, and factors related to the animal like species and physiological stage, the dietary CT might have both adverse and beneficial effects in ruminants (Makkar *et al.* 2007; Waghorn 2008; Piluzza *et al.* 2014). Adverse effects of CT are usually antinutritional, and decrease of the feed intake, digestibility of fibre and nitrogen, and animal performance (Min *et al.* 2003; Waghorn 2008; Piluzza *et al.* 2014). Conversely, tannins may prevent bloat, improve the digestive utilization of feed proteins, reduce the internal parasites, enhance the growth performance, wool growth, and milk production, improve the animal antioxidant status and oxidative stability of products due to its antioxidant properties, and increase the health FA levels in ruminant fat (Min *et al.* 2003; Waghorn 2008; Vasta and Luciano 2011; Piluzza *et al.* 2014; Jerónimo *et al.* 2016).

Our research team performed several *in vitro* and *in vivo* studies (Table 1) to explore the application of aerial part of *C. ladanifer* or its extracts in ruminant diets, particularly as way to enhance the digestive utilization of dietary protein, to protect the protein during ensiling and to improve the FA profile and the oxidative stability of the ruminant products. The possible adverse effects of application of *C. ladanifer* or its CT extract in ruminant diets on animal performance and overall quality of products constitute a major restriction to the practical application of these nutritional strategies, whereby the growth performance and quality of carcass and meat were monitored in all production trials with lambs.

In production trials, aerial parts of *C. ladanifer* (soft stems and leaves) were incorporated at levels ranging between 50 and 250 g/kg DM, in oil unsupplemented and oil-supplemented diets composed by different proportions of forage and concentrate. In addition to the use of aerial parts of *C. ladanifer*, one trial tested also a *C. ladanifer* CT extract. *Cistus ladanifer* and *C. lada*nifer CT extract levels, basal diets, and type and amount of lipid supplementation used in production trials are present in Table 3.

Table 3. Diets used in productive studies with lambs

Basal diet	*Cistus ladanifer* (g/kg DM)	Condensed tannins (g/kg DM)	Type and level (g/kg DM) of oil supplementation	Crude protein level (g/kg DM)	Ether extract level (g/kg DM)	Vitamin E (mg/kg DM)	References
Dehydrated Lucerne: wheat bran (90:10)	0	8.48	0	149	19	–	Vasta *et al.* (2010)
	0	8.17	60 of sunflower and linseed oils (1:2 v/v)	143	69		
	250	21.0	0	150	34		
	250	20.7	60 of sunflower and linseed oils (1:2 v/v)	128	88		
Concentrate: Dehydrated Lucerne (50:45,40 or 30)	50	2.5	0 of soybean and linseed oils (1:2 v/v)	165	23	22.5	Francisco *et al.* (2015)
	50	2.9	40 of soybean and linseed oils (1:2 v/v)	157	63		
	50	2.6	80 of soybean and linseed oils (1:2 v/v)	164	105		
	100	6.5	0 of soybean and linseed oils (1:2 v/v)	162	30		
	100	6.9	40 of soybean and linseed oils (1:2 v/v)	156	70		
	100	7.4	80 of soybean and linseed oils (1:2 v/v)	159	112		
	200	16.3	0 of soybean and linseed oils (1:2 v/v)	165	38		
	200	14.5	40 of soybean and linseed oils (1:2 v/v)	162	78		
	200	16.1	80 of soybean and linseed oils (1:2 v/v)	155	116		
Concentrate (Cereal): Dehydrated Lucerne (50:50 or 35)	0	0.4	60 of soybean oil	171	81	22.5	Francisco *et al.* (2018)
	150	3.5	50 of soybean oil	168	80		
Concentrate (dehydrated citrus pulp): Dehydrated Lucerne (50:50 or 35)	0	0.8	60 of soybean oil	178	78		
	150	5.6	50 of soybean oil	170	80		
Hay: Concentrate (15:85)	–	–	–	160		22.5	Dentinho *et al.* (2018b)
	–	–	–	120			
	–	15	–	120			
Dehydrated Lucerne	0	4.1	60 of soybean oil	162	77	–	Guerreiro *et al.* (2020)
	125	15.3	60 of soybean oil	147	82.7		
	250	26.6	60 of soybean oil	133	98.3		

5.1 *Cistus ladanifer* Condensed Tannin Extract as Additive to Improve the Soybean Meal Protein Efficiency in Lamb Diets

One of the most important effects of CT ingestion by ruminants is associated with their ability to improve the digestive utilization of feed proteins. Condensed tannins bind proteins under the rumen pH conditions (pH 5.5 to 7.0), preventing their excessive microbial degradation. The tannin-protein complexes are dissociated in the acidic pH of the abomasum (pH 2.5 to 3.5) and in alkaline conditions of the distal small intestine (pH > 7.5), releasing protein for digestion, absorption and metabolic processes (Jones and Mangan 1977; Mueller-Harvey 2006). *In vitro* and *in vivo* studies have confirmed the reduction of the effective degradability of protein induced by the presence of CT in diet, mainly due to a marked depression of the initial solubilisation and of the fractional rate of protein degradation, increasing the flux of undegraded dietary protein into the post ruminal compartments, without detrimental effects in the post-ruminal digestion (Min *et al.* 2003; Theodoridou *et al.* 2010).

To explore the use of a CT extract from *C. ladanifer* as additive to protect soybean meal (SBM) protein from degradation in the rumen, Dentinho *et al.* (2014) performed a metabolic trial using rumen-cannulated Merino rams in a 3×3 Latin square design. The rams were fed with diets consisting of 600 g of oat straw, 300 g of manioc and 100 g of SBM/kg DM, which SBM was treated with 0 (control), 15 and 30 g of *C. ladanifer* CT/kg of SBM. It was evaluated the *in situ* rumen degradability and *in vitro* intestinal digestibility (ivID) of SBM protein, the apparent digestibility of the experimental diets, the nitrogen (N) balance, the ruminal fermentation characteristics and the rumen microbial protein supply.

Treatment of SBM with *C. ladanifer* CT extract did not affect the effective degradability of SBM protein at low levels of feeding (maintenance, $k = 0.02\ h^{-1}$). However, at high levels of feeding with faster outflow rates ($k = 0.08\ h^{-1}$), a depression was observed (53% in control vs. 50% in SBM treated with 15 and 30 g of CT). This decrease was mainly due to a strong reduction in initial protein solubilisation (27% lower in SBM treated with 15 and 30 g of CT than in control). The slowly degraded fraction increased and the fractional degradation rate was not affected. The effect of CT in reducing degradability has been attributed to a direct complexation with feed proteins, reducing its solubilisation, to a reduction on the ability of microbes to attach the feed particles and to an inhibition of the microbial growth and activity (Aharoni *et al.* 1998; Min *et al.* 2003). In this study the results obtained suggest that *C. ladanifer* CT affect the degradability of SBM protein mainly by forming complexes with protein, reducing its soluble or rapidly degraded fraction with no effect on microbial activity, once that the degradation rate was unaffected. A slight decrease on NH_3-N concentration in ruminal fluid was observed ($P = 0.08$) reflecting the lower protein solubility and deamination with CT treatment. The microbial nitrogen supply (expressed in g/day) tended to decrease ($P = 0.06$) with SBM treated with CT. However, the VFA concentration in ruminal fluid was similar among treatments, suggesting that overall rumen microbial activity was not affected by CT inclusion. The ivID decreased from 77% in control to 74% in SBM treated with 15 and 30 g of CT suggesting no complete dissociation of CT-protein complexes in the lower digestive tract. The apparent N digestibility, the N retained and absorbed were not affected by CT addition suggesting that the reduction of ivID and microbial protein synthesis were balanced by an increase in protein absorption in lower digestive tract.

Treatment of SBM with 15 g/kg of *C. ladanifer* CT seems to be a valuable approach to reduce rumen protein effective degradability and to increase the amount of feed protein

absorbed in the small intestine, without compromising the digestibility of the diet. So, a production trial was conducted to evaluate the effect of SBM treated with 15 g/kg on DM of *C. ladanifer* CT on lamb´s growth, carcass and meat quality (Dentinho *et al.* 2018b). In this experiment, lambs received a diet composed by hay and concentrate (15:85). The concentrates were formulated to contain: 16% of crude protein (CP) with untreated SBM (control); 12% of CP with untreated SBM (Restricted protein); and 12% of CP with SBM treated with 15 g/kg on DM of *C. ladanifer* CT. Lambs were fed with moderate restriction, corresponding to a daily dry matter intake level of 4% live weight. The daily DM intake was similar among treatments but the CP intake was 21% lower in both diets with reduced protein content. Lambs fed with restricted protein diet without treatment with C. *ladanifer* CT had lower average daily gain and lower feed efficiency than lambs fed with the other diets, reflecting the lack of protein to supply growth requirements. Treatment of SBM with *C. ladanifer* CT induced to a positive response on lamb growth and on feed efficiency, with intermediate results between untreated SBM diets with 12 and 16% of CP. The carcass traits and meat quality of lambs were not affected by treatments. Moreover, treatment of SBM with *C. ladanifer* CT led to similar protein feed efficiency to control diet and higher than restricted protein diet. These positive responses suggest that *C. ladanifer* CT effectively bind to SBM protein resulting in an increase of dietary protein rumen outflow that was available to absorption in the small intestine.

It is known that diets with high protein concentration produce higher amount of NH_3-N in the rumen. The NH_3-N that is not used by microorganisms is absorbed from the rumen and transported to the liver where it is converted to urea, which then enters in the bloodstream, being excreted through the urine or recycled to gastro-intestinal tract. So, diets with high level of protein are associated to high nitrogen excretion in urine as urea, with negative economic and environmental impact. In this trial it was observed higher level of urea N in the blood in animals fed Control diet than with both restricted protein diets. The results obtained in this study suggest that *C. ladanifer* CT might be a promising approach to increase the digestive efficiency of dietary protein, maintaining productivity and decreasing the feed costs and the environmental damage.

5.2 *Cistus ladanifer* Condensed Tannins as Additives in Silages

The proteolysis that occurs during ensiling of high protein forages is one of the main causes for poor utilization of the silage N by ruminants (Mckersie 1985; Albrecht and Muck 1991; Coblentz and Grabber 2013). During ensiling forage, proteins are hydrolyzed, originating amino acids, peptides and NH_3-N due to the action of plant proteases and microbial activity. This shift from protein to non-protein N reduces the efficiency of N use by ruminants, increasing the NH_3-N concentration in the rumen above the metabolic capacity of microbes, leading to an increase of urinary N losses (Leng and Nolan 1984). This reduces the N retention in animal products and greatly contributes to environmental pollution.

Some positive effects were reported when CT were used as additives to protect proteins from hydrolysis during ensiling (Salawu *et al.* 1999; Tabacco *et al.* 2006; Coblentz and Grabber 2013; Copani *et al.* 2014). When CT are added to forages before ensiling, plant proteins may be protected from proteolysis by forming stable complexes with tannins (Salawu *et al.* 1999) and by the inhibition of plant and microbial proteases (Waghorn

2008). Moreover, the effect of the CT can be maintained after the ingestion of the silage, protecting protein from the excessive microbial degradation in the rumenl, increasing the flux of amino acids to the small intestine (Barry and Manley 1986; Dentinho *et al.* 2014).

In a study performed by our team (Dentinho *et al*. 2018c), an extract of *C. ladanifer* CT was used as additive in lucerne silage to reduce the proteolysis *in silo* and improve protein utilization by rams. In this study, lucerne forage was sprayed with different solutions of *C. ladanifer* CT extract in 60 ml of water in order to dose 0, 40, 80 and 120 g of CT/kg of lucerne DM and was ensiled in lab-scale silos, with 3.5 L of capacity. After 35 days of ensiling, silos were opened and determined the chemical composition of the silages and the *in situ* ruminal degradability in rams

The inclusion of CT in the silages caused an important dose dependent reduction in soluble-N, NH_3-N and a large increase in true protein content and NDF-N, which indicates an effective proteolysis reduction during ensiling (Fig. 4). The reduction of the rumen effective protein degradability (ED), estimated for a rumen outflow rate of 0.08/h, was 9.5, 9.9 and 13%, respectively in the silages with 40, 80 and 120 g of CT, relatively to the control silage without CT. This decrease was mainly due to a strong reduction of protein solubility that is consistent with the results achieved in soybean meal treated with *C. ladanifer* CT (Dentinho *et al.* 2014). Consequently, an increase on rumen undegradable protein (RUP) was achieved. However, the *in vitro* organic matter digestibility (IVOMD) linearly decreased with CT addition, which might neutralize the beneficial effect of increased RUP (Fig. 5).

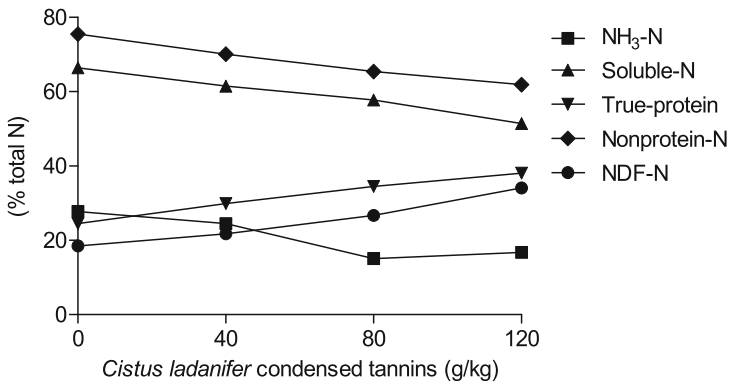

Fig. 4. Fermentative parameters of lucerne silage treated with 0, 40, 80 and 120 g/kg of *Cistus ladanifer* condensed tannins. Data from Dentinho *et al.* (2018c).

Results showed that *C. ladanifer* CT can be used as silage additives to reduce proteolysis of high-protein forages during ensiling. A level of CT of 40 g/kg DM seems to be the best compromise between the gains achieved by the protection of protein degradation in silo and in the rumen and the losses associated with the depression of the digestion and absorption.

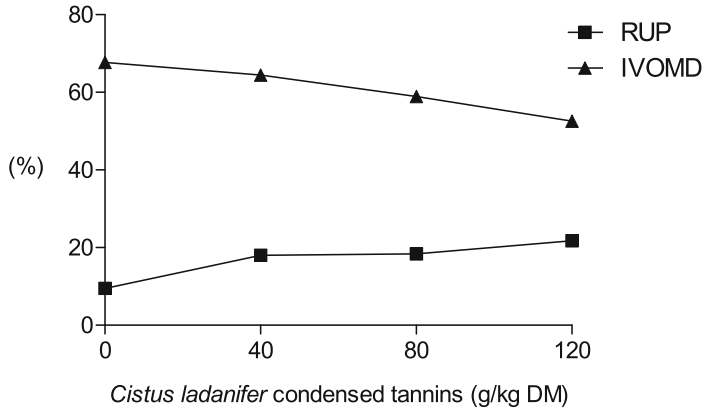

Fig. 5. Rumen undegradable protein (RUP) and *in vitro* organic matter digestibility (IVOMD) of lucerne silage treated with 0, 40, 80 and 120 g/kg of *Cistus ladanifer* condensed tannins. Data from Dentinho *et al.* (2018c).

5.3 Modulation of the Ruminal Biohydrogenation and Impact on Meat Fatty Acid Profile

Fatty acid composition of ruminant fat is directly linked to intense ruminal metabolism to which dietary lipids are subject. In the rumen, dietary lipids are rapidly hydrolyzed and then the non-esterified unsaturated FA are extensively biohydrogenated (Harfoot and Hazelwood 1997). During rumen biohydrogenation (BH), unsaturated FA are isomerized, hydrated or hydrogenated by action of microbial population (Bessa *et al.* 2015). Through of the BH process, the dietary unsaturated FA (mostly C18 PUFA) are transformed into SFA (mostly 18:0) and as result of the incomplete BH of unsaturated FA into a great diversity of *cis*- and *trans*-C18 FA isomers (Harfoot and Hazelwood 1997; Bessa *et al.* 2015), known as biohydrogenation intermediates (BI). The occurrence of this complex biological process in rumen explain the characteristic FA profile of ruminant fat, composed by high levels of SFA, low content of PUFA, and variable amounts of *trans*-FA (Wood *et al.* 2008). This FA profile is generally considered to be harmful to human health, being associated with an high cardiovascular diseases risk. However, the presence of health promoting FA in ruminant fat, such as conjugated linoleic acid (CLA) isomers, is also a result of the ruminal BH process.

Conjugated linoleic acid isomers are a group of positional and geometric isomers of linoleic acid with conjugated double bonds. The occurrence of more than 16 different CLA isomers was described in ruminant fat (Parodi 2003), being the majority produced during ruminal BH, and some of them can also be originated by endogenous synthesis in tissues from the *trans* monoenes, which are also produced during BH (Shingfield and Wallace 2014). Rumenic acid (*c*9, *t*11-18:2) is the major CLA isomer found in ruminant fat, representing about 70–80% of total CLA isomers (Kuhnt *et al.* 2016). The potential health effects of *c*9, *t*11-18:2 has been extensively studied, and is very well demonstrated its anticancerinogenic activity (Dilzer and Park 2012). Rumenic acid is formed as intermediate during BH of 18:2*n*-6 (Harfoot and Hazelwood 1997), and as the product of the endogenous conversion of vaccenic acid (*t*11-18:1) by the action of

stearoyl-CoA desaturase (SCD) in tissues (Griinari *et al.* 2000). Vaccenic acid is an intermediate product of the BH of both 18:2*n*-6 and 18:3*n*-3 (Harfoot and Hazelwood 1997). It is estimated that up to 80% of the *c*9, *t*11-18:2 present in ruminant milk and tissues results from endogenous synthesis (Corl *et al.* 2003; Palmquist *et al.* 2004), whereby the enrichment of rumenic acid in ruminant fats is mainly dependent of the rumen outflow of *t*11-18:1 and the endogenous conversation of *t*11-18:1 to *c*9, *t*11-18:2 by the SCD (Bessa *et al.* 2015).

In order to improve the FA profile of ruminant fat several nutritional strategies has been explored to modulate the ruminal BH, promoting either the protection of dietary unsaturated FA against BH or the increase of healthy FA that derived, directly or indirectly, from rumen BH. It is known that several dietary factors are able to modulate the ruminal BH, such as the amount and type of lipid supplement (Jenkins *et al.* 2008), basal diet (Bessa *et al.* 2015) and plant secondary compounds (Vasta and Bessa 2012). Numerous studies about the use of plants or plant extracts-rich in secondary compounds as modulators of the ruminal biohydrogenation have been published in the last years, and results from *in vitro* and *in vivo* studies has demonstrated that CT can be a promising strategy to modulate the ruminal biohydrogenation and improve the FA profile of ruminant fat (Vasta and Bessa 2012; Jerónimo *et al.* 2016). Impact of *C. ladanifer* on ruminal BH was tested for the first time 10 years ago in a production trial with lambs. In this experiment, 250 g of *C. ladanifer* leaves and soft stems DM per kg was included in a diet composed of dehydrated lucerne supplemented or not with 60 g/kg of vegetable oil blend (Jerónimo *et al.* 2010). The FA profile of abomasum digesta showed that *C. ladanifer* had no major effects on the BH patterns when added to oil unsupplemented diet, while with oil supplemented diets led to depression of 18:0 (−37%) and to large increase of *t*11-18:1 (+100%), without change the *c*9, *t*11-18:2 (Fig. 6). Reprocessing the FA profile of abomasal digesta according to the calculations of BH estimates and BH completeness presented by Alves *et al.* (2017), is clear that the inclusion of *C. ladanifer* in both unsupplemented and oil supplemented diets did not affect the BH percentage of *c*9-18:1, 18:2*n*-6 and 18:3*n*-3 (Table 4) but that the association of *C. ladanifer* with oil supplementation resulted in the lowest values of BH completeness (Table 4). This is in agreement with the high accumulation of *t*11-18 and reduction of 18:0 observed in abomasal digesta, suggesting an inhibition of the last reductive step of BH. The higher rumen outflow of *t*11-18:1 suggested with oil supplemented diet with *C. ladanifer*, resulted in higher levels of *t*11-18:1 and *c*9, *t*11-18:2 in intramuscular fat when compared with other experimental diets, including with oil supplemented diet that *per se* yet induced to higher *t*11-18:1 and *c*9, *t*11-18:2 levels in intramuscular fat. The higher availability of *t*11-18:1 for endogenous desaturation can explain the higher deposition of *c*9, *t*11-18:2 in intramuscular fat of lambs fed oil supplemented diet with *C. ladanifer*, once this diet did not increase the ruminal production of *c*9, *t*11-18:2.

However, in this trial we observed that the *c*9, *t*11-18:2 increase in muscle fat (+41%) was not equivalent to increase of 18:1 *trans*-11 (+86%), which could be related to down regulation of SCD (Francisco *et al.* 2016). Three factors might have contributed to SCD inhibition: *i*) it was reported that secondary compounds as tannins are able to modulate the lipogenic gene expression (Vasta *et al.* 2009; Rana *et al.* 2012), and *C. ladanifer* might have affected the SCD despite the effect *C. ladanifer* on SCD to be

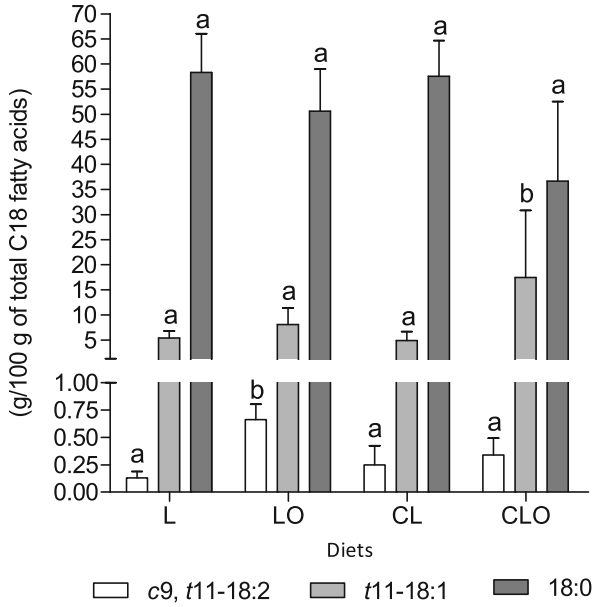

Fig. 6. Effect of inclusion of *Cistus ladanifer* and oil blend in high-forage diet on *c*9, *t*11-18:2, *t*11-18:1 and 18:0 (g/100 g of total C18 fatty acids) of the abomasal digesta of lambs. L – dehydrated lucerne:wheat bran (90:10); LO - L with 60 g/kg DM of oil blend (sunflower and linseed oils (1:2 v/v)); CL – L with 250 g/kg DM *Cistus ladanifer*; CLO – L with 250 g/kg DM *Cistus ladanifer and* 60 g/kg DM of oil blend (sunflower and linseed oils (1:2 v/v)). Data from Jerónimo *et al.* (2010).

inconsistent (Francisco *et al.* 2016; Francisco *et al.* 2018); *ii*) high PUFA availability can inhibit the SCD activity (Daniel *et al.* 2004), and *iii*) forage diets are associated to down-regulation of SCD expression (Daniel *et al.* 2004) and activity (Smith *et al.* 2009). Inclusion of *C. ladanifer* to diets with lower forage content could be a good strategy to obtain an high ruminal production of *t*11-18:1 without SCD down-regulation, promoting the endogenous synthesis of *c*9, *t*11-18:2 (Francisco *et al.* 2016). So, in second production trial with lambs, we tested the incorporation of increasing levels of *C. ladanifer* (50, 100 and 200 g/kg DM) in a diet containing 1:1 forage-to-concentrate supplemented with 0, 40 or 80 g/kg of oil blend composed by soybean and linseed oil (1:2), in a factorial arrangement. Contrarily to our expectations, in basal diets with 1:1 forage-to-concentrate ratio, all combinations of *C. ladanifer* and oil levels failed to increase the *c*9, *t*11-18:2 content in muscle fat (Francisco *et al.* 2016). Moreover, we also did not observed significant increase of *t*11-18:1 levels in rumen and abomasal digesta (Fig. 7) (Alves *et al.* 2017). Conversely, the increasing levels of *C. ladanifer* in diets resulted in increase of the ruminal production of *t*10-18:1 (Fig. 7), which was the main *trans* monoene isomer present in rumen and abomasum digesta, plasma and muscle fat (Francisco *et al.* 2016; Alves *et al.* 2017). Basal diet used in this study might have create favorable ruminal conditions to production of *t*10-18:1 instead *t*11-18:1 (known as *t*10-shift), which was exacerbates by simultaneous inclusion of PUFA and *C. ladanifer* in diets (Fig. 7). The

Table 4. Effect of *Cistus ladanifer* (CL) in C18 rumen biohydrogenation (%) and completeness (%) in abomasal digesta of lambs

Basal diet	*Cistus ladanifer* (g/kg DM)	Oil level (g/kg DM)	Biohydrogenation (%)			Completeness (%)
			*c*9-18:1	18:2*n*-6	18:3*n*-3	
Dehydrated Lucerne:wheat bran (90:10)[a]	0	0	58.9	79.8	78.4	78.9[a]
	0	60	49.0	82.6	86.4	66.3[b]
	250	0	58.8	80.6	76.5	77.6[c]
	250	60	49.4	79.6	81.6	49.6[d]
SEM			5.20	3.13	3.19	2.87
P value						
C. ladanifer (CL)			0.975	0.741	0.318	0.006
Oil (Oil)			0.082	0.783	0.058	<0.001
CL × Oil			0.955	0.549	0.638	0.016
Concentrate: Dehydrated Lucerne (50:45,40 or 30)[b]	50	0	69.5	80.2	60.8	76.1
	50	40	70.0	77.8	73.6	69.1
	50	80	75.0	83.7	73.3	64.1
	200	0	63.8	74.4	68.1	70.1
	200	40	71.7	80.3	79.1	38.4
	200	80	73.3	84.1	83.8	44.8
SEM			3.40	2.59	4.30	5.03
P value						
C. ladanifer (CL)			0.500	0.661	0.038	<0.001
Oil (Oil)			0.115	0.052	0.007	0.001
CL × Oil			0.555	0.267	0.846	0.064

[a]Calculations performed using data from Jerónimo et al. (2010)
[b]Data from Alves et al. (2017).

occurrence of *t*10-shift is associated with consumption of high-starch low forage diets (Aldai *et al.* 2013), and constitute a limitation to CLA enrichment in ruminants fat once the production of a CLA isomer from *t*10-18:1 is not possible.

Regarding to overall impact of *C. ladanifer* on ruminal BH, results obtained from FA profile of abomasum digesta showed that high levels of *C. ladanifer* in diets increase the BH percentage of 18:3*n*-3 and reduce the estimated BH completeness in abomasum, with very low values when diets are supplemented with oil (Table 4). Moreover, high *C. ladanifer* incorporation in diets led to reduction of 18:0 yield and an accumulation of BI, with more pronounced impact in oil supplemented diets (Fig. 7) (Alves *et al.* 2017). Such results suggest that *C. ladanifer*, particularly when associated with oil supplementation

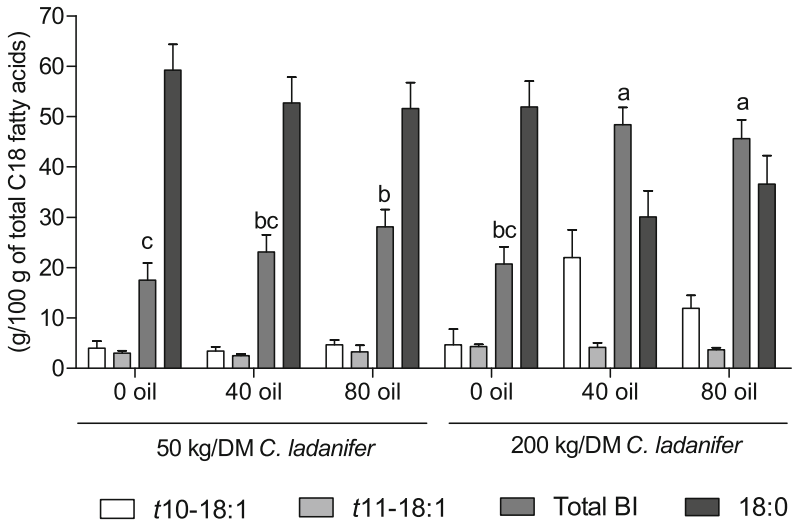

Fig. 7. Effect of inclusion of *Cistus ladanifer* and oil blend in 1:1 forage:concentrate diet on *t*10- and *t*11-18:1, total biohydrogenation intermediates (BI) and 18:0 (g/100 g of total C18 fatty acids) of the abomasal digesta of lambs. Data from Alves *et al.* (2017).

reduce the last step of rumen BH, which is in agreement with results of the first trial (Jerónimo *et al.* 2010). Apparently, there is a synergic effect between C. *ladanifer* and oil supplementation that promote the inhibition of the last step of rumen BH. The cause for this synergic effect is not known, but an excessive stress induced by both CT and PUFA in microbial population responsible for the last step of rumen BH might to be appointed as possible explanation (Alves *et al.* 2017). *Cistus ladanifer* is rich in CT, and either tannins as PUFA affect the microbial membrane stability and integrity (Smith *et al.* 2005; Maia *et al.* 2007). It is reported that some bacteria, as response to toxins that affect membrane integrity, are able to reduce its membrane fluidity through enrichment of their membrane lipids with *trans*-FA (Keweloh and Heipieper 1996; Endo *et al.* 2006). In fact, has been suggested that one the main purpose of rumen BH may be related with production of the *trans*-FA and their incorporation into microbial cell as protective mechanism against toxicity of PUFA (Bessa et al. 2000). So, the accumulation of *trans*-18:1 in detriment of 18:0 can be a response of microbial population to toxic effect induced by high levels of PUFA and CT, increasing the availability of *trans*-18:1 to incorporation into microbial cell membranes (Alves *et al.* 2017).

Several strategies to mitigate the *t*10-shift in intensively finished systems based on cereal diets was explored. So, in the next step we hypothesized that replacing cereal by dehydrated citrus pulp in an oil supplemented diet might be prevent the *t*10-shift and increase the *t*11-18:1 and *c*9, *t*11-18:2 deposition in muscle fat, and that the inclusion of *C. ladanifer* would amplify that response (Francisco *et al.* 2018). So, in an experiment with factorial treatment arrangement, 0 or 150 g/kg DM of *C. ladanifer* leaves and soft stems was included in a cereal and dehydrated citrus pulp diets, both supplemented with soybean oil. Contrary to the expectations, high levels of *t*11-18:1 and *c*9, *t*11-18:2 were

observed in muscle fat from all diets, and were not affected by inclusion of *C. ladanifer* of both cereals and dehydrated citrus pulp diets, while *t*10-18:1 was the second major *trans*-monoene. However, the effect of *C. ladanifer* on ruminal BH was dependent of the basal diet. When combined with cereals, *C. ladanifer* led to increase of the 18:0 and reduced the BI intermediates in muscle fat, suggesting that in this conditions *C. ladanifer* increase the ruminal BH completeness. Unfortunately, in this trial the C18 FA profile in ruminal or abomasal digesta was not evaluated to verify this and confirm the apparent lack effect of *C. ladanifer* on ruminal BH in dehydrated citrus pulp diets. However, these results are contradictory with those obtained on two first trials, where *C. ladanifer* when associated to oil supplementation increase the accumulation of either *t*11- or *t*10-18:1 and reduce the 18:0 production. Distinct ruminal conditions induced by basal diets resulted in distinct BH pattern, but the differences in secondary metabolites content from *C. ladanifer* batches used in different experiments could also explains the inconsistency of the results throughout the experiments.

Both *in vitro* as *in vivo* studies indicate that plant secondary metabolites are able to influence the ruminal BH pattern (Vasta and Bessa 2012). *Cistus ladanifer* present diverse secondary metabolites, and is not clear which specific group of compounds is responsible to the effects of *C. ladanifer* on rumen BH. In order to clarify which *C. ladanifer* compounds are responsible by the changes in rumen BH, we isolated several fractions from the whole plant (essential oils, CT, non-tannin phenols and lipophilic extract) and tested their effects on *in vitro* rumen BH (Guerreiro *et al.* 2016a). *Cistus ladanifer* fractions were incubated for 6 h with buffered ruminal fluid, using as subtract a mixture of dehydrated lucerne and concentrate (70:30) containing sunflower oil (60 g/kg DM). Although, several *C. ladanifer* fractions have induced changes in FA profile of ruminal fluid, the CT fraction was the most effective fraction in modulating the ruminal BH. Condensed tannins fraction of *C. ladanifer* induced the largest increased on the BH of 18:2*n*-6 and 18:3*n*-3, and the largest accumulation of *t*11-18:1 and 18:0. Costa *et al.* (2017), using same incubation conditions, also reported higher disappearance of 18:2*n*-6 and 18:3*n*-3 and an increased accumulation of *t*11-18 without reduction of the 18:0 in *in vitro* incubation of CT extract from *C. ladanifer* when compared to control incubation. In both *in vitro* experiments, no reduction in 18:0 production was observed in incubations with *C. ladanifer* CT, suggesting that increase of the *t*11-18:1 results from the increased activity of the initial steps of the BH and not by inhibition of the last reductive step of BH (*i.e.* formation of 18:0), which is contrary to observed in *in vivo* trials when *C. ladanifer* plant was incorporated into lamb diets. Other *C. ladanifer* fractions also showed ability to affect the BH, such as dichloromethane extract that induced to highest yield of *c*9, *t*11-18:2 and reduced the production of other BI presents, suggesting that this *C. ladanifer* fraction reduce the diversity of BH pathways. Despite the inconsistence results among *in vivo* and *in vitro* trials, results from *in vitro* studies (Guerreiro *et al.* 2016a; Costa *et al.* 2017) reinforces the hypothesis that the modulatory effects of *C. ladanifer* plant on rumen BH observed *in vivo* (Jerónimo *et al.* 2010; Alves *et al.* 2017) are due to its high CT content.

Basal diet, CT level and presentation form, and possible interactions with other components of *C. ladanifer* can also contributed to explain the differential response among trials. The CT effect on ruminal BH seems to be dependent of the dose (Carreño

et al. 2015), and the district effect on ruminal BH observed can be related with distinct CT levels applied in each trial. Modulation of the ruminal BH by inclusion of *C. ladanifer* plant in diet only was evident for CT levels between 15 and 21 g/kg DM (Jerónimo *et al.* 2010; Alves *et al.* 2017), and in *in vitro* experiments was used 100 g/kg DM of *C. ladanifer* CT extract (Guerreiro *et al.* 2016a; Costa *et al.* 2017). In *in vivo* experiment where *C. ladanifer* did not induced relevant changes in ruminal BH, the CT level in diets with *C. ladanifer* leaves a soft stems varied between 3.5 and 5.6 g/kg DM (Francisco *et al.* 2018), which may not enough to modulate the ruminal BH. The effect on different BH step can also be due to CT levels, with inhibition of the initial BH step with higher *C. ladanifer* CT levels, while lower CT levels affect the last reductive BH step. However, independently of the BH step which has been affected, either in *in vivo* trials with *C. ladanifer* plant as in *in vitro* trials with *C. ladanifer* CT extract occurred an accumulation of *trans*-FA in detriment of the 18:0 production. This corroborates the need of rumen microbial population enriched their membrane lipids with *trans*-18:1 to support the toxic effects induced by both CT and PUFA.

Evaluation of the parameters as volatile fatty acid (VFA), odd- and branched-chain fatty acids (OBCFA) and dimethyl acetals (DMA) in ruminal digesta or fluid can reflect the dietary effects on rumen microbiota (Vlaeminck *et al.* 2006; Alves *et al.* 2013). Only in one production experiment the impact of *C. ladanifer* on VFA concentration in rumen digesta was analysed (Alves *et al.* 2017), and was observed the reduction of VFA concentration with increase of *C. ladanifer* levels in diets from 50 to 200 g/kg DM, suggesting the inhibition of the fermentative activity. Moreover, in this experiment *C. ladanifer* appears to have affected the growth and activity of acetate- and butyrate-producing bacteria. Conversely, in *in vitro* studies where high levels of *C. ladanifer* CT extract (100 g/kg DM) were used, the total VFA concentration in ruminal fluid was not affected (Guerreiro *et al.*, 2016a; Costa *et al.* 2017). It is reported that dietary CT can affect the growth and activity of rumen microorganisms, with negative impact in ruminal activities as BH and fermentation, effect that is dependent of the CT level and source (Piluzza *et al.* 2014). Regarding to *C. ladanifer* CT the available results about its effect on general microbial metabolism in rumen are still few, but not suggest a worrying impact on ruminal function.

Overall our results suggest that CT from *C. ladanifer* might be used as a tool to induce beneficial changes in the ruminal BH pattern and consequently in the FA profile of edible products from ruminants. However, for this purpose further works are needed to optimize the practical conditions for *C. ladanifer* CT application in ruminant diets.

5.4 Impact on Lipid Oxidation in Meat

Lipid oxidation is one of the main causes of meat quality loss, leading to degradation of sensory attributes as colour and flavour, and formation of potentially toxic compounds (Morrissey *et al.* 1998). The oxidative stability of meat lipids is depend of the balance between antioxidant and pro-oxidant components in the muscle, which can be affected by several intrinsic and extrinsic factors (Bekhit *et al.* 2013). Amount and type of fat in the diet affect significantly the lipid stability in meat, and nutritional strategies that promote the increase of the PUFA levels in meats may also increase its susceptibility to oxidation as PUFA are more prone to oxidation (Morrissey *et al.* 1998; Faustman *et al.*

2010). Adequate supply of antioxidant compounds to animals is essential to prevent the lipid oxidation, which is particularly important in PUFA-enriched diets. Vitamin E has been widely used as antioxidant in animal nutrition. However, in last years the interest by use of natural antioxidants in animal nutrition has increasing, and plants or plant extracts rich in secondary metabolites has been appointed as possible sources of antioxidants for ruminant diets.

Use of aerial part of *C. ladanifer* for limit the lipid oxidation in meat was tested in four production experiments with lambs (Table 1), where samples of *Longissimus* muscle collected seventy-four hours after slaughter were individually placed on polystyrene trays, over-wrapped with oxygen permeable polyvinyl chloride film and maintained during 7 days at 2 °C. First results, obtained by a methodology that consist of the initial induction of oxidation followed by the measurement of oxidized lipids, showed that incorporation of 250 g/kg DM of *C. ladanifer* in a high-forage diets supplemented or not with vegetable oils enhance the meat resistance against lipid oxidation (Fig. 8) (Jerónimo *et al.* 2012). As expected, lipid oxidation increased with storage time and dietary lipid supplementation, as result of the loss capacity of meat to resist against lipid oxidation during storage time and the higher PUFA content in meat (Fig. 8). However, throughout storage period, both in PUFA-enriched and no-enriched meats, we observed lower lipid oxidation levels in meats from lambs fed with *C. ladanifer* than in those from lambs fed diets without *C. ladanifer* (Fig. 8). Although these results showed the potential of *C. ladanifer* to improve the oxidative stability of meat, the methodology used did not allow verify the real lipid damage in meat, because the lipid oxidation was determined after oxidative induction (Mercier *et al.* 2004). In another work, was possible verify the effectiveness of the *C. ladanifer* incorporation in lamb diets in reducing the lipid oxidation in meat without oxidative induction (Francisco *et al.* 2015). In this work, *C. ladanifer* was incorporated in oil-unsupplemented and oil-supplemented diets (0, 40 and 80 g/kg DM of blend of soybean and linseed oils (1:2 v/v)) composted by 1:1 of forage and concentrate, where forage (dehydrated lucerne) was partially replaced by *C. ladanifer* at levels of 50, 100 and 200 g/kg DM. Independently of the oil supplementation level, incorporation of 200 g/kg DM of *C. ladanifer* in diets reduced in about 45% the lipid oxidation in meat stored during 7 days when compared with utilization of 50 g/kg DM of *C. ladanifer* (1.74 vs. 0.96 mg MDA/kg fresh meat in meat from lambs fed diets with 50 and 200 g/kg DM of *C. ladanifer*, respectively). Incorporation of 100 g/kg DM of *C. ladanifer* in diets resulted in equal level of lipid oxidation in meat than use of 50 and 200 g/kg DM of *C. ladanifer*. In this experiment, all diets included vitamin E (22.5 mg/kg), and even with the expected prevention of the lipid oxidation in meat by vitamin E, the *C. ladanifer* resulted in a significant protection of the meat against lipid oxidation.

However, in more recent works we did not observed a reduction of the lipid oxidation in meat with increasing *C. ladanifer* levels in diets. Partial replacement of dehydrated lucerne with 150 g/kg DM of *C. ladanifer* in an oil supplemented diets composed by 1:1 forage and concentrate did not resulted in reduction of the lipid oxidation in meat (1.34 mg MDA/kg fresh meat) (Francisco *et al.* 2018). In this experiment all diets were also supplemented with vitamin E (22.5 mg/kg). Moreover, the increasing levels of *C. ladanifer* (0,

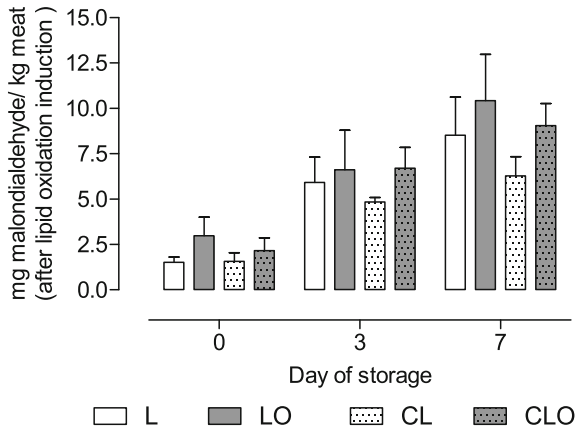

Fig. 8. Effect of inclusion *of Cistus ladanifer* and oil blend in high-forage diet and storage on lipid oxidation (TBARS) determined after oxidation induction in muscle. L – dehydrated lucerne:wheat bran (90:10); LO - L with 60 g/kg DM of oil blend (sunflower and linseed oils (1:2 v/v)); CL – L with 250 g/kg DM Cistus ladanifer; CLO – L with 250 g/kg DM Cistus ladanifer and 60 g/kg DM of oil blend (sunflower and linseed oils (1:2 v/v)). Data from Jerónimo et al. (2012).

125 and 250 g/kg DM) in a diet composed by dehydrated lucerne, did not affect the meat lipid oxidation (0.38 mg MDA/kg fresh meat) (Soldado *et al*. unpublished data).

Differences in basal diets (dehydrated lucerne and blend of concentrate and dehydrated lucerne), levels (0, 4, 5, 6 and 8%) and type of lipid supplementation (blend of linseed and soybean oils (2:1) and soybean oil), inclusion of other antioxidant compound in diets, or other uncontrolled factors as environmental temperature and stress-inducing situations, might have create different balance between antioxidant and pro-oxidant agents in muscle among experiments, resulting in inconsistent effect of dietary *C. ladanifer* on meat lipid oxidation. However, the lack of *C. ladanifer* effect on meat lipid oxidation in the last two experiments may not correspond to inefficiency of *C. ladanifer* to protect the meat against lipid oxidations. In these experiments the oxidative pressure may not have exceed the antioxidant capacity of meat, not evidencing the protective effect of *C. ladanifer*. The natural antioxidants of the raw materials and supplementation with α-tocopherol of both diets with or without *C. ladanifer*, associated to a low oxidative pressure may have masked a possible beneficial of *C. ladanifer* on lipid stability.

The Fig. 9 presents the TBARS results from the three experiments where was measured the real lipid oxidation (without oxidize induction).The lowest TBARS values was found in diets composed exclusively by dehydrated lucerne, even supplemented with 60 g/kg DM of soybean oil (Soldado *et al*., unpublished data). Greater protective effect of grass-diets against lipid oxidation in meat comparatively to concentrate diets is in agreement with several studies (Mercier *et al.* 2004; Descalzo *et al.* 2005; Wood *et al.* 2008). Grass-feeding systems provide higher levels of natural antioxidants to animals, which may protect meat lipids against oxidation (Descalzo *et al.* 2007). Although, grass-based feeding systems may increase the concentration of PUFA that are more prone to oxidation, as 18:3*n*-3, this natural antioxidant compounds are effective to limit the lipid oxidation (Faustman *et al.* 2010). In our experiment (Soldado *et al*. unpublished data),

increasing levels *C. ladanifer* were added to dehydrated lucerne basal diets. The natural antioxidants supplied by the forage seems to have been *per se* enough to prevent the lipid oxidation independently of the peroxidation index in meat (Fig. 10). A positive relation between TBARS values and PUFA levels in meat from concentrate-fed steers was reported by Wood *et al.* (2008). Contrarily, we did not observe a consistent relationship between TBARS values and peroxidation index in muscle when C. *ladanifer* was incorporated to forage:concentrate diets (Fig. 10), which can be explained by the protective effect of *C. ladanifer* from lipid oxidation in meat independently of the PUFA level.

The higher oxidative stability observed in meat from lambs fed *C. ladanifer* was associated with the presence of antioxidant compounds in *C. ladanifer*. Condensed tannins are known by its antioxidant activity, and are appointed as possible responsible for beneficial effect of *C. ladanifer* on meat oxidative stability. However, the results of inclusion of CT-rich plants and plant extracts in ruminant diets on meat lipid stability has been contradictory (Valenti *et al.* 2018), and many doubts subsist on mechanisms by which dietary CT can limit the lipid oxidation in meat, as previously reviews by our team (Jerónimo *et al.* 2016).

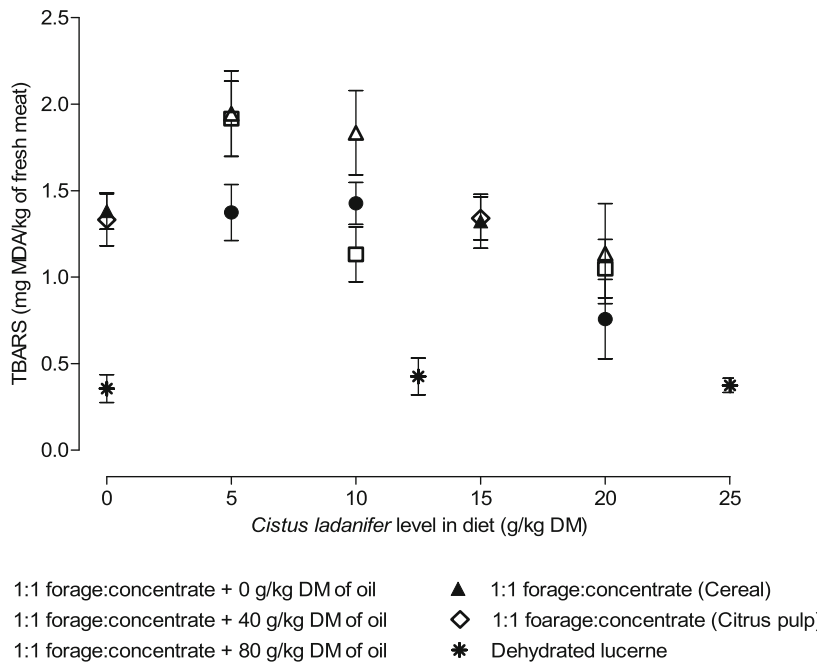

Fig. 9. Effect of *Cistus ladanifer* levels in diets on real lipid oxidation (TBARS) of muscle after 7 days of storage at 2°C. Data from Francisco *et al.* (2015), Francisco *et al.* (2018) and Soldado *et al.* (unpublished data).

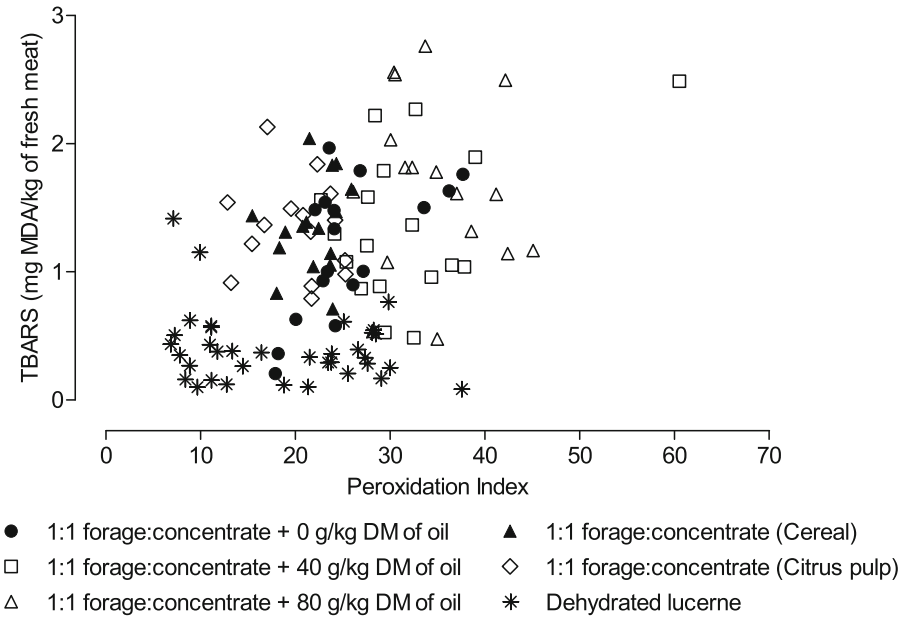

Fig. 10. Relationships between lipid oxidation (TBARS) and muscle peroxidation index. Data from Francisco *et al.* (2015), Francisco *et al.* (2018) and Soldado *et al.* (unpublished data)

5.5 Impact on Growth Performance and Quality of Carcass and meat

Due to high levels of anti-nutritional compounds presented in *C. ladanifer*, it could be expected that the incorporation of 250 g/kg DM of *C. ladanifer* in lamb diets would depress growth performance of lambs. However, the inclusion of *C. ladanifer* in diets has resulted in inconsistent results on lamb productivity. In the our first three lamb experiments, application of *C. ladanifer* at levels between 50 and 250 g/kg DM in lamb diets, did not affect the animal growth performance (Jerónimo *et al.* 2010; Francisco *et al.* 2015; Francisco *et al.* 2018). In these experiments, different levels of *C. ladanifer* incorporation in diets resulted in CT levels between 3.5 to 21 g/kg DM (Table 3). However, in the last experiment (Guerreiro *et al.* 2020) the application of 250 g/kg DM of *C. ladanifer* in a dehydrated lucerne diet, resulted in a dietary CT concentration of 26.6 g/kg DM in diets, and reduced the lamb average daily gain comparatively to diets with 0 and 125 g/kg DM of *C. ladanifer* (15.3 g/kg DM). Contrary to the previous experiments, in this last experiment, the DM intake was reduced at level of 250 g/kg DM of *C. ladanifer* in diet (905 vs. 1269 g/day in diet with 250 and 0–125 g/kg DM of *C. ladanifer*, respectively). The quite higher dietary CT content attained in the last experiment probably explains the negative impact on lamb weight gain and DM. Generically, the CT are known by their potential negative effects on animal performance, due to its ability to form indigestible complexes with several molecules, as proteins, polysaccharides or minerals, decreasing nutrient digestibility and feed intake. However, our results suggest that levels of *C. ladanifer* CT up to 20 g/kg DM are safe to prevent detrimental effects on lamb productivity.

Regarding carcass composition, the effect of *C. ladanifer* are also inconsistent. Inclusion of 250 g/kg DM of *C. ladanifer* in high-forage diet, supplemented or unsupplemented with oil, increased in 22% the subcutaneous fat proportion and decreased in 7.2% the muscle proportion in chump and shoulder cuts (Jerónimo *et al.* 2010). Moreover, *C. ladanifer* increased the percentage of kidney and knob channel fat (Jerónimo *et al.* 2010). However, in the two subsequent trials where *C. ladanifer* levels between 50 and 200 g/kg DM were used, we did not observed effect on carcass fat deposition (Francisco *et al.* 2015, 2018). The higher fat deposition in carcasses observed in the first experiment can be related to the ether extract content in diets, which increased significantly with inclusion of *C. ladanifer* in diets (Table 3), while in other experiments occurred a smaller increase of the ether extract (Francisco et al. 2015) or remained constant with incorporation of *C. ladanifer* in diets (Francisco *et al.* 2018).

Consistently with higher subcutaneous fat deposition, the incorporation of the 250 g/kg DM in oil supplemented diets also increased the intramuscular fat (Jerónimo *et al.* 2010, 2012), while in the other works the intramuscular fat content was not affected by inclusion of *C. ladanifer* in diets (Francisco *et al.* 2015, 2016, 2018). Usually dietary lipid supplementation does not increase intramuscular fat in ruminants, once high availability of diet derived preformed FA can depress *de novo* FA synthesis pathways (Chilliard 1993). Applying gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) to our samples, it was possible verify that the increase of the intramuscular fat deposition in lambs fed diets containing oil and *C. ladanifer* is sustained by *de novo* FA synthesis, while in diets without *C. ladanifer* the oil supplementation resulted in inhibition of *de novo* FA synthesis (van Leeuwen *et al.* 2017). Our results indicate that the inclusion of *C. ladanifer* in the diets blocked the inhibition effects of lipid supplementation on *de novo* FA synthesis in intramuscular fat (van Leeuwen *et al.* 2017). The mechanism involved in such effect is not known, however was previously reported that dietary tannins are able to modulate the lipogenic gene expression (Vasta *et al.* 2009; Rana *et al.* 2012; Francisco *et al.* 2016).

It has been reported that dietary tanniferous plant species affect the lamb meat colour, originating lighter meat (Priolo *et al.* 2002; Priolo *et al.* 2005). However, the inclusion of *C. ladanifer* in lamb diets did not affect the meat colour parameters at the day of carcass processing, or after 7 days of storage at 2 °C (Jerónimo *et al.* 2012; Francisco *et al.* 2015; Francisco *et al.* 2018). Meat quality traits as pH and cooking loss were not affected by dietary *C. ladanifer* (Francisco *et al.* 2015; Francisco *et al.* 2018). However, the results regarding to meat shear force are variable among experiments. The meat shear force increased 25.6% with inclusion of 150 g/kg DM in diets and consistently the tenderness score was reduced (Francisco *et al.* 2018). On the contrary, increasing levels of *C. ladanifer* in diets between 50, 100 and 200 g/kg DM did not affect the meat shear force (Francisco *et al.* 2015). The impact of *C. ladanifer* inclusion in lamb diets on sensory meat properties has also been inconsistent, as only in one trial was observed negative impact of *C. ladanifer* on meat overall acceptability (Francisco *et al.* 2018). However, our results showed that inclusion of 250 g/kg DM of *C. ladanifer* in lamb diets induce to a distinct volatile compounds profile of meat, with presence of compounds that are directly transferred from *C. ladanifer* to meat (Vasta *et al.* 2010), that apparently are not detected by consumers once consumer panel were not unable to distinguish between

meats from lambs fed diets with *C. ladanifer* from meats of lambs fed diets without *C. ladanifer* (Jerónimo *et al.* 2012).

6 Conclusions

Results from the series of experiments conducted by our team showed evidences that utilization of *C. ladanifer* or its CT extract in ruminant nutrition may be an effective approach to improve the feed nutritional value and the quality of edible products, promoting a sustainable livestock production system and the valorisation/control of this endogenous resource. The research work developed by our team enhanced significantly the knowledge on this plant and on its application in ruminant diets, providing valuable tools to implementation of new production strategies with improved feed efficiency and generators of the products with quality differentiated and with higher value. However in a different research topics, further works are needed with particular relevance on secondary compounds composition and action mechanisms, as well as optimization of the conditions of *C. ladanifer* and *C. ladanifer* CT use in ruminant diets. Considering its complex composition on secondary metabolites it can be expected that *C. ladanifer* or its CT may have other advantages when applied in ruminant diets besides the beneficial effects already demonstrated. Further work should be conducted to continue to explore other benefits of the use of *C. ladanifer* and/or its tannins in ruminant nutrition.

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Ameliorating Pork Marbling and Quality with Novel Feeding Approaches

M. S. Madeira[1], C. M. Alfaia[1], P. A. Lopes[1], J. Pestana[1], D. Coelho[1], C. M. G. A. Fontes[2], and J. A. M. Prates[1(✉)]

[1] Quality of Animal Products Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon , Lisbon, Portugal
japrates@fmv.ulisboa.pt

[2] Animal Nutrition and Biotechnology Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
cafontes@fmv.ulisboa.ot

Abstract. Although pork is the most consumed meat in the European Union, its production and quality are currently facing big challenges, including feeding sustainability, lower sensory quality and unhealthy image of fat. This chapter addresses the most efficacious and promising nutritional strategies to mitigate these issues. In pigs, genetic selection towards reduced subcutaneous fat has hugely reduced intramuscular fat (IMF), with a strong negative effect on the sensory properties and consumers' acceptability of pork. Dietary protein reduction, alone or in combination with other feed ingredients, improves fat partitioning with increased IMF content. This increased IMF may improve pork sensory traits, and the mechanisms underlying this relation are discussed here. These feeding strategies could satisfy consumer requirements and enhance the competitiveness of meat industry. In addition, microalgae are currently included in the diets of pigs only at low levels since their recalcitrant cell walls are largely indigestible by monogastric animals. However, the use of microalgae in pig feeding should represent a promising approach for the maintenance and development of livestock sector, as an environmental friendly alternative to balance food-feed-biofuel industries. The use of exogenous carbohydrate-active enzymes (cazymes) represents a promising cost-effective strategy to disclose the high nutritional value of algae for pigs.

Keywords: Pork quality · Pork marbling · Intramuscular fat · Fatty acid profile · Reduced protein diets · Microalgae · Cazymes

Abbreviations

AA	Arachidonic acid
ALA	Alpha-linolenic acid
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
IMF	Intramuscular fat
LA	Linoleic acid

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LDL Low-density lipoprotein
MUFA Monounsaturated fatty acids
PUFA Polyunsaturated fatty acids
RPD Reduced protein diets
SFA Saturated fatty acids
TFA *Trans* fatty acids

1 Introduction

The European Union (EU) is facing major challenges in its farm animal production sector. Pork production and quality are confronting the biggest challenge, as it is the most consumed meat in EU and the second most consumed worldwide. Feeding sustainability, pig welfare, wide use of antibiotics to control gastrointestinal diseases, excessive pork leanness and low pork lipids quality are key challenges. Therefore, the development of integrated strategies to solve these issues is of utmost relevance. The importance of these concerns is well expressed under the two following Horizon 2020 Societal Challenges: "Food security, sustainable agriculture and forestry, marine and maritime and inland water" and "Health and well-being".

As the consequence of genetic selection towards reduced subcutaneous fat, the amount of intramuscular fat (IMF) in pigs has also been dramatically reduced (<2.5%), with a strong negative effect on sensory properties and consumers' acceptability of pork. Therefore, the production of pork with higher amounts of IMF, without an increase in subcutaneous fat (improved fat partitioning), is eagerly awaited for the pig industry and consumers. The current efficacious nutritional strategies to increase pig fat partitioning and pork quality will be presented in this chapter.

Moreover, the nutritional profile of microalgae is species-specific but has, in general, contents of protein, carbohydrate and minerals that are comparable to conventional feedstuffs. Moreover, they have a high number of bioactive compounds. However, microalgae cell walls are composed by remarkably complex polysaccharides that are recalcitrant to degradation and, thus, largely indigestible by monogastric animals. Therefore, microalgae are currently included in the diets of pigs only at low levels (<1–2%), thus largely limiting the exploitation of their beneficial effects. The current and potential use of microalgae as feed ingredients for pork production and quality will be also discussed in this chapter.

Figure 1 provides a schematic overview of the novel feeding strategies addressed in this chapter and their major impact on pork quality.

2 Pork Production and Quality

Pork production has increased over the years and represents a strong market worldwide. According to US Department of Agriculture baseline projections (http://www.ers.usda.gov/topics), pork production will expand steadily between 2016 and 2025, driven by lower feed costs and strong meat demand domestically and abroad (Westcott and Hansen

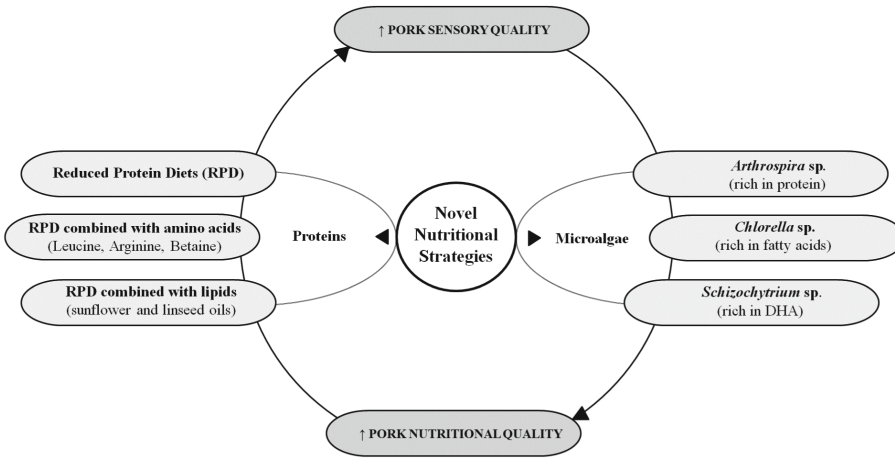

Fig. 1. Schematic overview of the novel feeding approaches on pork quality. DHA, docosahexaenoic acid; RPD, reduced protein diets.

2016). Pork production is projected to grow by 10.3% and, thus, *per capita* consumption of pork is projected to rise by 1.7% during the same period. Indeed, pork is one of the most consumed meats and it has, therefore, a significant contribution to nutrients intake. Besides high quality protein, trace minerals and vitamins, pork fat is also a major determinant of the nutritional value influencing consumer preferences.

The main pork quality attributes include water-holding capacity, colour, fat content and composition, uniformity and oxidative stability (Rosenvold and Andersen 2003). Regarding fat, pork has, in general, a lower content (between 1.5 and 2%) when compared with ruminant meats (5 to 6% of lipids). Multiple interacting factors, such as age (or weight), gender, genotype, castration and feeding, influence both total fat and fatty acid deposition in the various fat depots (Wood *et al.* 2008; Kouba and Sellier 2011). Dietary fatty acids are quickly deposited in pig subcutaneous adipose tissue (backfat) than in muscle (IMF), probably due to a less deposition of absorbed fat in muscle or due to the greater amount of membrane lipids in IMF containing high amounts of unsaturated fatty acids (Wood *et al.* 2008). Among the different fat depots, IMF content is an important economic and quality trait in pork production (Wood *et al.* 2003).

As consequence of genetic selection towards reduced subcutaneous fat, the amount of IMF, particularly in the case of white European breeds (Large White and Landrace), has been strongly reduced in crossbred commercial pigs (Jeremiah *et al.* 1999). Pigs reared in intensive systems have become leaner and faster growing due to the reduced subcutaneous fat content with the associated decreasing of IMF, which has well known adverse effects on pork eating quality (Hocquette *et al.* 2010). Still, approximately 84% of the carcasses from commercial pig genotypes have a muscle fat content below the level necessary for suitable eating quality (Daszkiewicz *et al.* 2005). Moreover, some pig breeds (*e.g.* Alentejano and Iberian) have higher amounts of subcutaneous and IMF, which are accompanied by higher sensory panel ratings (Daza *et al.* 2007; Madeira *et al.* 2013a; Madeira *et al.* 2013b). Indeed, IMF content positively influences

meat quality traits and the sensory properties of pork, such as juiciness, tenderness and overall acceptability, which are negatively affected when IMF is reduced below 2–2.5% (De Vol *et al.* 1988; Eikelenboom and Hoving-Bolink 1994). Consequently, a minimum value of 2.5% for IMF content was suggested in order to achieve a positive effect on eating quality and sensory acceptability of pork (DeVol *et al.* 1988; Fernandez *et al.* 1999). Nowadays, the knowledge on the specific mechanisms regulating IMF and subcutaneous fat deposition in pigs is particularly reinforced thanks to the studies in genomics, transcriptomics and lipidomics, which could help to improve production efficiency and pork quality traits (Corominas *et al.* 2013).

Fatty acid composition of IMF plays also an important role on pork quality. IMF includes the sum of polar lipids (phospholipids), mainly found in cell membranes, neutral lipids (triacylglycerols), the main forms of energy reserves, and cholesterol. While polar lipids amounts remain fairly constant, or increase little as the fat content of the animal and meat increase, neutral lipids amounts predominate in total fatty acid composition. The fatty acid profile in pork reflects both the tissue fatty acid biosynthesis and the fatty acid composition of the diets (Kouba and Mourot 1999). There is an increasing *de novo* tissue synthesis of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) and a relatively decreasing of the direct incorporation of linoleic acid (LA, 18−2*n*−6) from the diet in pig neutral lipids of both tissues (muscle and adipose tissue). For example, IMF from native pig genotypes, such as Alentejano breed, genetically similar to the Iberian pig, had a higher MUFA/SFA ratio compared to crossbred pigs. These genotypes display a large predisposition to deposit MUFA, especially oleic acid (18:1*c*9), due to the activity of the lipogenic enzyme stearoyl Co-A desaturase, whereas high-performing pigs show higher amounts of SFA (Pugliese and Sirtori 2012). Usually, oleic acid is the major fatty acid in pork being, however, more abundant in neutral lipids than in phospholipids. In opposition, LA, as well as the *n*-6 and *n*-3 long-chain polyunsaturated fatty acids (PUFA) are mainly found in phospholipids than in triacylglycerols. The greater incorporation of LA into pig muscle yields higher proportions of arachidonic acid (AA, 20:4*n*−6) and the final result is a higher *n*−6/*n*−3 PUFA ratio in comparison to ruminants (Enser *et al.* 2000; Cooper *et al.* 2004). Conversely, the PUFA/SFA ratio is much higher, and so beneficially, in pigs and other monogastrics compared to ruminants.

Fatty acid profile of meat has attracted substantial interest because of its implications for human health (Duran-Montgé *et al.* 2008). In fact, nutritional recommendations with a major focus on cardiovascular health have emphasised the requirement to decrease consumption of some SFA since dietary saturated fat and cholesterol play a crucial role in the development of metabolic disorders, like atherosclerosis and coronary heart disease. Nonetheless, the relationship between dietary saturated fat and coronary heart disease is controversial because the heterogeneous biological properties of the different individual SFA (Mozaffarian 2015; Liu *et al.* 2017). Particular SFA, such as lauric (12:0), myristic (14:0) and palmitic (16:0) acids have been recognised to exert atherogenic effects by rising plasma cholesterol and low-density lipoprotein (LDL) levels (Siri-Tarino *et al.* 2015). In contrast, stearic acid (18:0) has been shown to be neutral with respect to serum LDL-cholesterol concentrations. Considering PUFA, it is well known that these fatty acids are integral structural components of cell membranes of tissues involved in a wide range of physiological and pathophysiological processes (Simoupoulos 2001; Kouba

and Mourot 2011). Both *n*-6 and *n*-3 PUFA participate in cell regulation, act as direct modulators of gene expression, and contribute to signal transduction (Ma *et al.* 2016). LA and alpha-linolenic acid (ALA, 18:3*n*−3), the metabolic precursors from the *n*-6 and *n*-3 PUFA families, respectively, cannot be synthesised *de novo* and, consequently, must be supplied by the diet due to the absence of biosynthetic enzymes (delta12 and delta15 desaturases) in mammals.

Essential fatty acids (LA and ALA) are absorbed into the bloodstream and mainly transported to the liver, in which they undergo desaturation and/or elongation through delta5 and delta6 desaturases and elongase enzymes for the synthesis of long-chain PUFA. Unfortunately, Western diets are low in *n*-3 PUFA, notably in eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*−3), and rich in SFA and *n*−6 PUFA, mainly LA (Prates and Bessa 2009). While diets rich in *n*−3 PUFA are associated with potential human health benefits, decreasing the risk of heart disease and cancer, diets rich in *n*−6 PUFA are effective in increasing AA content in membrane phospholipids, which then outcomes in the overproduction of eicosanoids of the series 2 and 4, and so, incrementing arterial and other chronic detrimental conditions (Simopoulos 2002; Sioutis *et al.* 2008). The high *n*−6 PUFA level in the human food supplies have always been a cause for concern because these fatty acids can interfere with the conversion of ALA to *n*−3 long-chain PUFA (EPA and DHA). Furthermore, low PUFA/SFA and high *n*−6/*n*−3 PUFA ratios of some meats are known to contribute for the fatty acid intake imbalance in consumers (Wood *et al.* 2008).

International health organizations and many government agencies encourage reduction of the overall fat consumption to 30% of total energy intake and the reduction of saturated fat consumption to 10% of total energy intake (FAO/WHO 2008). More recently, it was recommended a total fat intake between 20 and 35% of total energy (FAO/WHO 2010). For instance, within the context of a healthy dietary pattern, consumers have been advised to limit the consumption of saturated fat (< 10% of energy intake) and *trans* fatty acids (TFA, less 1%), as well as increasing the intake of PUFA (up to 6–11% of total energy), namely 0.5–2% of total energy from *n*−3 PUFA and 2.5–9% of total energy from *n*-6 PUFA (EFSA 2012; USDA 2015). The Joint FAO/WHO Expert Consultation on Fats and Fatty Acids in Human Nutrition also recommends that MUFA intakes should be 15–20% of energy, according to total fat intake. MUFA intakes should be determined by calculating the difference between total fat (% of energy) and fatty acid composition of the diet (SFA, PUFA and TFA, % of energy). Finally, dietary guidelines for PUFA/SFA and *n*-6/*n*-3 PUFA ratios in Western diets are 0.4 or above and less than 5, respectively (ISSFAL 2004).

3 Pork Production with Low Protein Diets

The genetic selection towards reduced subcutaneous fat, mainly in the case of white European breeds (Large White and Landrace), has dramatically reduced the amount of IMF, or marbling, in commercial crossbred pigs (Jeremiah *et al.* 1999). However, IMF content is the main attribute affecting meat sensory characteristics and an important economic trait in pork production (Wood *et al.* 2003). In addition, fatty acid composition of IMF plays an important role on meat quality and, as a result, an adequate proportion

of SFA, MUFA and PUFA should be maintained in order to assure high standards of nutritional value and eating quality (Wood *et al.* 2008). Therefore, the production of pork with moderate amounts of IMF and a balanced fatty acid composition, without an increase in subcutaneous adipose tissue, is highly desirable for the meat industry and pork consumers. Thus, in last years, some nutritional feeding strategies to increase pig fat partitioning have been developed by the pig industry to produce meat with high quality to satisfy consumers' demands, and to make pork production more efficient with low costs. The most efficacious of these feeding strategies are based on reduced protein diets (RPD), alone or combined with amino acids or other ingredients, mainly oils. Table 1 summarises the main findings on the influence of dietary reduced protein on pork production.

It is well known that RPD (protein reduction from 15 to 20%) increase IMF content in the growing finishing phase of pigs and, consequently, can improve pork sensory scores. The reduction in protein content in diets results from a decrease concentration in all dietary amino acids. This is particularly important to the lysine content, which is the first limiting amino acid for muscle protein synthesis in pigs, i.e., the synthesis is limited if there is no available lysine for metabolism. Castell *et al.* (1994) reported that a reduction in dietary protein levels (11.9%, 13.3%, 14.8%, 16.2% and 17.6%) increased marbling and IMF contents in *longissimus dorsi* muscle of pigs. Different dietary low protein levels in different commercial lean crossbred pigs, like Large White, Landrace and Duroc, increased IMF content in *longissimus dorsi* muscle (D'Souza *et al.* 2003; Wood *et al.* 2004; Teye *et al.* 2006; Wood *et al.* 2013; Madeira *et al.* 2013a; Madeira *et al.* 2014a; Madeira *et al.* 2015; Suárez-Belloch *et al.* 2016).

The increase of IMF content can improve sensory attributes, like tenderness and juiciness. Some studies reported that RPD increase tenderness and juiciness in *longissimus lumborum* muscle (Castell *et al.* 1994; Wood *et al.* 2004; Wood *et al.* 2013; Madeira *et al.* 2013a; Madeira *et al.* 2015). Carcass characteristics and meat quality traits are also affected by RPD. Teye *et al.* (2006) reported that RPD increased muscle colour and lipid oxidation. Furthermore, RPD decreased loin weight and hot carcass weight, but increased colour parameters (L* and b*) (Madeira *et al.* 2014a; Madeira *et al.* 2015).

Doran *et al.* (2006) reported that reduced dietary protein level increases total fatty acids in porcine muscle, and this fact was related with higher activity of a key lipogenic enzyme, the stearoyl-CoA desaturase, indicating that MUFA synthesis played a role when IMF accumulation was promoted by low protein levels. Madeira *et al.* (2013) reported that reduced protein, equilibrated or not with lysine, had no influence on IMF content in Alentejano purebred, a fatty genotype, which was about twofold greater in the crossbred than in Alentejano pigs. The mechanisms underlying these effects are tissue- and genotype-specific and seem to be associated with the regulation of key lipogenic genes by RPD, which can lead to the increase of *de novo* fatty acid synthesis (Madeira *et al.* 2013b; Madeira *et al.* 2014b; Madeira *et al.* 2016). The genotype-specific effect of RPD on IMF content is related to lysine limitation and occurs via upregulation of the lipogenic enzyme stearoyl-CoA desaturase. Lysine requirements of growing pigs are directly proportional to protein accretion rate (NRC 1998).

Madeira *et al.* (2017) reported that although Alentejano pigs have higher IMF in comparison with crossbred pigs, RPD increased IMF content in the *psoas major* muscle

Table 1. Effect of low protein diets alone or combined on pork quality. The studies are presented by chronological order.

Low protein diet, alone or combined	Genotype	Trial weight/age	Main findings	References
18–12% CP	Landrace × Hampshire pigs	25–98 kg live weight	- Increased marbling, IMF content, tenderness and juiciness in LD muscle	Castell *et al.* (1994)
19–17% CP	Large White × Landrace × Duroc female pigs	68–124 days (grower) 125–159 days, 100 kg (finisher)	- Increased IMF content	D'Souza *et al.* (2003)
20–16% CP 1.14–0.68% Lys	Two modern breeds: Duroc and Large White and two traditional breeds: Berkshire and Tamworth male pigs	9–21 week age	- Produced fatter carcasses, especially in the two modern breeds - Increased IMF content in LD and *psoas major* muscles - Increased pork juiciness and tenderness - Decreased pork flavour	Wood *et al.* (2004)
21–18% CP with oils (palm kernel, palm and soybean)	50% Duroc × 25% Large White × 25% Landrace intact male pigs	40−100 ± 10 kg live weight	- Increased IMF, MUFA, muscle colour, juiciness, tenderness and lipid oxidation of the LD muscle - Decreased PUFA/SFA ratio of LD muscle	Teye *et al.* (2006)
19.5–15.3% CP Combined with linseed or sunflower oil	Male Landrace pigs (castrates)	60–120 kg live weight	- No effect on IMF content of the *longissimus* muscle - Increased *n*−3 fatty acids and decreased *n*−6/*n*−3 PUFA ratio for pigs fed linseed oil-containing high- and RPD	Dannenberger *et al.* (2012)
18.9–16.6% CP 1.1–1.1% Lys 17.1–14.5% CP 1.0–0.7% Lys 15.2–12.7% CP 0.8–0.5% Lys	Entire male pigs (Large White × Landrace)	40–60 kg live weight 60–85 kg live weight 85–115 kg live weight	- Increased subcutaneous and intermuscular fat in the carcass and IMF in *longissimus* and *semimembranosus* muscles, and oleic acid - Increased tenderness and juiciness - Decreased linoleic acid in both muscles	Wood *et al.* (2013)
17.5–13% CP 0.7–0.4% Lys	Alentejano purebred and commercial Large White × Landrace × Pietrain crossbred male pigs	60–93 kg live weight	- RPD equilibrated for lysine had no effect on backfat thickness - RPD not equilibrated for lysine increased IMF content and pork sensory traits in crossbred pigs - No effect on IMF content in Alentejano genotype	Madeira *et al.* (2013a)

(*continued*)

Table 1. (*continued*)

Low protein diet, alone or combined	Genotype	Trial weight/age	Main findings	References
16% CP, 13% CP, 13% CP with 2% Leu, 16% CP with 1% Arg, 13% CP with 1% Arg and 13% CP with 2% Leu and 1% Arg	Entire male pigs Duroc × Pietrain × Large White × Landrace crossbred	59–92 kg live weight	- Arg had no effect on IMF content - Arg produced meat off-flavour, increased meat tenderness and overall acceptability - RPD increased IMF content, back fat thickness, 18:1*c*9, MUFA, SFA and PUFA - RPD decreased loin weight - Leu had no effect on IMF content, back fat thickness or loin weight - RPD and Leu increased juiciness	Madeira *et al.* (2014a)
16.0% CP, 13.0% CP, 13.0% CP with 0.33% betaine, 13.0% with 1.5% Arg, 13.0% CP with 0.33% betaine and 1.5% Arg	Entire male pigs from a commercial crossbreed (Duroc × Large White × Landrace)	60–93 kg live weight	- RPD increased IMF, juiciness, L* and b* - RPD decreased HCW and loin weight - RPD, betaine and Arg had no effect on IMF - RPD, betaine and Arg increased overall acceptability - Arg increased tenderness - No effect on fatty acid composition	Madeira *et al.* (2015)
21.6% CP and 1.10 Lys 17.7% CP and 0.91% Lys 14.7% CP and 0.78% Lys 13.5% CP and 0.52% Lys	Duroc × (Landrace × Large White) pigs, 50% barrows and 50% gilts	26–123 kg live weight	- Increased IMF, MUFA and meat colour - Decreased PUFA	Suárez-Belloch *et al.* (2016)

CP, crude protein; IMF, intramuscular fat; LD, *longissimus dorsi*; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; RPD, reduced protein diets; SFA, saturated fatty acids.

in Alentejano and commercial crossbred pigs. This increase could be mediated by shifting the metabolic properties of fibres from glycolytic type to oxidative type, as described by Pires *et al.* (2016) for the *longissimus lumborum* muscle. This process was based on a significant enrichment of contractile proteins, calcium-signalling proteins and glycolytic enzymes in the skeletal muscle (Pires *et al.* 2016). Moreover, Alentejano purebred had a higher percentage of oxidative fibres and a lower percentage of glycolytic fibres (Madeira *et al.* 2017). This finding points towards a muscle-specific effect of RPD in different types of skeletal muscle, red and white.

Several studies revealed that RPD also change the fatty acid composition of muscle. Teye *et al.* (2006) reported that feeding a low protein diet resulted in higher concentrations of myristic acid, palmitic acid, total SFA and MUFA but in lower concentrations of individual and total PUFA and PUFA/SFA ratio, which reflects the higher content of IMF. In addition, Dannenberger *et al.* (2012) reported that muscle fatty acid concentration is affected by diet, resulting in higher *n*-3 fatty acid levels. However, in another study, the percentage of LA was lower, while oleic acid was higher in IMF from RPD (Wood *et al.* 2013). RPD equilibrated for essential amino acid increased IMF in *longissimus*, but not in *semimembranosus* muscle. When pigs were fed RPD with lower amino acid levels, both muscles showed reduced percentages of LA (Wood *et al.* 2013). In addition, Madeira *et al.* (2014a) reported that protein reduction increased meat deposition of oleic acid, MUFA, SFA and PUFA, but decreased loin weight. In addition, Suárez-Belloch *et al.* (2016) reported that these diets increased MUFA and decreased PUFA content. This effect is likely a consequence of the distinct distribution of fatty acids between triacylglycerols (richer in SFA) and phospholipids (richer in PUFA) and the increasing proportion of triacylglycerols with increasing IMF content (De Smet *et al.* 2004). The differences of fatty acid composition between pigs fed high and low protein diets may be due to the variance of stearoyl Co-A desaturase activity.

The combined effects of RPD with some amino acids or oils are not very conclusive. However, RPD with leucine supplementation increased juiciness (Madeira *et al.* 2014). In turn, RPD combined with betaine and arginine had no effect on IMF content, but increased the overall acceptability of meat. Finally, Dannenberger *et al.* (2012) reported that IMF did not increase in animals fed RPD with vegetable oils.

4 Pork Production with Dietary Microalgae

Pork products have been associated with an unhealthy image due to the relative lower and higher proportions of PUFA and SFA, respectively (Morgan *et al.* 1992). The possibility of changing fatty acid composition in pork via diet has been widely established (Václavková and Bečková 2007), being much easier than in ruminants (Nurnberg *et al.* 1998). The level of feed intake and composition regulates the rate of fatty tissue growth and the composition of lipids (Václavková and Bečková 2007). Changing the pigs´ diet provides an effective method of altering the fatty acid composition of pig fat depots, thereby modifying the human dietary fat intake from pork (Wood and Enser 1997). In fact, mathematical relationships were developed fifteen years ago by Nguyen *et al.* (2003) between the intake of $n-6$ and $n-3$ PUFA and their contents in the adipose tissue of growing pigs. The strong correlations on PUFA content observed between feed and fat tissue clearly indicate that fatty acid composition of tissues may be used as an index of fatty acid composition of the diet, and *vice versa* (Nguyen *et al.* 2003). The ultimate goal is to turn meat into a healthier product for the consumer. In this sense, a number of studies have attempted to increase pork PUFA content by increasing the $n-3$ fatty acids. Functionally, the most important $n-3$ fatty acids are EPA and DHA, although the roles for docosapentaenoic acid (DPA, 22:5$n-3$) are now also emerging (Kaur *et al.* 2011). In most Western populations, the distribution of fatty fish consumption is bimodal, with a smaller proportion of the population being regular fish consumers (Calder 2017). Yet,

in general, the intakes of EPA and DHA are typically small and much lower than what is recommended (Calder 2006; Calder 2012). This fact raised substantial interest in food enrichment with EPA and DHA from marine origin.

For livestock production, the nutritional value of microalgae is extremely variable. Up to 30% of the current world algae production is commercialised for animal feed (Becker 2004). The predominant microalgae species are *Schizochytrium* sp., *Chlorella* sp., *Arthrospira* sp., *Isochrysis* sp. and *Porphyridium* sp. In this respect, *Arthrospira* sp. is responsible solely for 50% of worldwide production as feed supplement (Yamaguchi 1997) due to its excellent nutritional composition and easy digestibility attributed to a low content of carbohydrates (Yaakob *et al.* 2014). Several species of heterotrophic microalgae have been acknowledged as biofactories for commercial *n*-3 long-chain PUFA. Particularly, whereas heterotrophic microalgae are well established as an alternative source of DHA, autotrophic microalgae have been used for EPA production. For example, *Thraustochytrium* and *Schizochytrium limacinum* contain DHA between 30–40% of total fatty acids when cultivated heterotrophically, whereas *Phaeodactylum tricornutum* and *Nannochloropsis* sp., which are autotrophic, display an EPA content up to 39% of total fatty acids (Adarme-Vega *et al.* 2012). These results are very promising and unexpectedly surpass those found in marine wild fish. In general, the use of small amounts of microalgae biomass in feed can benefit animals' physiology by improving immune response, disease resistance, antiviral and antibacterial action, gut function and probiotic colonization stimulation, resulting in a wide spectrum of benefits, including animals' weight control, growth promotion, improved feed conversion ratio and reproductive performance (Harel and Clayton 2004).

Some nutritional experiments using microalgae supplementation have successfully improved pork quality (Table 2). In general, different microalgae, mainly *Arthrospira platensis*, *Chlorella* sp. and *Schizochytrium* sp., have no effect on pork quality traits, such as colour, pH, meat oxidative stability and cooking loss (Sardi *et al.* 2006; Banoch *et al.* 2012; Simkus *et al.* 2013; Vossen *et al.* 2017). In order to study the effect of fresh blue algae *Arthrospira platensis* biomass on pigs fattening rate and meat quality, Simkus *et al.* (2013) carried out an experiment with 85-days-old crossbreds of Landrace and Yorkshire. Two groups, control and experimental, were formed, each containing 16 pigs. When daily ration of pigs was supplemented by 2 g of 75% humidity of fresh blue algae *Arthrospira platensis* biomass, dressing percentage in the experimental group of pigs was increased by 2.0% relative to the control group. In turn, the amount of IMF in meat decreased by 0.33% (Simkus *et al.* 2013). The non-variation in meat quality traits reported by Banoch *et al.* (2012) was probably due to the small level (0.0002%) of *Chlorella* spp incorporated. Moreover, iodine-enriched *Chlorella* spp. has been shown to retain iodine in the muscle at a higher extent than inorganic potassium iodine (KI). Even so, iodine concentrations in meat remained low, limiting the recognition of *Chlorella* spp. as a significant iodine source for human nutrition (Banoch *et al.* 2012).

The inclusion of dietary *Schizochytrium* sp., a marine algae containing a high level of DHA, increased DHA levels in both loin and subcutaneous fat and decreased *n*-6/*n*-3 ratio in barrows with 118 kg of body weight (Sardi *et al.* 2006). No significant differences in the relative proportions of fatty acids were observed between pigs receiving the marine algae product supplement at the lower level (2.5 g/kg) over an 8-week period and pigs

Table 2. Effect of dietary microalgae on pork quality. The studies are presented by chronological order.

Microalga	Level in the diet and duration	Animal and initial weight/age	Main findings	References
Schizochytrium sp.	32.5−65 g of DHA/pig over the last 4 weeks prior to slaughtering	Landrace × Large White barrows with 118 kg live weight	- No effect on pork quality traits, as pH and meat colour - Increased DHA and decreased *n*-6/*n*-3 ratio in loin and subcutaneous fat	Sardi *et al.* (2006)
Schizochythrum sp.	0, 0.125 and 0.250 kg of DHA from microalga/pig from day 22 to 42	Market hogs	- Increased eicosapentaenoate, docosahexaenoate, and docosapentaenoate $n-6$ and $n-3$ in ham, loin and shoulder muscles by 0.250 kg of DHA - Increased docosapentaenoate $n-6$ and docosahexaenoate by 0.125 kg of DHA	Marriott *et al.* (2007)
Chlorella sp.	2 mg iodine/kg from *Chlorella* spp for 3 months	Landrace × Czech Large White female pigs with 3 months age	- No differences in meat quality traits - Iodine from microalga retained in muscle in a higher extent than inorganic KI	Banoch *et al.* (2012)
Arthrospira platensis	2 g of 75% fresh alga biomass with forage until pig's weight reached 95 kg	Landrass × Yorkshire crossbreds with 85-days-old	- Decreased intramuscular fat by 0.33%	Simkus *et al.* (2013)
Schizochytrium sp.	0.3, 0.6, or 1.2 g microalga/100 g feed for 45 days	Crossbred female pigs with 75 kg live weight	- No effects on carcass quality and fresh meat oxidative stability - No effect on consumers' acceptance and sensory analysis of dry cured hams - Increased concentrations of DHA in loin and dry cured hams	Vossen *et al.* (2017)

DHA, docosahexaenoic acid.

receiving the higher marine algae product supplement (5.0 g/kg) over a 4-week period prior to slaughter (Sardi *et al.* 2006). In the experiment of Marriott *et al.* (2007), market hogs were fed a ration from 22–42 days prior that contained DHA supplemented at 0.125 and 0.250 kg/pig in the form of *Schizochythrum* sp. From the 21 fatty acids analysed, the concentration of only five was affected by DHA supplementation. Muscles from the ham, loin and shoulder of pigs that received 0.250 kg of DHA contained more EPA, DHA, DPA *n*-6 and *n*-3 than either the controls or those receiving solely 0.125 kg. Supplementation of DHA in the ration at 0.125 kg caused an elevation of DPA *n*-6 and DHA. These data suggest that addition of DHA to the diet can elevate the amount of this *n*-3 fatty acid in pork with potential health benefits to consumers (Marriott *et al.* 2007). Vossen *et al.* (2017) produced dry cured hams and loins enriched with DHA using three doses of *Schizochytrium* in the diet of pigs: 0.3, 0.6, or 1.2 g algae/100 g feed. Two control treatments were included: a diet containing soybean oil and a linseed oil diet high in ALA. *Schizochytrium* microalgae supplementation allowed increasing the concentration of DHA in the loin and dry cured ham (Vossen *et al.* 2017). The nutritional value of these meat products was considerably high, without affecting carcass quality and oxidative stability of the fresh meat. The increased DHA content of the dry cured hams had no influence on the consumers' acceptability in terms of appearance, aroma, texture and flavour. However, the oxidative stability, colour and instrumental texture parameters were slightly affected by the algae treatments (Vossen *et al.* 2017).

In general, microalgae are endowed with recalcitrant cell walls, composed by an incredibly diverse and complex matrix of cross-linked insoluble carbohydrates, which make them only digestible by monogastric animals in small amounts (<1–2% in the diet). Therefore, it is necessary to develop novel technologies to improve microalgal nutrient utilization and facilitate the cost-effective use of microalgae for animal feed industry (Lum *et al.* 2013). The organisms specialised in the utilization of the energy stored in these cell wall structures produce carbohydrate-active enzymes (cazymes), which are complex multi-modular enzymes in which the catalytic module(s) is(are) appended to one or more non-catalytic carbohydrate binding modules (CBM) (Fontes *et al.* 2010). Exogenous cazymes are now well accepted as a class of feed additives in diet formulations for pigs and poultry to overcome the negative effects of anti-nutritional factors, and to improve digestion of dietary components and animal performance (Ravindran *et al.* 2011). Therefore, the use of cazymes is, in some circumstances, a cost-effective strategy to improve the nutritional value of cereal-based diets for monogastrics, which remains to be established for microalgal biomass. In the last few years, our research group has been testing specific cazymes for use in feeds incorporated with high amounts (5–15%) of microalgae (Provisional Patent number 115108/2018, INPI, Portugal).

5 Conclusion and Future Perspectives

Dietary protein reduction, alone or combined with other ingredients, contributes to satisfy consumer requirements and to enhance the competitiveness of the meat industry with higher pork quality and lower production costs. RPD have an important effect on fat partitioning modification leading to increased IMF content. This increased IMF can improve pork quality traits, mainly sensory traits.

Data described above revealed that the mechanisms regulating fat deposition in pigs are genotype- and tissue-specific. It was found that RPD improve fat partitioning and meat sensory score due to lysine limitation, in all muscle types of lean pig genotypes but only in red muscles of fatty pig genotypes. It was revealed that the increased IMF is mediated by shifting the metabolic properties of fibres from glycolytic to oxidative, by the up-regulation of key lipogenic enzymes and related transcription factors.

The use of microalgae is a major nutritional challenge in the near future. Overall, the inclusion of microalgae in feed represents a promising approach for the maintenance and development of the livestock sector, as an environmental friendly alternative to balance food-feed-biofuel industries. Although conversely, feeding sources of *n*-3 fatty acids to pigs increase their content in pork, but results have been highly variable.

The use of exogenous cazymes is a very promising cost-effective strategy to degrade the recalcitrant polysaccharides of microalgae cell walls and to improve the nutritional value of algae for pigs. In addition, the degradation of microalgae cell walls by cazymes provides oligosaccharides (prebiotics) that may substitute antibiotics, mainly in weaned piglets.

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Growth and Development of the *Lusitano* Foal on Extensive Systems

M. J. Fradinho[1(✉)], R. J. B. Bessa[1], R. M. Caldeira[1], and G. Ferreira-Dias[2]

[1] Animal Production Systems Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
mjoaofradinho@fmv.ulisboa.pt

[2] Reproduction and Development Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal

Abstract. One of the main goals of horse breeding industry is to raise animals with a sound and adequate musculoskeletal system that withstands the rigors of training and competition. Therefore, the knowledge of the most adequate growth model for each breed and purpose, as well as the factors that influence skeleton development, is of high concern for breeders and users. The Lusitano horse is no exception. This Portuguese breed has become more popular as a sport and leisure horse due to its functional and behavior characteristics. In this review we will bring about an integrated approach on the characterization of the Lusitano production systems, both for the broodmare productive cycle and further foals' growth and development. In particular, the application of non-linear models to foals' growth data from birth to the onset of training, has enabled a comprehensive overview on growth patterns of the Lusitano horse when managed in extensive systems, which constitutes innovative information for this breed. Based on the achievements of our research team, the relationship among growth patterns, bone metabolism and osteoarticular quality are also highlighted.

Keywords: Lusitano breed · Skeleton development · Musculoskeletal system · Extensive systems

1 Introduction

In the horse, growth models have not been studied as much as in other animal species. After birth, and depending on the breed, growth and development can last three to five years, which represents a large proportion of its productive life (Martin-Rosset et al. 2015a).

Nowadays, sportive utilization is becoming more and more intensive and begins at an earlier age, when the skeleton development may not yet be completed. Therefore, the knowledge of the most adequate growth model for each breed and purpose, as well as the factors that influence skeleton development, is of high concern for horse breeding industry. The study of bone metabolism is particularly important in sport breeds, in order to provide an adequate muscular-skeletal condition throughout the horse life. The

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Lusitano horse is no exception. This Portuguese breed has been recognized as one of the world's best saddle horses, combining a good temperament with easy and comfortable gaits. Overall growth models have been proposed for other breeds (Staniar et al. 2004; Heugebaert et al. 2010). However, despite the common believe that Lusitano horse reaches its maturity at a later age, growth and development processes in the Lusitano foal were less characterized.

Lusitano stud farms are traditionally based on extensive grazing systems, where mares and foals are often bred outdoors throughout the year. In these systems, pastures represent a significant part of diets, but herbage production is commonly limited by Mediterranean climate conditions. Under such conditions and as other livestock females, mares store and mobilize body reserves during their productive cycle (Martin-Rosset et al. 2006). It is known that an adequate amount of body reserves may influence the reproductive performance of the mare and can also influence productive parameters such as milk production and the subsequent foals' growth (Henneke et al. 1984; Doreau et al. 1986). Therefore, a more accurate information regarding the nutritional status and body reserves management in the Lusitano mare, may contribute for better decisions on the most appropriate feeding plans and foaling seasons, in order to improve the efficiency and profitability of these production systems. In addition, a better knowledge of the growing process, in relation to bone metabolism and quality, will be pivotal to support more adequate management options during the growth period of the Lusitano foal.

2 The Lusitano Breed: A Brief Characterization

2.1 Origin

The presence of horses in the Iberian Peninsula, goes back to an age around the transition between the Early and the Middle Pleistocene (van der Made 1999, 2013). According to Andrade (1954), the horse began to be ridden in the Iberian Peninsula, even before the first millennium BC, continuing to be used in subsequent ages for warfare. From the times of Roman occupation until the Middle Age, the horses raised in the plains of rivers Tagus, Guadiana and Guadalquivir, in the southern Iberian Peninsula kept the shape, the size and the character despite the successive invasions. For Europe's Renaissance, the "Genet d' Espagne" (name used then), was the true blooded horse and was exported to everywhere as enhancer of other breeds in order to produce light horses (Andrade 1954).

The Lusitano designation was adopted in 1942 for horses born in Portugal, which presented the morphological and functional characteristics of the breed and with a known genealogy that allowed its acceptance under such denomination (Monteiro 1983). In 1967, the Lusitano Studbook was formalized (Monteiro 1983) and in 1989, the Portuguese Association of Lusitano Horse Breeders (APSL) was created in order to expand and to promote the breed. Currently, the Lusitano horse is spread around the world with approximately 5,000 registered breeding mares (Vicente et al. 2012). The growing interest in the Lusitano breed outside Portugal led to the formation of breeders' associations in 19 countries, with a significant number of animals in Brazil, France, Mexico, Spain, Italy, Belgium and United Kingdom, among others (Vicente 2015).

2.2 Morpho-Functional Characteristics

The great diversity of the existing horse breeds is mainly described based on characters related to their external morphology and proportions. Therefore, morphometry becomes an indispensable tool for an objective characterization of the breeds and the consequent definition of their standards (Monteiro 1983; Oom 1992). In addition to biometrical parameters, which are objective and quantifiable, the morphological characterization of the horse includes also some qualitative and subjective features regarding conformation (*e.g.* head profile, type of neck or orientation of the ears) (Vicente 2015).

There are several methodologies that can be used in order to quantify biometrical variables. The direct measurements on live animals with simple instruments like a height measuring stick and flexible measuring tapes are the most common methods reported in biometric studies, both in growing (Kavazis and Ott 2003; Valette et al. 2008; Fradinho et al. 2016) and in adult horses (Oom 1992; Zechner et al. 2001; Cabral et al. 2004; Gomez et al. 2009; Komosa et al. 2013). Other methodologies like photogrammetric methods (Thompson 1995; Barrey et al. 2002) or the 3-D video morphometric measurements have been applied (Weller et al. 2006; Santos 2008; Kristjansson et al. 2013).

The breed Standard describes an ideal model for the Lusitano horse in which a large set of morphological characteristics are described (APSL 2016). According to this standard, the Lusitano is a medium shaped horse, with a mean weight of 500 kg and with a sub-convex profile throughout the body (rounded outlines), which silhouette can be fitted into a square. The height at withers, measured at six years old should be about 1.55 m for females and 1.60 m for males (APSL 2016).

In the horse, morphologic and conformational traits have important implications on the limits or range of movements and function. Therefore, these characteristics will have an impact on performance (Barrey et al. 2002; Santos 2008; Vicente 2015). The selection of the Lusitano horse was traditionally based on functional features that arose from its continued use in the fieldwork, handling of wild cattle, and in bullfight on horseback. It is also widely accepted that the former way of combat on horse, called "gineta", which required agility and quickness of turn back was fundamental for the maintaining of functional characteristics that were later used in bullfighting (Andrade 1954; Cordeiro 1997). Nowadays, the selection of the Lusitano horse for functionality has become increasingly important as the breed has been more used in high-level sport competitions, achieving very good results, in dressage, driving and working equitation (Solé et al. 2014; Vicente 2015). Recent studies in the Lusitano breed aiming at evaluating quantitative kinematic traits, identified some positive results in what concerns the swing phase duration and the range of motion of the elbow, hock and pelvis joints, characteristics that are objectively relevant to sports performance (Solé et al. 2014). In a previous study, it was also reported a relation between Lusitano morphologic traits like the length of the fore pastern and the slope of the hind pastern, and the ability for bullfighting, associated with a maximum elevation of fore and hind limbs (Santos 2008). All these morphological and functional features, together with its behavioural and temperamental characteristics, reinforce the recognition of the Lusitano as one of the best saddle horses in the world.

2.3 The Lusitano Production Systems

Historically, Lusitano horse production in Portugal was mainly located in the former regions of Ribatejo and Alentejo, and it was generally associated to big livestock farms with large agricultural surfaces (Monteiro 1983). Data from the "Registo Nacional de Equinos" showed that between 1987 and 2012, the average number of horses born and registered by breeder (including the Lusitano) was nine in the NUT 2 region of Lisboa and 14 in the NUT 2 region of Alentejo. These data confirm that nowadays, those regions still represent the most important centers of horse production in our country (Fontes and Jorge 2013). According to information provided by APSL, there are about 350 Lusitano active stud-farms in Portugal, totalizing around 700 worldwide.

The majority of Lusitano stud farms are based in extensive grazing systems (EGS). In these systems, mares are often bred outdoors throughout the year, being pastures a significant part of diets. Pastures can be natural or sown, temporary or permanent (renewed when necessary), irrigated or rainfed, and they are essentially constituted by plants of two botanical families: grasses and legumes (Paço and Fradinho 2011). For southern regions under Mediterranean climate, rainfed pastures are based in annual species and have a peak production in spring, a pause in summer and a smaller peak in autumn, which further decreases due to low temperatures in winter (Salgueiro 1984). When grass production is scarce, supplementary feeds (concentrate feeds and preserved forages) are generally used, but farm practices vary widely (Paço and Fradinho 2011). This type of systems contrasts to the majority of sport and race horse breeds in temperate and cold regions, where the mares stay indoors during the winter and only turn out to pasture in the spring (Miraglia et al. 2006; Martin-Rosset et al. 2015b).

In some Lusitano stud farms, where water availability is not a limiting factor during the summer, grass production from irrigated pastures has become also an important feed resource for the global feed management of the herd. The introduction of irrigated pastures in the production system has several advantages: it avoids the lack of green grass during the summer, providing forage with high nutritive value during a long period and, if well managed, could be also harvested for hay or haylage production, to be used as good quality preserved forage during the winter time. In this type of system, rotational grazing is often implemented and stoking rates are normally higher than in EGS. Whatever the grazing system, one of the challenges in horse production is to cope with the specific nutritional requirements of some stages of the mare productive cycle and foals' growth.

The ovarian activity of the mare is seasonal. In the northern hemisphere, an anovulatory phase starts in the Fall when the length of the day is decreasing, whereas ovarian cyclicity starts when day length is rising. Therefore, the breeding season occurs in the spring when the daylight, temperature and availability of feed increases (Ferreira-Dias et al. 2005; Guillaume et al. 2006). In our latitudes, most of the stud farms manage mare' reproductive cycle in order to concentrate foaling in spring, when pasture production is higher. In fact, the effects of feeding management and foaling season on nutritional status of Lusitano broodmares throughout the gestation/lactation cycle, were investigated by our team. Our results showed that changes in body weight and body condition (BC) in the Lusitano broodmare, when managed on extensive grazing systems, were mainly influenced by pasture availability and quality and the time when foaling season occurs in the year. Corresponding to pasture cycle, a general increase in body reserves was

observed during spring followed by a mobilization until winter, although this change does not represent more than half point of BC, in a 0–5 points scale (INRA-HN-IE 1997; Fradinho et al. 2013). Another important observation drawn from this study was the marked effect of the early foaling on mares' BC, in particular those that were mainly dependent on grazing resources. Mares that foaled early in the season showed a poorer or even no recovery of BC during the spring and a lower level of BC throughout the whole cycle. Nevertheless, higher growth performances through the first three months of life were observed in early born foals, which may indicate a higher milk production. In fact, the shift of nutrients for milk production at this stage, would justify a less effective recovery of mares' BC during the spring in comparison with mares that foaled later in the year (Fradinho et al. 2013).

The Lusitano foaling season is normally spread between January and May and mating usually takes place in the month following parturition. The type of breeding allowed for the Lusitano is the natural service and artificial insemination using fresh, refrigerated or frozen semen, although there is a fixed number of mated mares per stallion in the same year. In the last update of the Studbook Regulation also embryo transfer was allowed, but only a maximum of three animals from each donor mare may be registered per year (APSL 2016).

In ideal conditions, it is important that the mare conceives again during the first month after foaling in order to obtain a foal per year and to promote the efficiency of the system (Martin-Rosset et al. 2015b). But, the success of reproductive performance is generally limited because it is influenced by a wide range of factors. Among these, nutritional status and energy balance, which are reflected by BC and its changes, have been related with the reproductive efficiency of the mare (Henneke et al. 1984; Guillaume et al. 2006; Cavinder et al. 2009). In addition, on extensive systems the reproductive performance of the Lusitano broodmare showed to be highly influenced by the nutritional status in the early postpartum period (Fradinho et al. 2014). In this study, BC changes at conception had a strong effect on fertility outcome of the first two estrous cycles after foaling, being highly impaired by negative changes, whatever the BC score. On the contrary, best fertility results were obtained with positive and greater BC changes. According to our model, the best predictive results regarding the probability to conceive during the two first estrous cycles after foaling (above 85%) were obtained for a BC score at breeding between 3.0 and 3.75 (0–5 scale; INRA-HN-IE 1997) and with a positive variation of 0.25 points (Fradinho et al. 2014). Another relevant observation of this work was the effect of mares' nutritional status on the growth of suckling foals. Foal growth performance appears to be influenced by mares BC score changes during the first three months of lactation, with lower growth rates observed in foals which dams presented negative BC changes throughout this period (Fradinho et al. 2014).

The lactation peak in the Lusitano mare occurs between the first and the second postpartum month, reaching a milk yield of 14 kg/day and a daily average milk yield of 12 kg during the first four months of lactation (Santos and Silvestre 2008). Until weaning, Lusitano foals are kept with their mothers on pasture. During the first two months, they are mainly fed with mares' milk, but from this age on, grazing time increases and frequency of nursing decreases (Martin-Rosset et al. 2015a). In the Lusitano production systems, the last two or three months before weaning always matches with the period of

low or null pasture availability. During these periods, preserved forages are commonly distributed, but the use of concentrate feeds as creep-feeding is not wide spread. A recent survey conducted in 31 sport horse stud farms in Portugal, where more than half were Lusitano stud farms, showed that the creep-feeding is only practiced in 23% of the studs (Barbosa et al. 2015).

The weaning of foals occurs generally in the fall, between six to seven months of age, with the full separation from the dams on that day. After weaning, foals are normally kept together during an adaptation period where they are group or individually fed with preserved forages and concentrate feeds, and turn out to pasture before Spring. At this time, they are normally separated by sex. Up to three years of age, colts and fillies remain outdoors and likewise broodmares, when pasture amount and quality decrease, supplementary feeds are timely provided. It was also observed, in Lusitano stud farms, a growing tendency to supply a daily complement to the pasture, in all groups of growing foals (one, two and three years old) (Barbosa et al. 2015). At the end of the spring in which they complete three years old, they are normally stabled for breaking in and for the beginning of training (Paço and Fradinho 2011).

3 Growth and Development of the Foal

Growth is defined as the increase in live weight and body dimensions as a function of time (Martin-Rosset et al. 2015a). At a cellular level, growth is reflected by both the increase in cell number (hyperplasia) and cell size (hypertrophy) (Owens et al. 1993; Staniar 2013). Growth rate is usually measured by live weight expressed as mass unit per day (*e.g.* g or kg/day). Development occurs through a set of events from conception to adulthood. At its most basic level, development involves the coordinated regulation of cell proliferation, cell death (apoptosis), cell migration, and differentiation (Butler and Le Roith 2001). During gestation, the embryo evolves through various stages to become a fetus and then, at parturition, a foal. Until adult age, morphological, anatomical and chemical modification will follow, resulting in sexual and physical maturation. Development is measured by comparing weight, size or anatomical and chemical composition of a region or tissue at a given age to a reference element (Martin-Rosset et al. 2015a). From conception to maturity, the pattern of equine growth can generally be described as a sigmoid curve (Martin-Rosset 2005; Staniar 2013). However, the expression of growth is highly dependent on genetic background and environmental factors (Fitzhugh 1976; Staniar 2013; Martin-Rosset et al. 2015a). Among these, breeders' goals and local management, including feeding practices, has been shown to have a large influence on the lifetime growth pattern of the horse (Bigot et al. 1987; Pagan 2005). After birth, and according to the genetic type and use, growth period for light breed horses lasts from 3 to 5 years of age, which represent a large percentage of their productive life (Martin-Rosset et al. 2015a). Considering the importance of this period on the future performance and productivity of the horse, the knowledge and characterization of growth and development is of utmost importance for breeders and users.

3.1 Pre-foaling Growth and Development

Most of the studies reported in literature regarding fetal growth and development in the horse were based on data from foals that were aborted or stillborn either in light breeds or ponies (Meyer and Ahlswede 1976; Platt 1978, 1984; Giussani et al. 2005). According to these data several equations (power, exponential and polynomial models) were proposed for predicting BW of the fetus from the day of gestation (NRC 2007; Coenen et al. 2011; Martin-Rosset et al. 2015b). During the first half of the gestation period, there is only a small increase in weight of the fetus. The bigger increase in weight is observed in the last third of gestation whatever the model used (Fig. 1).

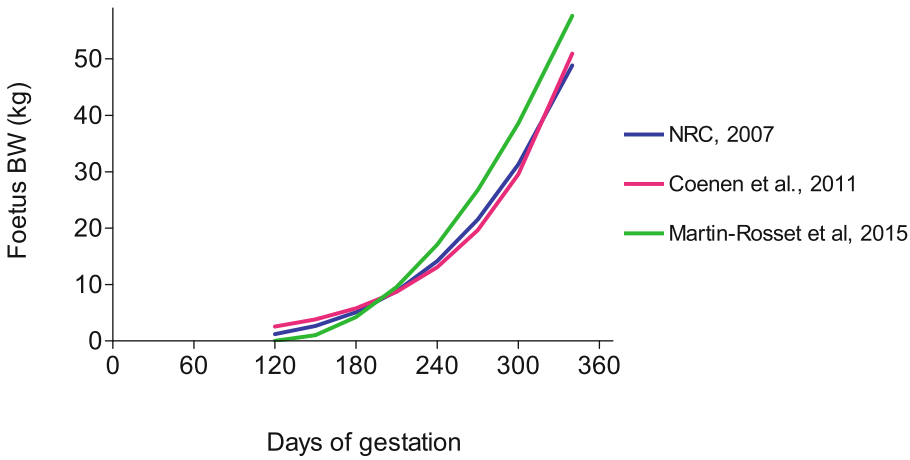

Fig. 1. Modeling of fetal body weight during gestation, considering foal birth weight as 10% of the mare body weight after foaling, and mare body weight as 500 kg. Equations: Fetal weight (as percent of birth weight) $= 1 \times 10^{-7}\ X^{3.5512}$ (NRC 2007); Fetal weight (as percent of birth weight) $= e^{0.0136\ X}$ (Coenen et al. 2011); Fetal weight (kg) $= 17.38 - 0.2885\ X + 0.001197\ X^2$ (Martin-Rosset et al. 2015b) X = days of gestation

The development in body length observed on Thoroughbred (aborted and stillborn fetus) and measured as crown-rump length tended to precede the increase in body mass and seems to be steadier from the beginning to the end of the gestation period (Platt 1978) (Fig. 2).

The influence of maternal size on fetal development was studied based on crosses between different size horse breeds (Walton and Hammond 1938; Tischner 1985; Peugnet et al. 2014). These studies showed that fetal growth can be either enhanced above, or restricted below, the normal genetic potential for the breed by varying maternal size. The rate and extent of fetal growth is also influenced by the area of placental interface, and birth weight is related with the mass, gross area and volume of placenta (Allen et al. 2002).

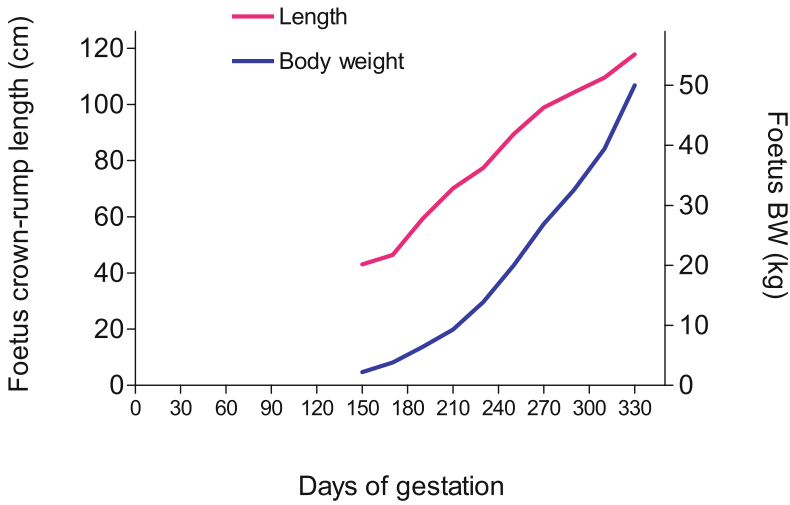

Fig. 2. Fetal growth observed in Thoroughbred (mean crown-rump length and body weight from 150 days to term of gestation (adapted from Platt 1978).

3.2 Growth and Development from Birth to Adulthood

Considering BW and body size at birth, the foal seems to be more advanced in terms of growth when compared to newborns from other livestock species, namely bovine (Martin-Rosset et al. 1983). According to the breed, BW at birth ranges from 8 to 12% of adult BW, representing average values of 15 to 35 kg for ponies, 45 to 55 kg for light breeds and 65 to 80 kg for draft horse breeds (Martin-Rosset et al. 2015a). In the light breeds, which includes most of the saddle horses, some birth weights were reported for Thoroughbred: 54 to 58 kg (Kavazis and Ott 2003; Brown-Douglas et al. 2005; Pagan et al. 2006; Elliot et al. 2009), Anglo-Arab and "Selle Français": 53 kg (Bigot et al. 1988), Purebred Arabian: 46 kg (Çilek 2009) and Lusitano: 52 kg (Fradinho et al. 2016). At birth, the skeletal of the foal is more developed than muscle or fat tissue (Martin-Rosset et al. 2015a). Therefore, for most breeds, height at withers (WH) measured at birth represents already approximately 60% of mature height (*e.g.* 63% for Anglo-Arab and "Selle Français", Bigot et al. 1988; 62% for Mangalarga Marchador and for the Lusitano, Cabral et al. 2004; Fradinho et al. 2016). Average WH values observed for Thoroughbred foals at birth were 102 to 103 cm (Kavazis and Ott 2003; Pagan et al. 2006), 96.7 cm for Arabian foals (Çilek 2009), and 100 cm for the Lusitano (Fradinho et al. 2016).

During the first months after birth growth rates are very high. Average daily gains (ADG) of 1.443 kg/day and 1.233 kg/day were observed, respectively, in Thoroughbred and in a group of Anglo-Arab and "Selle Français" foals during the first month of life (Bigot et al. 1988; Pagan et al. 2006). From birth to six months of age ADG values of 0.900 to 1.000 kg/day were observed in the same breeds (Bigot et al. 1988; Jelan et al. 1996). At weaning (six to seven months of age), BW of foals can be almost five times its birth weight, representing about 45% of its adult BW (Martin-Rosset 2005). After this age and depending on the breed and management conditions, ADG progressively

decline until maturity. The increase in WH is also very high during the first months, declining gradually after the six months (Jelan et al. 1996; Martin-Rosset et al. 2015a; Fradinho et al. 2016). For most of the light breeds, at one year of age WH represents already 88 to 90% of mature height, reflecting the earlier development of the skeletal tissue (Cabral et al. 2004; Martin-Rosset 2005; Fradinho et al. 2016). In fact, allometric coefficients obtained from the comparative slaughter method showed that bone, muscle and adipose tissue develop chronologically in this order, being respectively 0.74, 1.13 and 1.41 (Martin-Rosset et al. 2015a). Moreover, considering the different skeletal segments, it was observed that bones in the extremities (e.g. metacarpus) develop earlier in relation to the whole skeleton (Martin-Rosset et al. 1983).

In order to obtain a comprehensive overview on growth patterns of the Lusitano foal when managed in extensive systems, some non-linear growth functions (Brody, Logistic, Gompertz, von Bertalanffy and Richards) were applied to foals' growth data (BW, WH, girth (G) and cannon circumference (CC)) from birth to the onset of training (Fradinho et al. 2016). The Richards's function [$y = A(1 - b.exp(-kt))^M$] was the best fit model for all the variables, and parameter estimates are presented in Table 1.

Table 1. Parameter estimates of the Richards function fitted to body weight, withers height, girth and cannon circumference-age data sets in Lusitano foals (adapted from Fradinho et al. 2016)

Measure[a]	Parameters[b]				R^2 [c]	RSD[d]
	A (±SE[e])	b (±SE[e])	k (±SE[e])	M (±SE[e])		
BW (kg)	552.4 ± 22.0	0.986 ± 0.006	0.0010 ± 0.0002	0.570 ± 0.028	0.936	28.8
WH (cm)	158.6 ± 0.8	0.942 ± 0.012	0.0018 ± 0.0002	0.164 ± 0.008	0.954	3.23
G (cm)	197.8 ± 3.6	0.985 ± 0.004	0.0008 ± 0.0001	0.204 ± 0.007	0.958	5.08
CC (cm)	19.9 ± 0.2	0.893 ± 0.036	0.0022 ± 0.0003	0.215 ± 0.026	0.866	0.79

[a]BW – body weight; WH – withers height; G – girth; CC – cannon circumference.
[b]A – asymptotic value for BW/WH/G/CC as age approaches infinity (interpreted as mean BW/WH/G/CC at maturity); b – scaling parameter that defines the degree of maturity when age = 0 d (intercept on y axis); k – maturing index (rate that establishes the spread of the curve along time axis); M – determines the point of inflexion of the curve (for $0 < M < 1$, M is undefined).
[c]R^2 correspond to a pseudo R^2, calculated as 1 – (SS(Residual)/SS($\text{Total}_{\text{corrected}}$)).
[d]RSD – residual standard deviation.
[e]SE – approximate standard error.

In long-term growth studies with large volume of data, models which are nonlinear in their parameters adjust better to data and, usually, have an easier biological interpretation (Fitzhugh 1976). Thus, A is the asymptotic value of y as age (t) approached infinity, and is commonly interpreted as the mean mature size; b is a scaling parameter that adjusts for situations where y_0 and/or t_0 do not equal to 0 (for example, when only postnatal observations are available and t_0 is taken as birth); k is a maturing index, establishing the earliness with which y approaches A (large k values indicate early maturing individuals and small k values indicate late maturing individuals); M determines the point of inflection where the estimate growth rate changes from an increasing to a decreasing function

(for $0 < M < 1$, M is undefined) (Richards 1959; Brown et al. 1976; Fitzhugh 1976; Perotto et al. 1992; Staniar et al. 2004). For each body measure, the first derivative of Richards equation was calculated with respect to time ($\delta y/\delta t$), expressing the instantaneous rate of gain (ADG, kg/d or cm/d) at time t (t = days of age): $y' = MAkb.\exp^{(-kt)} (1 - b.\exp^{-kt})^{M} (1 - b.\exp^{-kt})^{-1}$.

The curve fit adjustments for BW, WH, G and CC using Richards equation and corresponding estimates of ADG as the first derivate of the function are presented in Fig. 3.

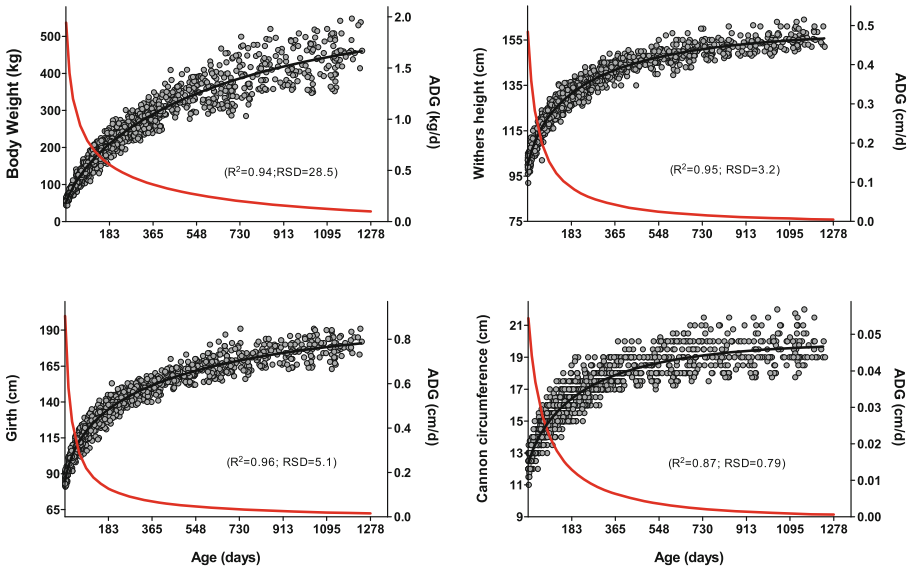

Fig. 3. Best fit adjustments (black lines) for individual data points regarding body weight, withers height, girth and cannon circumference using the Richards equation. Red line represents ADG (kg/d or cm/d) for each variable. (Fradinho et al. 2016)

The apparent late maturity of this breed, concerning BW, was confirmed by a lower maturing index and lower ADG, which were comparable with the moderate growth levels proposed for other sport horses (Table 2).

In contrast, the maturing index and the pattern of WH growth observed for the Lusitano were similar to those found in earlier maturing breeds like the Thoroughbred. These results showed that skeletal development may follow a between-breed similar pattern and confirms the recognized precocity of bone tissue.

The potential for growth and production performance of the horse is determined by its genetic background, but the expression of growth related traits is highly influenced by environmental factors. The individual genetic effect on body size and live weight is high, and huge differences can be found from small ponies to draft horse breeds (e.g. less than 150 kg in the Miniature horse or Shetland Ponies to 1100 kg in the Shire breed). Estimated heritability coefficients (h^2) for morphologic traits vary according to the breed and to body size parameter. In the Murgese, Noriker draught, Lipizzaner and Pura Raza

Table 2. Comparison of body weight average daily gain (g/day) estimates at standard age-periods for the Lusitano horse with published values for a moderate growth in sport horses and for Thoroughbreds (Fradinho et al. 2016)

	Measure[a]	Age-periods (months)				
		3–6	6–12	18–24	30–36	36–42
Lusitano[b]	ADG (g/d)	669	443	233	141	111
Sport horses[c]		650–750	400–500	300–350	50–100	50–100
TB[d]		939	681	247	91	51

[a]ADG – average daily gain (g/day).
[b]ADG values for Lusitano were estimated from the resolution of the first derivative of Richards function obtained in the present study.
[c]ADG range values indicated for a moderate growth of sport horses expected to mature at 500 kg (INRA 2012).
[d]TB – Thoroughbred; ADG values for TB were estimated from the resolution of the first derivative of Richards function obtained by Kocher and Staniar (2013).

Española breeds, observed h^2 for WH and girth perimeter range, respectively, between 0.24 and 0.67, and 0.26 and 0.48 (Molina et al. 1999; Zechner et al. 2001; Dario et al. 2006; Druml et al. 2008). Sexual dimorphism is also reported both for growing and adult horses. At the adult age, stallions are on average 10% heavier than mares (Martin-Rosset 2005). A study with Thoroughbred foals showed that colts were heavier than fillies at birth and the differences increased with age (Hintz et al. 1979). The effect of sex on BW and WH was also observed in a large study with Thoroughbred foals until weaning (Pagan et al. 2006). In the Finnhorse, sex of foal primarily influenced cannon bone circumference, and males tended to be taller, longer and wider than females (Saastamoinen 1990). The same result was observed in the Lusitano horse, with a significant effect of sex reflected in higher mature values for males, in what concerns BW, WH, girth or cannon circumference (Table 3) (Fradinho et al. 2016). The difference between the mature BW estimated for Lusitano male and female was even slightly higher than the 10% value indicated for light breeds (Martin-Rosset 2005).

Nutrition plays a major role among the environmental factors that influence growth and development. Body weight and body size increases with feed intake level (Cymbaluk et al. 1990; Donabédian et al. 2006) and is influenced by the nature of diet, namely by the type of forage or concentrate composition (Bigot et al. 1987; Ott and Kivipelto 2002; Ott et al. 2005). The introduction of creep-feeding during mid-lactation can improve growth performances of the nursing foal and improve general condition after weaning (Coleman et al. 1999; Rezende et al. 2000). Growth of the nursing foal is also influenced by the month of foaling or season of the year, which is closely linked with pasture availability (Hintz et al. 1979; Pagan et al. 2006; Kocher and Staniar 2013; Fradinho et al. 2013; 2016) and mare nutritional status (Fradinho et al. 2014). Thoroughbred foals born in January and February had lower BW than foals born in March, April and May during the first three months of age, but these differences disappeared at five months of age (Pagan et al. 2006). This observation was explained by an increase in the ADG of the

Table 3. Parameters estimates and standard errors of Richards equation fitted to body weight, withers height, girth and cannon circumference as function of age (days), according to sex in the Lusitano breed (Fradinho et al. 2016).

Measure[a]	Sex	Parameters[b]				R^{2} [c]	RSD[d]
		A (±SE[e])	*b* (±SE[e])	*k* (±SE[e])	*M* (±SE[e])		
BW (kg)	Males	610.5 ± 39.9	0.982 ± 0.009	0.0010 ± 0.0002	0.61 ± 0.04	0.956	25.4
	Females	525.3 ± 25.0	0.988 ± 0.007	0.0010 ± 0.0002	0.54 ± 0.03	0.932	28.3
WH (cm)	Males	161.5 ± 1.5	0.944 ± 0.016	0.0017 ± 0.0002	0.17 ± 0.01	0.961	3.06
	Females	157.6 ± 1.0	0.945 ± 0.015	0.0017 ± 0.0002	0.16 ± 0.01	0.956	3.10
G (cm)	Males	202.9 ± 6.4	0.985 ± 0.006	0.0008 ± 0.0002	0.21 ± 0.01	0.969	4.52
	Females	196.3 ± 4.7	0.987 ± 0.005	0.0008 ± 0.0002	0.20 ± 0.01	0.952	5.34
CC (cm)	Males	21.4 ± 0.4	0.926 ± 0.035	0.0016 ± 0.0004	0.21 ± 0.03	0.895	0.77
	Females	19.3 ± 0.1	0.883 ± 0.048	0.0026 ± 0.0004	0.21 ± 0.03	0.893	0.64

[a]BW – body weight; WH – withers height; G – girth; CC – cannon circumference.
[b]*A* – asymptotic value for BW/WH/G/CC as age approaches infinity (interpreted as mean BW/WH/G/CC at maturity); *b* – scaling parameter that defines the degree of maturity when age = 0 d (intercept on y axis); *k* – maturing index (rate that establishes the spread of the curve along time axis); *M* – determines the point of inflexion of the curve (for $0 < M < 1$, *M* is undefined).
[c]R^2 correspond to a pseudo R^2, calculated as 1 – (SS(Residual)/SS($\text{Total}_{\text{corrected}}$)).
[d]RSD – residual standard deviation.
[e]SE – approximate standard error.

lighter foals coinciding with spring pasture growth in April. The influence of the season on growth rates may be also observed in the Lusitano foal when individual ADG are plotted against the corresponding day of year, (Fig. 4). This influence is particularly noticeable in BW and girth ADG from the second spring onwards. Apparently, WH growth rate was not so influenced by the season and, although with a bigger dispersion, CC followed a similar pattern.

In fact, like other livestock species, growing horses are able to undergo compensatory growth periods when the quantity and quality of feeds, namely pasture, becomes available after a period of energy deprivation. However, this ability to undergo compensatory growth will decrease with age (Bigot et al. 1987; Staniar 2013; Martin-Rosset et al. 2015a).

Another environmental factor that has been reported as having an effect on growth and development of the foal is the exercise conditions (Martin-Rosset 2005; Martin-Rosset et al. 2015a). However, as this effect is mainly observed at bone tissue level, it will be discussed in the next point.

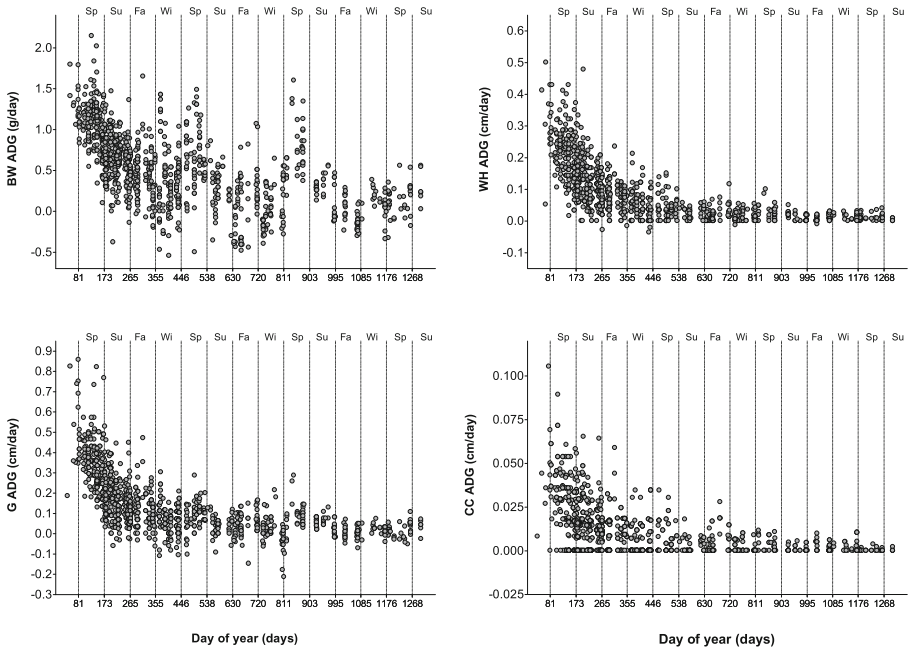

Fig. 4. Average daily gains for body weight (BW ADG), withers height (WH ADG), girth (G ADG) and cannon circumference (CC ADG) based on day of year, considering zero as the 1st of January of the birth year. The second and the third years start, respectively, at 366 and 731 days. Each dotted line represents the beginning of a season based on spring (Sp) (March 20th) and fall (Fa) (September 23rd) equinoxes and summer (Su) (June 21st) and winter (Wi) (December 21st) solstices in northern hemisphere (Fradinho et al. 2016).

3.3 Growth Patterns, Bone Development and Implications on Osteoarticular Quality

The musculoskeletal structure of the horse is adapted to maximize locomotor efficiency at an early age. Foals can gallop a few hours after birth and, in most breeds, training for athletic performance starts when full growth has not yet been achieved (Firth 2006).

Bone tissue development and quality is highly influenced by feed intake level and diet composition (Thompson et al. 1988a, b; Ott and Asquith 1989, 1995; Hoffman et al. 1999; Trillaud-Geyl et al. 2004; Martin-Rosset 2005), but bone characteristics like mineral content, mineral density and the morphology of the mineralized tissue are also highly influenced by exercise conditions (Firth and Rogers 2005; Firth 2006). Several studies showed that free or controlled exercise increases bone mineral density and content, and increase cannon bone circumference either in the weanling foals or young horses (Raub et al. 1989; McCarthy and Jeffcott 1992; Bell et al. 2001; Hiney et al. 2004). In addition, stall confinement of young foals or even conditioned adult horses favors a decrease in bone mineral content (Porr et al. 1998; Hoekstra et al. 1999).

One of the main goals of horse breeding industry is to raise animals with a locomotor system that withstands the rigors of training and competition. Nevertheless,

growth-related disorders associated to bone tissue development remain one of the biggest problems that breeders face until today (McIlwraith 2004; Jeffcott 2005).

3.3.1 Developmental Orthopaedic Diseases

The widely accepted term "Developmental orthopaedic diseases" (DOD) appeared in 1986 in order to group into a single category all the orthopaedic problems observed in the growing foal (McIlwraith 2004). It encompasses a spectrum of conditions that are mainly associated to disturbances in the development and maturation of the musculoskeletal system, in particular in the articular and metaphyseal cartilage, during skeletal growth (Jeffcott 2005). The DOD designation includes all the conditions grouped under the general agreed terms of: osteochondrosis (OC); physitis; angular limb deformities; flexural deformities; cuboidal bone deformities or tarsal bone collapse; cervical vertebral malformation (Wobbler syndrome); and acquired vertebral deformities (McIlwraith 2004; Jeffcott 2005). Recently, the new term "Juvenile osteochondral conditions" was proposed to describe the subgroup of DOD that are related to juvenile disorders of either the articular-epiphyseal cartilage complex and/or the physis, resulting in articular (joint) or physeal (metaphyseal growth plate) disorders, and thus, excluding tendon-related flexural limb deformities and the neurological disorder, Wobbler Disease (Denoix et al. 2013).

The time of onset and the predisposed sites for each of the conditions varies, and in many instances, multiple sites and multiple limbs of the foals can be affected. However, a number of factors is thought to be involved in their complex aetiopathogenesis.

Among the different conditions under the common DOD designation, OC has been the main research subject in the growing horse as it affects almost all breeds and because of the economic losses it generates to the worldwide horse industry (van Weeren and Brama 2005; van Weeren and Jeffcott 2013).

Osteochondrosis is a dynamic disturbance in the natural process of endochondral ossification during growth, with a complex multifactorial etiology (Dik et al. 1999; van Weeren and Brama 2001; Denoix et al. 2013). Genetic predisposition and environmental factors such as exercise/biomechanical stress, nutrition, growth rate and local/endocrine disturbances in the cartilage have been generally implicated in this condition (Barneveld and van Weeren 1999; Donabédian et al. 2006; Ytrehus et al. 2007; Distl 2013).

A detailed discussion regarding all the factors that have been recognized as having an influence on the manifestation and course of OC is beyond the scope of this revision. However, some studies have explored growth parameters as a potential risk factor for this condition. In a controlled experiment in Selle-Français foals with different nutritional levels, Donabédian et al. (2006) showed an association between OC and fast development of some skeletal segments, being WH at early ages, the variable most frequently implicated. Nevertheless, in this experiment, a high but balanced feeding level per se was not a sufficient factor for the occurrence of DOD. Fast growth was also positively correlated with the occurrence of OC lesions in Thoroughbred foals (Gee et al. 2005) and a predisposition of taller horses has been established for OC in young Hanoverian (Sandgren et al. 1993; Stock et al. 2006).

In a cohort study conducted in Normandy, a high growth in girth perimeter together with irregular exercise conditions was associated with a poor osteoarticular status in

sport breed weanlings (Lepeule et al. 2013). Previously, the same group of researchers had identified WH at 30 days of age and the slope of WH growth curve in the first six months after birth, as one of the risk factors for the presence of juvenile osteochondral conditions (which includes OC) (Lepeule et al. 2009). The relationship between growth patterns of the Lusitano horse and long-term changes on bone quality, bone metabolism, growth factors and metabolic related variables, were recently investigated by our research team, in a longitudinal field study. In this study the presence of radiographic findings compatible with OC-like lesions at the onset of training was associated with changes in BW and WH growth patterns. The larger maturing index obtained in the BW growth model of positive foals (using the Richards function) indicated that these animals had higher growth rates in early stages when compared to negative OC foals (Fradinho et al. 2019).

Despite all the complex etiology ascribed to OC condition, the body of evidences drawn from these studies emphasized the importance of an early monitoring of foals' growth. In particular, sudden changes to the average growth rates should be avoided. These results may complement the current knowledge regarding some management practices in our production systems, including the prevention of inadequate diets, in order to promote a sound skeletal development and a better osteoarticular quality of the Lusitano horse.

4 Final Remarks

In the horse, growth and development are particularly high during the last stage of the gestation and the first 6 months of life. After birth, Lusitano foals' growth performance appears to be influenced by mares BC score changes during the first three months of lactation, with lower growth rates observed in foals which dams presented negative changes of BC scores during this period. Therefore, foaled mares should be gaining body reserves throughout the postpartum period in order to enhance fertility and to support an adequate milk production for the growth of the suckling foal.

The detailed characterization of BW, WH, G and CC growth patterns based in the adjustment of non-linear models, provided innovative information for the Lusitano breed. Compared with other sport breeds, the Lusitano foal managed on grazing systems presented moderate BW growth rates. In contrast, it appears to have similar WH growth patterns of earlier maturing breeds. Body weight and G growth rates were prone to short-term deviations, with ADG changes generally observed during winter and springtime. These ADG changes showed the well-known seasonal compensatory growth ability related with pasture availability and quality.

In the light of the current knowledge regarding the multifactorial etiology of osteochondrosis, the implication of growth characteristics as a risk factor for a poor osteoarticular status was also shown for the Lusitano breed. Foal's growth should be carefully monitored early after birth and, in particular, during the first year of life, in order to prevent the effects associated to sudden changes of average growth rates. Thus, an early implementation of adequate management practices, including the inclusion of balanced diets is vital for breeders, in order to promote a sustained growth and a better osteoarticular quality of the Lusitano horse.

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Assisted Reproductive Technologies (ART) Directed to Germplasm Preservation

R. M. L. N. Pereira[1,2(✉)], C. C. Marques[2], J. Pimenta[1,2], J. P. Barbas[1,2], M. C. Baptista[2], P. Diniz[1], A. Torres[1], and L. Lopes-da-Costa[1]

[1] Reproduction and Development Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
rosa.linoneto@iniav.pt

[2] Genetic Resources and Biotechnology Unit, National Institute of Agrarian and Veterinary Research (INIAV), Santarem, Portugal

Abstract. The preservation and sustainable management of animal genetic resources became an international priority due to the worldwide decline of biodiversity and animal fertility, to the climatic change and to the society and market behaviors. The *in situ* conservation, allied to the *ex situ* cryopreservation of gametes and embryos are ancillary tools for the widespread and maintenance of animal biodiversity, including the preservation of endangered native breeds. Developments in assisted reproductive technologies (ART) opened new possibilities for the preservation of germplasm and fertility. Germplasm quality is a key limiting factor in both male and female fertility preservation. As such, knowledge on the cellular and molecular mechanisms behind germplasm competence may highlight tools to improve its successful preservation. This review focus on animal germplasm evaluation and preservation, supported by studies addressed at CIISA's Reproduction and Development Laboratory and INIAV.

Keywords: Animal conservation · Biodiversity · Reproductive biotechnologies · Cryopreservation · Oocyte · Spermatozoa · Embryos

1 Introduction

Concern superimposed by the world population growth, and global climate change, threatening decline of biodiversity and fertility is ever growing. Farm animal genetic diversity is critical for food provision, ecosystem homeostasis, and sustainable agriculture and rural development. However, in the past decades, farm animal genetic resources have been facing an alarming rate of extinction and genetic erosion, as hundreds of uniquely adapted breeds became extinct. Therefore, the conservation and sustainable management of animal genetic resources became a challenging as well as a vital goal of modern society (FAO 2015; 2018; Pereira 2017).

Advances in assisted reproductive technologies (ART) allowed novel solutions for germplasm preservation and infertility management. Germplasm quality is a key limiting factor for its successful cryopreservation. Improvement of germplasm (gametes and

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embryos) cryotolerance is imperative to improve its subsequent developmental competence in order to achieve acceptable fertility rates after artificial insemination (AI) and embryo transfer (ET). This review discusses recent strategies devoted to this goal. The review covers CIISA's Reproduction and Development Lab and INIAV studies in the fields of germplasm lipid metabolism, membrane permeability and cryobiology, gene transcription and protein expression of gametes and embryos, and embryo-maternal communication.

2 The *In Situ* and *Ex Situ* Preservation of Animal Genetic Resources

Human progress has been dependent on livestock since at least the advent of agriculture, and even the most modern post-industrial societies remain critically reliant on animals for human nutrition. However, due to the world population growth, the climate change, the economic globalization, and financial constraints, the genetic resources of domestic animals are facing an alarming rate of extinction and genetic erosion (FAO 2018; Zhang et al. 2018). Current efforts to conserve biodiversity comprise a patchwork of international and national goals and plans, and local interventions that, overall, are failing. Nowadays, most indicators of the state of biodiversity show declines, whereas indicators of pressures on biodiversity are increasing. The global decline of biodiversity does not appear to be slowing and reports on the extinction of species and breeds, including those of domestic animals, continue to arrive (Butchart et al. 2010; FAO 2015; Arlidge et al. 2018). According to the Second Report on the State of the World's "Animal Genetic Resources for Food and Agriculture" (2000–2014), approximately 561 uniquely adapted breeds became extinct and up to 17% of world livestock breeds are currently endangered. Horses, cattle and sheep are the domestic species with the highest number of breeds at risk. Domestic breeds are considered endangered when attain the threshold of less than 1000 females and 20 fertile males alive. Further below this threshold the breed is considered critically endangered. In these cases, ART, including gamete and embryo cryopreservation, AI, ET and *in vitro* fertilization (IVF) may represent an option to increase the reproductive rate of the endangered population (FAO 2015).

The richness in animal genetic resources of the Mediterranean area places this region in a key position regarding overall biodiversity. Relative to the world number of livestock breeds, Mediterranean breeds represent about 16% for cattle, 18% for goats, 21% for sheep, 16% for horses and 18% for pigs (Gama 2006). Portugal owns an animal inheritance with a high genetic diversity, which includes 61 recognized domestic breeds, most of them now considered endangered. In this scenario, the preservation and management of Portuguese animal genetic resources becomes a national priority in compliance with international organizations. Facing this distressing reality, different breed conservation strategies have been implemented. To meet FAO recommendations, conservation programs should include three categories of actions - *in situ*, *ex situ in vivo*, and *ex situ in vitro* conservation - implemented in a coordinated way due to their complementary roles (FAO 2012). *In situ* conservation strategies alone are expensive and may be unable to ensure the maintenance of animal genetic resources, due to natural disasters or even human activity (Gandini et al. 2007). Therefore, the cryopreservation of gamete and

embryos in animal germplasm banks allows for the *ex situ* conservation of genetic biodiversity, also contributing to dynamically maintaining live populations in their native environments. For semen banks, to maximize genetic diversity, in each breed, a minimum of 25 sperm doses collected from each of 25 unrelated males must be cryopreserved. For somatic cells or embryo banks, 25 unrelated males and 25 unrelated females should be considered. If the number of available animals is below this threshold, then they should all be included in the program, irrespective of the relationships among them (FAO 2012, 2015). All the above rationale was consolidated in the establishment of the Portuguese Bank of Animal Germplasm in 2005 at INIAV-Santarém (Pereira and Marques 2011; Pereira 2017).

3 Cryopreservation of Gametes and Embryos

3.1 Cryobiology Principles

Cryopreservation is the long time preservation of cells and tissues at sub-zero temperatures ideally without loss of viability after warming. Low temperatures can induce the crystallization of water, creating ice crystals and changes in intracellular solute concentrations that may injure the cells. The mechanical damage induced by ice crystals can be avoided when the freezing process is accelerated, whereas the use of cryoprotectants (CPAs) can decrease chemical damage (Sieme et al. 2015; Arav and Saragusty 2016). These CPAs are classified according to their ability to cross cell membranes, as penetrating or non-penetrating. Various combinations of penetrating (e.g. dimethyl sulfoxide (DMSO), glycerol, 1,2 propanediol (PrOH), ethylene glycol (EG)) and non-penetrating (e.g. sucrose, glucose, fructose, trehalose) CPAs can be used.

In the early 50s bovine spermatozoa were among the first to be successfully cryopreserved, which gave rise to worldwide AI programs. Spermatozoa cryopreservation protocols are now available for most domestic species (Barbas and Mascarenhas 2009). Sperm kinematics and response of sperm subpopulations during cryopreservation provide critical information for the optimization of cryopreservation protocols (Barbas et al. 2018). For female gametes and embryos two main cryopreservation techniques are currently used: slow freezing and vitrification (reviewed by Pereira and Marques 2008). In slow freezing cells are exposed to a low concentration of CPAs, whereas vitrification requires a high concentration of CPAs. The higher the concentration of CPAs, the higher the glass transition temperature (Tg), lowering the chance of ice nucleation and crystallization (Arav 2014). Vitrification aims to eliminate ice crystal formation in both the extra- and intracellular compartments, where it induces the formation of a non-crystalline amorphous solid (Mandawalla et al. 2016). However, vitrification associated high concentrations of CPAs are potentially toxic. To circumvent toxicity, exposure to CPAs must be brief and in a multistep fashion, combining increasing concentrations of penetrating CPAs with non-penetrating CPAs, followed by an ultra-rapid cooling into liquid nitrogen (−196 °C), inside small media volumes to allow better cold transfer. Thawing requires an ultra-rapid warming to near body temperatures, exposing germplasm to non-penetrating CPAs (usually sucrose), to avoid hypoosmotic shock during rehydration. Failure to proceed through these steps may cause irreversible plasma membrane damage and/or disruption of organelles (Agca et al. 1998a; Karlsson et al. 2014). Similar

steps are used in slow freezing, although applying much lower CPA concentrations and cooling rates. Therefore, in slow freezing the osmotic stress and CPA toxicity is less significant, but the ability to prevent ice-crystal formation is limited.

3.2 Permeability Features

During cryopreservation cells are submitted to extreme volume changes due to the movement of water and CPAs through the plasma membrane. This induces a cumulative osmotic stress that may compromise cell viability (Agca et al. 1998a, b; Matos et al. 2015). In the presence of penetrating CPAs, the water flux outside the cells and the CPA flux inside the cells are expected to occur simultaneously, inducing cell volume changes (swelling and shrinkage). These flux patterns change during development and are species specific (Agca et al. 1998b; Pedro et al. 2005; Jin et al. 2011). When crossing the plasma membrane, water and CPAs can follow two different pathways: diffusion through the lipid bilayer and/or facilitated diffusion through channels (Agca et al. 1998a, b; Jin et al. 2011, 2013). In oocyte and cleavage-stage embryo membranes, water and CPAs mainly move through the lipid bilayer and the flow rates are low and dependent on temperature. However, in bovine oocytes, the expression of aquaporin 3 (AQP3) also allows movement of water and some CPAs through channels. In contrast, in morulae and blastocyst membranes, water and CPAs mainly move through facilitated diffusion via channels (Agca et al. 1998b; Edashige et al. 2006; Jin et al. 2011). Aquaporins (AQP3, AQP7 and AQP11) detected in boar and bovine spermatozoa were related to sperm cryotolerance (Prieto-Martinez et al. 2017; Fujii et al. 2018). Besides species and developmentally specific, fluxes are CPA dependent. In bovine morulae, glycerol and EG mainly move by facilitated diffusion via channels, mostly through AQP3, whereas DMSO predominantly moves by diffusion through the lipid bilayer. The facilitated diffusion via channels markedly increases permeability, which becomes less dependent on temperature. In this case, a long exposure to CPAs will increase toxicity (Jin et al. 2011). Concentration and exposure time of CPAs must be balanced to reduce toxicity and osmotic stress (Szurek and Eroglu 2011).

The multistep equilibration process using increasing levels of penetrating CPAs allows the cell to shrink and to re-expand in steps, avoiding the critical minimum cell volume, but allowing a sufficient efflux of intracellular water to prevent ice formation (Lai et al. 2015; Marques et al. 2018). For instance, although EG plus DMSO and PrOH permeate into bovine oocytes at comparable rates, PrOH induced the oocyte highest minimum volume (Marques et al. 2018; Fig. 1), implying that water was not completely depleted, and insufficient concentrations of CPA were loaded. Oocytes cryopreserved using this PrOH protocol resulted in the lowest embryo rates after IVF. Therefore, controlling the minimum cell volume (above critical but low enough) and the flux rates is crucial for minimizing damage during the addition and removal of CPAs (Lai et al. 2015; Matos et al. 2015).

3.3 Lipids in Cryopreservation

The lipid content of gametes and embryos is related to their cryotolerance (Pereira and Marques 2008; Awasthi et al. 2010; Prates et al. 2014), as a high intracellular lipid

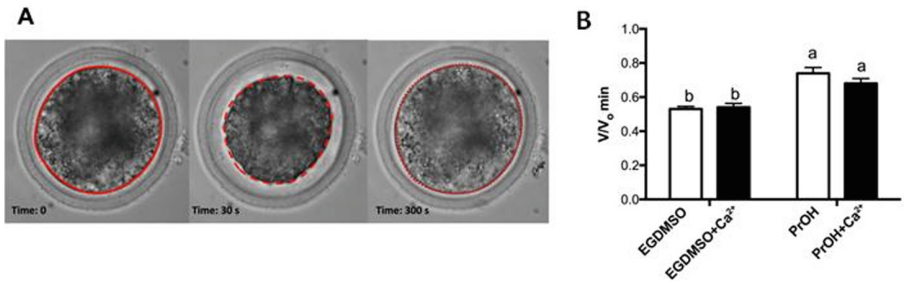

Fig. 1. Oocyte membrane permeability of different cryoprotectants in calcium free or -containing solutions (adapted from Marques et al. 2018). A) Representative illustration of oocyte volumes along a cryoprotectant permeability assay; initial equilibrium volume Vo (left panel), minimum volume Vmin (middle panel) and final equilibrium volume V∞ (right panel) after an osmotic challenge with ethylene glycol plus dimethylsulfoxide (EGDMSO 7.5% + 7.5%) to evaluate EGDMSO membrane permeability. B) Oocytes minimum volumes (V/Vo min) attained after the addition of cryoprotectant (EGDMSO (7.5% + 7.5%) and PrOH 2M) before oocyte reswelling. The lower minimum volume was observed for EGDMSO independently of Ca^{2+} in the media. Different small letters indicate significant differences between groups ($p < 0.05$).

content is negatively related to the cryoresistance of germplasm (Pereira et al. 2007, 2008; Romão et al. 2015, 2016a). In fact, changes occurring in lipids during cryopreservation are referred as major causes of cellular cryodamage (Arav et al. 2000; Zeron et al. 2002a, b; Pereira et al. 2007; Arav 2014; Romão et al. 2016a, b). During chilling, the fatty acid composition of the membrane influences the transition from the liquid crystalline to the gel phase, a phenomenon known as lipid phase transition (Arav et al. 2000; Zeron et al. 2001). The supplementation of polyunsaturated fatty acid (PUFA) to the diet of ewes increased the proportions of long chain PUFA in the plasma and cumulus cells phospholipids as well as of oleic acid (c9-18:1) in oocytes (Zeron et al. 2002a). This improved the quality and integrity of oocyte membranes, following chilling, which was related to changes in the membrane properties, namely its fluidity and thus, the ability to resist chilling (Zeron et al. 2002a).

Spermatozoa require a high PUFA content to provide the plasma membrane with the necessary fluidity to participate in the membrane fusion events associated with fertilization. Specifically, sperm have a high percentage of lipids with long PUFA, whereas oocytes mostly have lipids with fully saturated fatty acids (Zeron et al. 2002b; Wathes et al. 2007; Lapa et al. 2011; Prates et al. 2013b). This contributes to the different lipid phase transition profiles of male and female gametes and their associated differences in cryoresistance (Zeron et al. 2002b; Wathes et al. 2007).

The main cellular damages associated with cryopreservation are disruption of cell membranes, decrease of the number of microvilli and of inter-cellular junctions (cattle: Ohboshi et al. 1998; Cavusoglu et al. 2016; sheep: Romão et al. 2016a; Barbas et al. 2018; pig: Fujihira et al. 2004; mouse: Nishizono et al. 2004; chicken: Olexikova et al. 2018). Cryoinjury also induces a reduction in mitochondria number and maturity, changes in mitochondrial cristae and matrix, swelling of rough endoplasmic reticulum, rupture of cytoskeleton, premature cortical granule exocytosis and hardening of the zona pellucida,

and a grey color tone of lipid droplets (Fujihira et al. 2004; Cavusoglu et al. 2016; Romão et al. 2016a; Marques et al. 2018).

Lipid droplets (LD) are storage depots of neutral lipids, mainly triacylglycerols and sterol esters, which may be mobilized for the generation of energy by β oxidation, or for the synthesis of membrane lipids and signaling molecules (Digel et al. 2010). The LD content is relevant in the context of lipid homeostasis, energy metabolism, metabolic disorders and infertility (Awasthi et al. 2010; Digel et al. 2010; Purcell and Moley 2011; Prates et al. 2014). In oocytes and embryos, LD occupy a considerable portion of cell volume and mass (Pereira et al. 2007, 2008; Prates et al. 2013a; Romão et al. 2016a). They are located in association with other organelles linked to cellular metabolism, such as mitochondria, endoplasmic reticulum, endosomes, peroxisomes, and cytoskeleton (Sturmey et al. 2006; Walter and Farese 2009; Prates et al. 2014; Romão et al. 2016a; Abe et al. 2017), and interact with the intermediate filaments of the cytoskeleton (Zehmer et al. 2009). Therefore, during cryopreservation, physical changes of LD lipids lead to irreversible damages in the cytoskeleton (Fujihira et al. 2004).

Oocytes and embryos of livestock species such as the porcine, bovine and ovine have a very high cytoplasm content of LD, which confer them a dark appearance (Pereira et al. 2007; Prates et al. 2013a; Romão et al. 2015). Cattle gametes and embryos are more cryotolerant compared to pigs. This may be due to differences in the number and structure of LDs, and in lipid composition (McEvoy et al. 2000; Lapa et al. 2011; Prates et al. 2013a, b). Analysis of fatty acid (FA) composition of cattle and pig oocytes during i*n vitro* maturation showed that the palmitic acid (16:0), followed by the oleic (c9-18:1) and stearic (18:0) acids were the most abundant in immature and mature oocytes, followed by n-6 PUFA, specifically, the linoleic (18:2 n-6) and arachidonic (20:4 n-6) acids. The phospholipid fraction consistently accounted for a quarter of all FAs, but pig oocytes had a higher complement of PUFA (34%) in this fraction compared to ruminant oocytes (14–19%). Pig oocytes, compared to ruminant oocytes, also had three-fold higher FAs in the triglyceride fraction. The FA profile of cattle and pig oocytes may change due to culture media or dietary lipid supplementation, and oocyte FA composition was related to oocyte developmental competence and cryosurvival (McEvoy et al. 2000; Zeron et al. 2002a; Lapa et al. 2011; Prates et al. 2013b).

With the goal of enhancing cryosurvival, several approaches to change germplasm total FA content and profile were attempted. One approach was delipidation of oocytes and embryos through chemical or physical (microaspiration, ultracentrifugation or both) methods. Chemical delipidation increased the cryotolerance of bovine and porcine vitrified oocytes and embryos (phenazine ethosulfate: Barceló-Fimbres and Seidel 2007; resveratrol: Abe et al. 2017; forskolin: Prates et al. 2013a, b). Physical delipidation of pig embryos by centrifugation and microaspiration of polarized lipids increased embryo cryotolerance (Nagashima et al. 1994; Jin et al. 2013). On the contrary, sheep embryos submitted to an ultracentrifugation process to displace LD presented a reduced viability mostly due to zona pellucida and membrane fracture (Romão et al. 2015). This was overcome by addition of cytochalasin D, a cytoskeleton relaxant/stabilizer, during the ultracentrifugation.

Another strategy to improve cryosurvival is the modulation of plasma membrane constituency and/or permeability. The supplementation of trans-10 cis-12 conjugated

linoleic acid (CLA) to oocytes and embryos during culture increased their developmental competence following cryopreservation (Pereira et al. 2007, 2008; Prates et al. 2013a, b; Romão et al. 2015; Matos et al. 2015). This was related with a decrease in membrane permeability and fluidity caused by CLA incorporation into membrane phospholipids. Compared to untreated oocytes, CLA treated oocytes showed a slower influx of the CPAs solutions, a slower shrinkage and lower water permeability with increased resistance to osmotic stress (Fig. 2), which might have helped to minimize damage during cryopreservation.

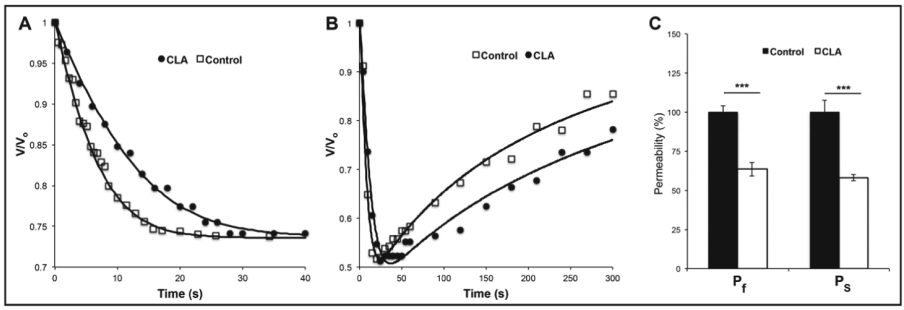

Fig. 2. Effect of trans-10, cis-12 conjugated linoleic acid (CLA) on oocyte membrane permeability to water and cryoprotectants (adapted from Matos et al. 2015). A) Time course of the oocyte volume change after the addition of sucrose (200 mM) to the extracellular media (oocyte shrinkage). The rate at which the oocyte shrinks is visibly slower when oocytes are matured in the presence of CLA. B) Time course of the oocyte volume change after the addition of penetrating cryoprotectants to the extracellular media. The first water outflow (shrinkage) is followed by an influx of cryoprotectant with consequent oocyte reswelling. The rate of cryoprotectant influx is slower for oocytes matured in the presence of CLA. C) Oocyte membrane permeability (%, relative to control) for oocytes control and oocytes matured with CLA. Pf, permeability to water. Ps, permeability to cryoprotectants. *** indicates significant difference ($p < 0.001$).

Other approaches to improve germplasm cryotolerance included addition of liposomes to modify plasma membranes constituency, altering their lipid phase transition and chilling sensitivity (Zeron et al. 2002b), the reduction of Ca^{++} from the vitrification media (Marques et al. 2016, 2018), and the blockage of membrane P2Y2 purinergic receptors (Pereira et al. 2016).

In conclusion, modulation of lipid content is envisaged as a very promising strategy to improve the efficiency of germplasm cryopreservation.

3.4 Cryosurvival and Developmental Competence

The discovery of the protective effects off egg yolk and glycerol against sperm cold shock and freeze-thaw damages, allowed for long term storage of gametes and the use of frozen thawed spermatozoa in artificial breeding programs (Barbas and Mascarenhas 2009; Konyali et al. 2013). Despite more than 60 years of optimization, the freezing process still remains highly damaging for spermatozoa. In several domestic species, a

sperm cryopreservation protocol that returns suitable fertility rates for commercial AI programs is yet to be achieved (Barbas et al. 2013, 2018). In AI centres, 40–50% of sperm cells do not support cryopreservation, which generates high economic losses. Sperm cryosurvival vary among species, because of sperm differences in shape, volume, organelles size and composition. Also, individual variation within species/breeds is a well known phenomenon (goats; Barbas et al. 2006, 2018; sheep: Marques et al. 2006; pig: Roca et al. 2006; cattle: Mostek et al. 2018). This individual variation is attributed to several factors. Differences in specific DNA sequences were identified among boars with thawed semen quality classified as poor or good (Thurston et al. 2002). Similarly, prion protein testis specific (PRNT) gene polymorphisms and transcript level were related to ram sperm freezability, fertilization and embryo development (Pereira et al. 2018). Bull low quality ejaculates had a high carbonylation level in proteins (Mostek et al. 2018). Worldwide research currently focuses on the identification of biomarkers (quantification of gene transcription and protein expression) of male fertility and sperm cryosurvival (Mostek et al. 2018).

Compared to spermatozoa, the cryopreservation of female gametes has a lower success rate. Current oocyte vitrification protocols are unsatisfactory (Lai et al. 2015; Matos et al. 2015; Arav and Saragusty 2016; Marques et al. 2018). Oocyte quality before cryopreservation is pivotal for subsequent developmental competence. In high yielding dairy cows, metabolic disorders during the negative energy balance of postpartum alter the endocrine and FA composition of blood and follicular fluid, with detrimental effects on oocyte and embryo quality (Leroy et al. 2008; Awasthi et al. 2010). Likewise, the dark appearance and high LD content of oocytes, typical of infertile women due to age and obesity, compromise their developmental competence (Walther and Farese 2009; Purcell and Moley 2011). Therefore, as the lipid profile of the oocyte is dynamic and largely dictated by the environment in which it develops, metabolic stress has major effects on the FA composition and consequently in oocyte quality.

3.5 Embryo-Maternal Interactions Leading to Pregnancy Establishment

Germplasm competence following cryopreservation reflects not only the resistance to cryodamage, but also its intrinsic quality to establish interaction within the female reproductive tract. Sperm-oocyte interactions are central for subsequent fertilization and embryo development, whereas embryo-maternal interactions are critical for subsequent pregnancy establishment. In this context, the deciphering of the cellular and molecular mechanisms that drive sperm-oocyte and embryo-maternal communication will have a major impact on ART and infertility control strategies. This has long been a goal of the Reproduction & Development Laboratory of CIISA and INIAV (Fig. 3). One research line considered the evaluation of the steroidogenic and prostanoid embryo-maternal interactions leading to embryonic development and survival (Pereira et al. 2009; Torres et al. 2013a, 2015). These i*n vitro* studies evidenced that blastocyst-stage bovine embryos show transcription of genes coding for enzymes of the prostaglandins (PGs) and progesterone (P4) synthesis pathways, and produce these molecules. Receptors for P4 are present in the bovine blastocyst (Clemente et al. 2009), oviduct (Kenngott et al. 2011), uterus (Okumu et al. 2010), and corpus luteum (Rueda et al. 2000), whereas PGs participate in maternal recognition and maintenance of pregnancy (Weems et al. 2006;

Dorniak et al. 2011). Therefore, these early embryonic-derived factors may induce subtle and localized, however potentially relevant autocrine and paracrine changes in the endometrium, resulting in enhanced embryonic development and uterine receptivity.

Another molecule that has received the attention of researchers is lysophosphatidic acid (LPA), a cell signaling lipid mediator of reproductive tissues (Wocławek-Potocka et al. 2014). Supplementation with LPA of oocyte maturation medium increased oocyte transcription of quality marker genes (FST and GDF9) and decreased the BAX/BCL2 ratio and cumulus cells transcripts associated with low viability (CTSs), indicating that an antiapoptotic balance was induced in the oocyte (Boruszewska et al. 2014) and potentially enhancing oocyte developmental competence. Also, transcription of mRNA and protein expression of enzymes involved in LPA synthesis (ATX and cPLA2) and of LPA receptors (LPAR1–4), and embryonic LPA production into culture medium were detected in Day 5 and Day 8 *in vitro* produced bovine embryos (Torres et al. 2014). Supplementation of culture medium with LPA had no effect on embryo yield, but affected transcription levels of embryo quality markers, decreasing BAX (apoptotic) and increasing BCL2 (antiapoptotic) and IGF2R (growth marker) gene transcription levels. This prompts for a LPA role in early embryo-maternal interactions leading to embryonic survival.

Progesterone-dependent regulation of anti-adhesion MUC1 mucin appears to be an important factor in determining endometrial receptivity. In fact, MUC1 plays a role in protecting the endometrium from microbial attack but must be lost for embryo implantation to occur. Accordingly, cell surface MUC1 gene expression was drastically reduced in endometrial tissue from cows with higher receptivity to the embryo (Mesquita et al. 2012; van Hoeck et al. 2014). Thus, the knowledge on how MUC1 expression can be regulated in uterine epithelium may aid improving pregnancy rates and reproductive efficiency, in domestic animals as well as in humans, by decreasing MUC1 and increasing the availability of the uterine cell surface to the embryo (Mesquita et al. 2012; Pereira et al. 2013).

Due to its unequivocal role in pregnancy establishment and maintenance, P4 based pharmacological strategies to enhance embryo survival have received great attention. Strategies designed to increase post-ovulatory peripheral concentrations of P4 include increasing the endogenous function of the primary corpus luteum, inducing secondary corpora lutea, directly supplementing P4, or to inhibiting the endometrial PGF2α-synthesizing enzymatic machinery, responsible for luteolysis, during the critical period of maternal recognition of pregnancy (Binelli et al. 2001; Inskeep 2004). Two *in vivo* models were used to evaluate the effects and mechanisms of these therapeutic approaches, designed to enhance embryonic survival following ET. Low developmental competence embryos (demi-embryos) were transferred either to a sub-normal fertility recipient (high yielding lactating dairy cow; Torres et al. 2013b) or to a high fertility recipient (virgin dairy heifer; Torres et al. 2013c). The effect of human chorionic gonadotrophin (hCG, a luteotrophic agent) treatment at ET on embryo survival was evaluated. Treatment with hCG induced formation of secondary corpora lutea, increased plasma P4 concentrations and survival rate of demi-embryos, which were rescued beyond maternal recognition of pregnancy. However, growth and further survival until implantation and placental secretion of pregnancy specific proteins were not affected. Therefore,

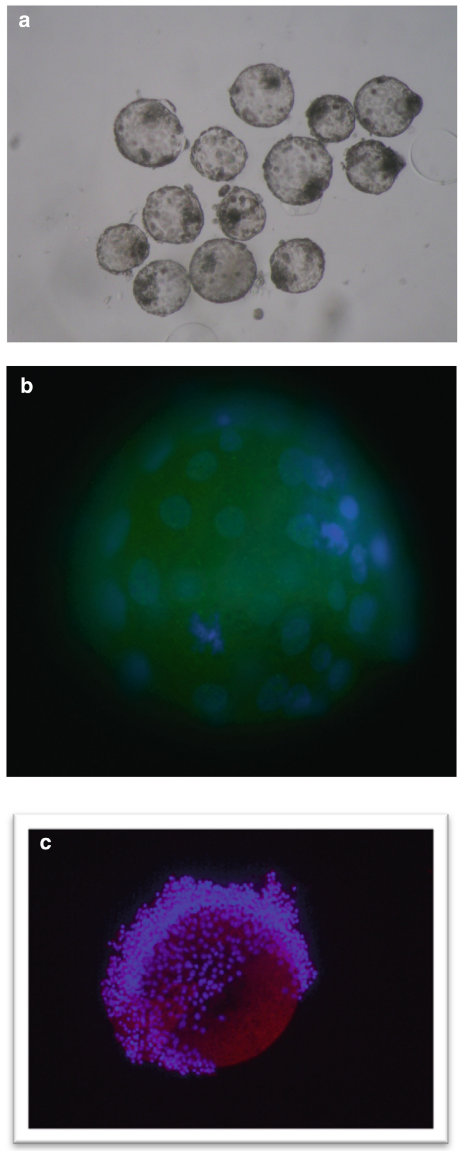

Fig. 3. **A** – Day 8 (Day 0 = *in vitro* insemination) *in vitro* produced bovine embryos at the expanded, hatching and hatched blastocyst stages. Oocytes were recovered *post-mortem* from the ovaries, and *in vitro* matured and fertilized with sperm previously cryopreserved, followed by *in vitro* culture of embryos until stages compatible with cryopreservation or embryo transfer. This methodology allows rescue of germplasm and obtaining offspring from shortly dead individuals. **B** – Day 8 *in vitro* produced bovine expanded blastocyst with cells expressing autotaxin (green fluorescence), an enzyme of the LPA synthesis pathway. This evidences the early production of this lipid mediator, which is involved in embryo-maternal communication leading to pregnancy establishment. **C** – Oocyte and granulosa cumulus cells expressing PGFR (red fluorescence), an enzyme of the prostaglandin F synthesis pathway.

embryonic survival following maternal recognition of pregnancy did not appear to be directly dependent on maternal P4 concentrations.

3.6 The Future of ART for Germplasm Preservation

Germplasm preservation is now envisaged a critical tool for the conservation of animal genetic resources and for fertility preservation. This requires the refinement of cryopreservation protocols that minimize the damages caused by the intra- and extracellular ice crystal formation, toxic effect of high concentrations of CPA and osmotic stresses. Besides strategies discussed above, another possibility is the use of antifreeze proteins, which are found in numerous species that have adapted to extreme negative temperatures (Hezavehei et al. 2018). These antifreeze proteins significantly increased the motility and viability of ram, bull and mouse spermatozoa after cryopreservation (Payne et al. 1994; Koshimoto and Mazur 2002; Prathalingam et al. 2006).

The lyophilization of germplasm represents an innovative and ecological non-cryogenic storage solution (Anzalone et al. 2018). Germplasm drying has many potential advantages. This process, leading to water removal, allows the conservation of specimens in an anhydrous state at ambient temperatures, lowering costs and labor (Arav and Saragusty 2016; Anzalone et al. 2018). Attempts to establish lyophilized sperm banks for endangered breeds and species are still in the infancy. However, oocytes injected with freeze-dried sperm developed to the blastocyst stage, and freeze-dryed stem cells differentiated into gametes (spermatozoa and oocytes) (Arav and Natan 2017; Olaciregui et al. 2017; Anzalone et al. 2018). This opens new avenues for the preservation of reproductive cells and tissues.

Long-term follow-up studies on offspring obtained from cryopreserved germplasm are necessary to fully assess the biological safety of these methodologies (Hezavehei et al. 2018).

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Tropical Livestock Development: Mitigating Seasonal Weight Loss and Health Conditions

L. A. Cardoso(✉), A. Almeida, S. van Harten, and S. Zúquete

Tropical Livestock Unit, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
alfarocardoso@fmv.ulisboa.pt

Abstract. Two main limiting tropical environment conditions in livestock production are concerned with seasonal dry periods leading to a decrease of foodstuff availability and with hyperthermia, both negatively affecting animal productivity. Seasonal weight loss (SWL) is an important constraint limiting animal production in the Tropics and the Mediterranean. Hence, our focus on the study of physiological and biochemical mechanisms by which domestic animal breeds respond to SWL and plants show resilience under heat or dehydration stress.

Since the last two years we have also been involved in tropical animal health issues research, concerning trypanosomoses and hard ticks biologic life cycles in livestock. These agents induce major health issues in livestock within tropical areas. Our work aims at contributing to alternative immunity pathways to mitigate their effects in animal production.

The definition of these main research terms of reference were much collected whilst implementing development livestock projects in African countries, as a previous and a parallel activity to our research work. So food security has also been a field of our interest.

We are also referring to results on our food security approaches. For the purpose of this review, we made a selection of our published papers so as to synthetize the presentation.

Keywords: Vectors · VBDs · Diagnosis · Risk assessment · Control · Portugal

1 Introduction

The work presented in this article was accomplished by a team of investigators at the Tropical Research Institute (IICT, Lisbon) and at the Faculty of Veterinary Medicine at the University of Lisbon which, since 2015 integrated most of IICT members. The activities also included partnerships with colleagues and facilities form other institutions identified in the bibliographic references. The work was implemented during MSc, PhD and Post Doc assignments of many members of the research group.

Tropical livestock development is a subject that binds the will and the efforts of many research workers, justified by strong arguments: Not only it corresponds to vast areas of developing countries, but also because climate changes are steadily imposing new

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agriculture approaches within temperate weather regions. For these reasons, mitigating main agrarian limitations concerning tropical parts of the globe will be a main working field enhancing sustainable food availability for human consumption. Hence the drive for our work.

2 Work on Animal Seasonal Feed Restriction and Metabolism

Our first approach to metabolic changes induced by feed restriction, one of the most frequent livestock constraints in tropical conditions, mainly due to the seasonal lack of pastures, was to study the metabolic effect of adrenergic stimulae in underfed animals. For this purpose, the β-agonist clenbuterol was given as a dietary admixture (4 mg/kg diet) to three groups of male Wistar rats housed individually in metabolism cages and fed for 15 d at 110, 160, and 235% (*ad libitum*) of the estimated requirement for energy maintenance. Untreated groups at each level of energy intake and a baseline group were also included. This adrenergic agonist induced significant increases in body weight (BW), feed efficiency, and carcass weight, dressing and protein content at all three levels of energy intake. This effect was particularly noticeable in the restricted animals (Cardoso and Stock 1996) in which energy balance was increased by the adrenergic treatment in the diet-restricted rats, with no significant changes occurring in the *ad libitum* fed rats. In the diet-restricted rats, the β-agonist induced greater and more persistent increases in nitrogen balance, biological value, and net protein utilization than it did in the *ad libitum*-fed rats. The protective effect of the adrenergic stimulae on the duodenal epithelium specially on the restricted fed rats may play a relevant role on these performances (Cardoso and Ferreira 2002) Compared with untreated rats, the adrenergic stimulae reduced blood glucose in all diet groups and serum insulin in the ad libitum and the moderately restricted (160%) rats. Serum IGF-I was increased in the highly restricted (110%) rats. Corticosterone levels were increased by clenbuterol treatment in all diet groups. These results are consistent with previous results showing that adrenergic stimulae can help improve recovery from organic depletion (Cardoso and Stock 1998)

We also showed that feed restriction in rats, corresponding to 34% energy maintenance needs, simulating livestock weight loss in frequent drought seasons significantly induced on the 23 day of the experimental period, the increase of % free C14:0 content in the plasma and decreased the % C16:0 incorporated in triacylglycerol's of muscle fat. C18:0 fatty acids suffered a relative increase in its free and in its esterified form in the adipocytes of the muscle. Feed restriction decreased the % of C18:1 as free fatty acid in the muscle and in plasma These results are consistent with the existence of a preferential degradation of long-chain fatty acids in underfed rats (Harten et al. 2003). We also found in Gastrocnemius muscle myofibrillar protein characterization higher concentrations of Ile, Thr, Met, Ser and Cys in the restricted fed group, with no preferable catabolism of individual amino acids observed, keeping muscle structure (Almeida et al. 2002).

We extended our research work on seasonal feed restriction metabolomic effects using rabbits as models.

We first aimed at the identification of relevant intermediate metabolism enzymes contributing to improved meat production due to genetic selection. A wild rabbit (WR) breed and a highly meat selected breed, New Zealand rabbit (NZ) was used. Control

(*ad libitum* feed) and feed restriction were experimental conditions so as to enhance differences within the metabolic pathways under study. During a period of 30 days, NZ and WR experimental breeds were subjected to, respectively, 40% and 60% ad libitum feed restriction leading to 17.7% and 21.1% initial weight reduction. Hepatic glycolytic, lipidic and protein regulatory enzyme activity, transcriptional and metabolite levels were determined. Insulin-like growth factor (IGF-1), triiodothyronine, and cortisol were also evaluated. In the glycolytic pathways, the NZ control rabbits presented a higher phosphofructokinase and pyruvate kinase activity level when compared to the WR, while the latter group showed a higher expression of glycogen synthase, although with less glycogen content. In the nitrogen metabolism, our results showed a lower activity level of glutamate dehydrogenase in WR when subjected to feed restriction. Within the lipid metabolism, results showed that although WR had a significantly higher mRNA hepatic lipase, non-esterified fatty acid levels were similar between the experimental groups. NZ rabbits presented a better glycemia control and greater energy substrate availability leading to enhanced productivities in which triiodothyronine and IGF-1 played a relevant role (van Harten and Cardoso 2010).

In the nitrogen metabolism, our results showed a lower activity level of glutamate dehydrogenase in WR when subjected to food restriction. Within the lipid metabolism, results showed that although WR had a significantly higher mRNA hepatic lipase, non-esterified fatty acid levels were similar between the experimental groups. NZ rabbits presented a better glycaemia control and greater energy substrate availability leading to enhanced productivities in which triiodothyronine and IGF-1 played a relevant role. On these experimental animals, we also studied physiological changes occurring during selection that contributes to an improved understanding of relationships leading to efficiencies in animal production. To investigate the effects of food restriction in gastrocnemius muscle protein expression was performed using one-dimensional gel electrophoresis and peptide mass fingerprinting. Lower expression levels of myosin heavy chains were found in the Wild Rabbits restricted group, while myosin light chain and alpha-crystalline proteins were not detected in restricted groups. Glyceraldeyde-3-phosphate dehydrogenase and glycogen phosphorylase expression levels were similar for all experimental groups. Phosphopyruvate hydratase beta was not detected in the wild rabbit restricted diet group. Pyruvate kinase levels were 50% lower in the New Zealand restricted group. LIM protein detection was absent in the control New Zealand group. Results also showed relevance of actin in preserving muscle structure in depressed food availability. The sensitivity of both myosin light chain and alpha-crystalline protein to restricted feed and the role of PK in the resistance of New Zealand rabbits to food restriction was also shown (Almeida et al. 2010).

The study of changes within the key agents regulating metabolism during genetic upgrading because of selection can contribute to an improved understanding of genomic and physiological relationships. This may lead to increased efficiencies in animal production. These changes, regarding energy and protein metabolic saving mechanisms, can be highlighted during feed restriction periods. For this propose we used the previously described experimental rabbits with a 20% weight reduction, applying techniques of two-dimensional gel electrophoresis and peptide mass fingerprinting. Results show that L-lactate dehydrogenase, adenylate kinase, beta enolase and alpha enolase, fructose bisphosphate aldolase A and glyceraldehyde 3-phosphate dehydrogenase, which

are enzymes involved in energy metabolism, are differentially expressed in restricted diet experimental animal groups. These enzymes are available to be further tested as relevant biomarkers of weight loss and putative objects of manipulation as a selection tool towards increasing tolerance to weight loss. Similar reasoning could be applied to 2D gel electrophoresis spots corresponding to the important structural proteins tropomyosin beta chain and troponin I. Finally, a spot identified as mitochondrial import stimulation factor seems of special interest as a marker of undernutrition, and it may be the object of further studies aiming to better understand its physiological role (Almeida et al. 2010a).

We also used gastrocnemius samples from these experimental animals to accomplish the establishment of a proteome reference map of a specific tissue, as it is found in several laboratory and production species. The rabbit is widely used as both a production and experimental animal. A lot of physiology research involving the gastrocnemius muscle of rabbit is described, although no reference proteome map is available. In this work, the first reference map of the rabbit's gastrocnemius muscle using 2D gel electrophoresis and the identification of proteins through peptide mass fingerprinting (PMF) was established. A total of 45 proteins were localized and identified with three major roles: cell structure and contractile apparatus; metabolic and cell defense proteins. A reference map of major proteins expressed is described enabling possible comparisons with other physiological studies (Almeida et al. 2009).

The effects of feed restriction on testicular angiogenic activity, microvascularization, tissue growth and regression were also studied, using the rabbit as a model. These experimental animals (*Oryctolagus cuniculus cuniculus*) were randomly assigned to a control group fed *ad libitum* (A), and to two feed restriction groups who suffered a 21.2% decrease in body weight in group B and 34.7% in group C. Testis explants were cultured for 24 h and conditioned media were tested for their ability to stimulate mitogenesis of bovine aortic endothelial cells (BAEC). There was an increase in testicular microvascular area and mitogenesis of BAEC in group C rabbits. Despite no change in testicular DNA concentration among groups, feed restriction decreased both RNA and protein compared with control. No treatment differences in the percentage of seminiferous tubules filled with all stages of spermatogenesis (spermatogonia, spermatocytes, and spermatids) and spermatozoa, as well as the area occupied by seminiferous tubules, were observed. Nevertheless, serum testosterone was markedly less in group C compared with groups A and B. These results suggest that angiogenesis may play a role in overcoming testicular nutritional impairment in rabbits subjected to feed restriction (Carvalho et al. 2009).

In this context, we conducted several essays including metabolomics approaches to the effect of seasonal feed restriction on small ruminants, much utilized in Africa namely by small scale farmers operating in harsh environments and in Australia.

The adrenergic stimulae effects in sheep under feed restriction was done. In Serra da Estrela sheep housed individually in metabolic cages and fed for 45 days at 65% of estimated requirement for energy maintenance, we showed that during the 4th week of the trial, β-agonist treated the animals showed increased mass gains, greater energy retention and serum IGF-1 levels. This study showed that the adrenergic stimulae induced a protective effect in sheep subjected to periods of feed deprivation, based on the body mass and digestible energy effects manifested by treated animals (Cardoso and Taveira 2002).

Such animals, 15 (6–8 months old), were allocated into two groups and fed *ad libitum* for a period of 29 days. The Winter hay restricted fed group (WH; $n = 8$) received a chopped diet consisting of grass hay, predominantly *Themeda trianda* grass (cut during the winter) from a natural pasture (veld). The WH + S control group (winter veld hay plus supplement; $n = 7$) received a chopped diet consisting of *Themeda trianda* veld hay, supplemented with maize meal, molasses meal and urea. In a study of mineral profile of these experimental animals, we found that non-supplemented animals had a significant decrease in live weight and lower whole quantities for each mineral analyzed (phosphorus, potassium, iron, zinc, magnesium and calcium). Concentrations of Ca, Mg, Fe and P were higher for non-supplemented animals as these minerals are mainly associated with the skeleton. Quantifications and concentrations for each carcass mineral for young Boer goat bucks are provided in this study (Almeida et al. 2006a).

The levels of thyroid and plasma insulin of these animals were also studied. The no supplemented group presented a lower insulin plasma level, characterising the nutritional status of both groups, indicating reduced protein synthesis and increased catabolism. Over the supplemented group. T3 and T4 were levels were the same in both experimental groups, showing that undernutrition did not influenced these hormones concentration (Almeida et al. 2002a).

In the same experimental boer goats, we studied the content of blood and muscle free fatty acids in the fraction of triacylglycerol and saw that the percentage of C16:0 as free fatty acid in the plasma suffered a significant increase with undernutrition, suggesting a priority in the hydrolysis of this fatty acid in feed restriction. C18:1 showed a relative decrease in muscle fatty acid incorporated in triacylglycerol in underfed goats. C18:2 revealed a relative increase in muscle fatty acid incorporated in triacylglycerol and a relative decrease in free fatty acid in the plasma in the restricted group, with no statistical effects of undernutrition detected in C14:0 and C18:0. (van Harten et al. 2003a)

Regarding these experimental animals serum aminoacids and myofibrillar protein profiles, we saw that control group animals had higher concentrations of Ala, Tyr and Cit; the restricted fed group showed higher concentration of Val, Ile, Leu, Thr, Met, Lys, Tau, Orn, Hyp and 3-metyl histidyne (Me3His), while Gly, Ser, Asp, Glu, Arg, His and Pro were similar in both groups. The restricted fed group showed myofibrillar protein degradation of protein C and α-actinin. From the results it can be concluded that serum amino acid and myofibrillar protein profiles in the goat are strongly affected by undernutrition. Amino acid results suggest that the degradation of small carbon chain amino acid has a higher efficiency than degradation of long carbon chain amino acid. Myofibrillar protein profiles suggest a disruption of muscle structure at the level of the second third of each half of the A band (protein C) and the matrix of the Z line (α-actinin) (Almeida et al. 2004).

The same animals were subjected to reproductive system evaluations. Our results show differences in sperm cell abnormalities (43% in the restricted fed group versus 24% in the control group), testicular volume (156 ml in the control group versus 104 ml for restricted fed animals) or scrotal circumference (20.7 cm in the control group versus 17.7 cm in the restricted fed group). It is essential to supplement the nutrition of small ruminants under winter environments, to maintain scrotal, testicular and semen characteristics, especially if the animals are to be used in the subsequent breeding season (Almeida et al. 2007).

The effect of feed restriction on gene expression of regulatory enzymes of intermediary metabolism was also studied in two sheep breeds (Australian Merino and Dorper) subjected to two nutritional treatments: feed restriction (85% of daily maintenance requirements) and control (*ad libitum* feeding), during 42 days. During the experimental period, the Merino Restricted animals lost 12.6% in BW compared with 5.3% lost by the Dorper lambs. Merino Control and Dorper Control rams gained, respectively, 8.8% and 14% during the same period. Through real-time PCR within the Dorper breed, restricted feed animals revealed a significant decrease over controls in the transcription of PFK phosphofructokinase (1.95-fold) and PK pyruvate kinase (2.26-fold), both glycolytic enzymes. The gluconeogenesis showed no change in the feed restricted animals of both breeds. Dorper Restricted (DR) feed group presented a significant decrease over the homologous Merino sheep group on GS glycogen synthase. In both experimental breeds, FAS fatty acid synthase mRNA expression was decreased in restricted feed groups. GDH glutamate dehydrogenase expression was decreased only in the DR animals (1.84-fold) indicating a reduced catabolism of amino acids in these animals. Finally, CPS (carbamoyl phosphate synthase) was significantly ($P < 0.05$) higher in the Dorper sheep, indicating a facilitated urea synthesis in this breed. These results indicate a better adaptation of metabolic intermediate regulatory enzymes and hepatic glucose production of Dorper sheep to feed restriction concurring with the BW results in the experimental groups (van Harten et al. 2013).

The role of the amino acid 3-methylhistidine as an indicator of protein breakdown and weight loss is often suggested. Despite existing information for other animal species, little is known about the actual levels of 3-methyhistidine in the serum of less studied domestic species such as the goat. We have evaluated the 3-methyhistidine serum concentrations in young Boer goat bucks subjected to two distinct feeding regimens: winter-grass hay with or without supplementation. Non-supplemented animals had a negative nitrogen balance and experienced weight loss throughout the experiment and significantly higher concentrations of 3-methyhistidine than supplemented animals that had a slight increase in live weight. This amino acid can be considered a valid indicator of protein breakdown and weight decrease in male goats. Serum 3-methylhistidine concentrations in adequately fed male goats were similar throughout the assay (20–40 μmol/l) whereas in weight-losing animals, concentrations of up to 170 μmol/l can be expected. (Almeida et al. 2008)

The study of the proteome has been instrumental in gathering important information on physiological mechanisms, including those underlying seasonal weight loss (SWL). In spite of that, little information is available concerning physiological mechanisms of SWL in production animals. Hence this study aiming at the determination of differential protein expression in the muscle of three different breeds of sheep, the Australian Merino, the Dorper and the Damara, each showing different levels of tolerance to weight loss (low, medium and high, respectively). Per breed, two experimental groups were established, one labeled "Growth" and the other labeled "Restricted." After forty-two days of dietary treatment, all animals were euthanized. Muscle samples were then taken. Total protein was extracted from the muscle, then quantified and two-dimensional gel electrophoresis were conducted using 24 cm pH 3-10 immobiline dry strips and colloidal coomassie staining. Gels were analyzed using Samespots® software and spots of interest

were in-gel digested with trypsin. The isolated proteins were identified using MALDI-TOF/TOF. Results indicated relevant differences between breeds; several proteins are suggested as putative biomarkers of tolerance to weight loss: Desmin, Troponin T, Phosphoglucomutase and the Histidine Triad nucleotide-binding protein 1. This information is of relevance to and of possible use in selection programs aiming towards ruminant animal production in regions prone to droughts and weight loss (Almeida et al. 2016).

Milk production in bovines is significantly affected by high temperatures. Our genomic approach into this subject aims at examining the expression of genes on the mammary gland, corresponding to various levels of adaptation or acclimatization to environmental stress. We utilized 18 cows from three genetic groups, Holstein Brazil (HB), Gyr and Gyrolando (GH), all in the same stage of lactation, and subjected them to the same management conditions. Venous and arterial blood were collected to determine the hormonal profile and blood chemistry. Mammary gland tissue was used for transcriptomic studies. Prolactin and GH plasmatic concentrations were higher in Holstein animals. There were no differences in IGF-1 concentrations among the experimental groups. T3 concentrations were similar among the Holstein and Gyr groups. From the 4608 transcripts in the BLO-Bovine EST (Michigan State University, US) databank that were used in this experiment, 105 differentially expressed genes were identified in at least one of the groups. Among these, the authors highlighted 14 genes that were related to the structure of the mammary gland (CRDGF, CD97, GH, endoglin, LTF, INPP, PTP), to response to thermal stress (Crh_11, v-Fos, Cdc37) and to milk protein (RPL35, κ-casein, β-casein, α-s2-casein). Eight of these were validated through real-time polymerase chain reaction. The HB animals, in comparison with the GH and Gyr groups, presented up-regulated genes associated with epithelium cellular differentiation and proliferation, milk productivity and decreased heat stress tolerance. Gyr animals presented up-regulated transcripts associated with cellular defence, apoptosis processes and increased tolerance to heat stress. The GH group showed intermediary results compared with the other two groups (Wetzel-Gastal et al. 2016).

We also aimed at identifying mammary gland genes expressed in Brazilian Holstein cattle produced under tropical conditions, as compared to the Portuguese Holstein cattle produced in a temperate region. For this purpose, cDNA microarrays and real-time (RT) PCR transcriptomic techniques were utilized in 12 Holstein cows from the same lactating phase and management systems divided into two groups: Holstein Brazil (HB) originated from Brazil and Holstein Portugal (HP) from Portugal. The genomic results show that from a total of 4608 genes available from the microarray slide (Bovine Long Oligo (BLO) library), 65 transcripts were identified as differentially expressed in mammary glands. The genes associated with mammary gland development and heat stress responses showed greater expression in HB animals. In the HP group, upregulated genes related with apoptosis and vascular development and downregulated genes related with resistance to heat stress were observed. Validation of microarray results was done using RT-PCR. HB animals had higher blood levels of growth hormone than HP animals. Blood levels of prolactin and T_3 were similar for both groups and GH levels were increased in the HB group. The results suggest a gene change towards long-term acclimatization of Brazilian Holstein cattle to cope with tropical heat stress conditions (Wetzel-Gastal et al. 2018).

3 Work on Animal Health

Ticks are obligate ectoparasites found in nearly all regions of the world. They possess the largest diversity of transmittable diseases of all arthropods causing worldwide economic losses (14–18.7 million US$) in livestock and they are the most significant vectors of animal and human diseases, after mosquitoes. Control of ticks and tick-borne diseases of livestock depends almost exclusively on the use of acaricides inducing a widespread occurrence of tick resistance to acaricides and environmental concerns prompting research into alternative methods for controlling ticks most of them consisting in of host vaccination against ticks,. These vaccination strategies include antigens against the tick/host chemical adhesion complex. During the tick natural evolution cycle, its host attachment system goes through significant changes allowing for the ectoparasite fall from the host so as to able to complete its life phases or reproduction periods. We are studying the involution of tick cement composition occurring on the final "feeding" stages of this parasite upon its host leading to the identification of an antigen against the tick/host chemical adhesion complex. At the present study we focused on the cement-cone formation and composition during *Hyalomma lusitanicum* feeding process.

We started this study by analyzing the histological time frame in skin lesions induced by *Hyalomma lusitanicum* infestation development on bovines in order to understand the role of such process in tick attachment and detachment from hosts. Samples of skin biopsies throughout of the complete tick cycle were collected. Both superficial and deep interstitial and follicular dermatitis were observed with eventual implications on tick predominance of inflammatory cells, providing information on these local events on tick attachment and detachment from hosts (Zúquete et al. 2016).

Artificial tick feeding systems are relevant to our work as they allow us to have aggregation complex samples free from host contaminants, facilitating the identification of the complex composition during the tick cycle. We have obtained in our lab both i*n vivo* and *in vitro* systems, adapted to *Hyalomma lusitanicum* (artificial feeding by silicon membranes), at five sequential time points: 1) unfed ticks; 2) early-feeding; 3) medium engorged; 4) detachment period and 5) drop-off. Microscopic observation of bovine biopsies collected at different sampling times enabled skin lesions characterization. Replicates collected *in vivo* failed to reproduce the same findings throughout the process, but it was possible to divide results in two phases (attachment and detachment). The *in vitro* results were more constant, yet less variable within sampling times (Zúquete et al. 2018).

Tick salivary glands produce a broad range of bioactive molecules responsible for anti-haemostasis, inflammatory responses suppression and for immune modulation. Hard tick fixation mechanism to host dwell on a ciment like plug, secreted by the tick salivary gland We proceeded with the study of cement collections, obtained both i*n vivo* and *in vitro* (artificial feeding by silicon membranes), at the five sequential time points mentioned above. We identified several proteins from the cement proteome by mass spectrometry. Proteins were assembled according to their gene ontology, showing different salivary products being produced during the progressive feeding moments. In the light of the associations found between protein subsets and feeding phases, insights into the cement plug assembly and formation are here focused. Understanding such molecular events may lead to the development of new control strategies (Zuquete et al. 2018a).

In Sub-Saharian Africa Trypanosomiasis is a public health problem and a serious constraint to livestock production. It is a zoonose caused by flagellated protozoa *Trypanosoma* spp transmitted by the bite of an infected tsetse fly (*Glossina* spp). About 50 million livestock heads are exposed to this disease in Africa annually inducing animal production losses of about US$ 4.5 million. This disease control consists on Tsé-Tsé fly population reduction (traps and insecticides), medicaments and vaccines. The latter face difficulties due to the parasite genetic variability, showing efficiency resistances. Thus new strategies against trypanosomiasis such as paratransgenesis have been studied including glossina bacterial symbionts. *Sodalis glossinidius,* a glossina symbionte bacteria is found in its midgut, muscle, fat body, milk glands, and salivary glands. This symbionte is not sensitive to the peptides Attacin and Defensin synthesized by glossina and integrating its immune system. *Sodalis glossinidius* has thus a favorable biology to be used in paratrangenesis. Few studies have search into this matter, particulary concerning the recombinant expression of Attacin and Defensine in symbiotes and *in vitro* and *in vivo* identifying and quantifying its tripanocide activity. In our lab we have achieved, to the best of our knowledge, for the first time, the syimbionte genome transformation so as to it individually expressed Attacin and Defensin recombinant proteins, through thermic shock, chemical treatment and electroporation (Zuquete et al. 2016a). In this work it was also developed the plasmid construction expressing, those proteins in a controled way, allowing their purification, and designed and optimized an essay quantifying through qPCR, the expression of Attacin and Defensin. On the sequence of this work, this project aims at studying the *in vitro* and *in vivo the* tripanocid expression and effect of Attacin and Defensine peptides in transformed *Sodalis glossinidius* maintaining this bacteria viability and also evaluating the tripanocide effect of the transformed symbiont inoculated in *Glossina spp.*

4 Work on Plant Adaptation to Hyperthermia and Draught Environments

Hyperthermia and draught are challenges for the development of pastures and forages, representing main limiting facture to livestock production in tropical regions. Mitigating plant adaptation problems to these conditions is therefore a relevant component of present and future animal production challenges within these areas. Regarding this matter, our work has concerned the possible role of trehalose, a non-reducing disaccharide of glucose in protecting plants against high temperatures and dry environments. Several strategies leading to this sugar accumulation have been envisaged in both model and crop. Trehalose is one of the most effective osmoprotectants plants using genes of bacterial, yeast and more recently, of plant origin. Significant levels of trehalose accumulation attempted through genetic engineering in model and crop plants using genes of bacterial and yeast origin have been shown to cause abiotic stress tolerance in transgenic plants. An alternative strategy for accumulation of trehalose seems to be the blocking of threhalase, the enzyme involved in this sugar breakdown. Our work within this area has its emphasis on the manipulation of trehalose metabolism to improve abiotic stress tolerance in plants, using as research models Arabidopsis thaliana and Nicotiana tabacum (Almeida et al. 2007a).

Working in maize, we aimed to genetically engineer it with the Arabidopsis thaliana trehalose phosphate synthase gene (AtTPS1), involved in trehalose biosynthesis via electroporation. A cassette harboring the AtTPS1 gene under the control of the CaMV35S promoter and the Bialaphos resistance gene Bar as a selective agent was inserted in the plasmid vector pGreen0229 and used to transform maize inbred line Pa91 via electroporation. Fifteen putative transgenic plants (T0 generation) were obtained. Transgene integration in T0 plants was analyzed by Southern-blot analysis. T0 plants had normal phenotypes, although smaller than wild type plants. Contrary to wild type plants, when sexual organs emerged, tassels appeared at least 15 days earlier than ears in the same plant, rendering impossible the self-pollination of the T0 plant. These plants were then crossed with wild type plants and in some cases T1 seeds were obtained. T1 seeds presented deformities, especially the lack of endosperm, but it was still possible to germinate some of these seeds. The so obtained plants were tested by Northern blot but no AtTPS1 gene expression was detected, a fact possibly due to the incomplete insertion of the AtTPS1 gene or an extremely low gene expression level (Almeida et al. 2007b).

In another work we aimed to improve desiccation tolerance in maize, one of the most agronomical important crops, by increasing trehalose accumulation through transformation with the *Arabidopsis thaliana* trehalose phosphate synthase gene (AtTPS1) is involved in trehalose-6-phosphate synthase and hence on trehalose biosynthesis. A cassette harboring the AtTPS1 gene under the control of the CaMV35S promoter and the Bialaphos resistance gene Bar as a selective agent (conferring resistance to the PPT) was inserted in the plasmid vector pGreen0229 and used to transform maize inbred line Pa91. Immature zygotic embryos were collected 14-20 days after pollination and embryogenic calli culture were initiated. Embryogenic calli were electroporated with 20 μg of plasmid DNA using a Biorad Gene Pulser II at 374 V, for 1 s. Embryogenic calli were electroporated and selected PPT. Eighty putative transgenic plants were obtained and analysed by PCR for the presence of the AtTPS1 gene (Almeida et al. 2003).

Concerning the genetic transformation of plants introducing AtTPS1 gene (trehalose-6-phosphate-synthasegene from Arabidopsis thaliana), a cassette harboring the *AtTPS1* gene under the control of the CaMV35S promoter and the Bialaphos resistance gene was inserted in the binary plasmid vector pGreen0229 and used for *Agrobacterium*-mediated transformation of tobacco (*Nicotiana tabacum*). T0 plants obtained were analyzed by PCR for the presence of *AtTPS1* gene. Thirty lines were positive and seeds were germinated on media with 6 mg/l PPT to obtain T1 plants that were grown in the greenhouse to obtain T2 seeds that were germinated on selective media. Lines which seeds showed a 100% survival rate were considered homozygous transgenic T1 lines. Three lines were selected and gene expression confirmed by northern and western blots. Transgenic seeds were germinated on media with different concentrations of mannitol (0, 0.25, 0.5 and 0.75 M) and sodium chloride (0, 0.07, 0.14, 0.2, 0.27 and 0.34 M) to score their tolerance to osmotic stress. Assays were conducted to test the tolerance of transgenic plants to drought (measurement of water percentage as a consequence of water withdrawal), desiccation (measurement of water loss as a consequence leaf detaching) and temperature stresses (germination at 15 °C and 35 °C). Transgenic tobacco plant lines registered higher germination rates under osmotic and temperature stress situations than did wild-type plants. Responses to drought and desiccation stresses were similar for all

plant lines. Hence it can be suggested that the heterologous expression of TPS1 gene from *Arabidopsis* can be used successfully to increase abiotic stress tolerance in model plants and probably in other crops (Almeida et al. 2005).

After obtaining several lines of tobacco transformed with a trehalose-6-phosphate synthase gene of plant origin (*Arabidopsis thaliana*), involved in the first step of the biosynthesis of trehalose, two of them showed distinct intensity of expression: high (B5H) and low (B1F). Such lines were analysed for trehalose-6-phosphate content and the obtained results demonstrated to be in accordance with the expression results. In order to study the responses of photosynthesis to water deficit of transgenic lines in comparison to wild type (WT), three experiments were performed under different conditions: (1) Relative water (2) Leaf gas exchange (3) Modulated Chlorophyll *a* Fluorescence. Different responses in Relative Water Content of plant lines to water withdrawal were detected, with transgenic line B5H indicating less water loss after the water withdrawal period. Similar responses to water deficit regarding the leaf gas exchanges were recorded for the three lines. When subjected to water deficit stress situations, higher F_v/F_m, Φ_{PSII} and qP were detected for the transgenic lines. Under a Soil Water Content of 20% higher values for such parameters were detected with special relevance for the B5H line, indicating a possible higher ability to withstand severe drought stress and to resist to prolonged periods without water than the B1F and WT lines (Almeida et al. 2006b).

Following the establishment of a transgenic line of tobacco (B5H) expressing the trehalose-6-phosphate synthase (TPS) gene from *Arabidopsis thaliana*, a preliminary immunolocalization study was conducted using leaves of adequately watered B5H and wild-type plants. Immunocytochemical staining, followed by electron microscopy showed that the enzyme could be detected in both B5H and wild-type plants at two different levels. Quantification showed the signal to be two to three times higher in transgenic plants than in the wild type. This enzyme was markedly present in the vacuoles and the cell wall, and to a lesser extent in the cytosol. Moreover, a high profusion of gold particles was detected in adjacent cells and in the sieve elements. Occasional spots were also detected in chloroplasts and the nucleus, especially in the transgenic B5H line. No labelling signal was detected in mitochondria. Protein localization seems to confirm the important role of TPS in sugar metabolism and transport through the plant, which could explain its role in plant stress tolerance. Finally, it can be expected that TPS from tobacco has a relatively high similarity to the TPS of *Arabidopsis thaliana* (Almeida 2007c).

5 Work on Food Security and Rural Development

As stated at the beginning of this paper, being involved in rural development projects, which started in 1976, in 11 African countries, has played a relevant part in the definition of our terms of reference concerning research work. A limited selection of articles highlight backgrounds and fundaments for our attachment regarding field development activities.

The changeable history of the fight against hunger is as old as humanity whose populations had constantly to adapt to changing environmental conditions, epidemics and other adversities. For the first time since the beginnings of agriculture, humanity now has the means at its disposal to overcome world hunger. Malnutrition remains one of

Sub-Saharan Africa's most fundamental challenges for improved human development. It is important to recognize the links between malnutrition, poverty and, at the aggregate level, broad economic growth and national development, namely agrarian production. Presently one person in four goes hungry. In Sub-Saharan Africa, the modest progress achieved in recent years up to 2007 was reversed, with hunger rising 2% per year since then. Progress towards the Millennium Development Goals 1 (MDG 1) target, is assessed not only by measuring undernourishment, or hunger, but also by a second indicator – the prevalence of underweight children under five years of age. A vast amount of International Organizations deal with this subject and publish comprehensive reports not only on estimates on the progress already achieved, but also identifying remaining problems, providing guidance on which policies should be emphasized in the future. However, their targets remain almost unchanged. The aim of the present short review is to enhance the need for improved agriculture productivity and trading systems closely related with persistent malnutrition (Cardoso et al. 2017).

Livestock production in sub-Saharan Africa (SSA) is not matching the annual 2.5% growth of its population. Regional per capita meat and milk production corresponds, respectively, to about 13 and 8% of developed countries indicators. Livestock performances in this region have decreased within the last 30 years. In fact, SSA, with a 12% bovine extraction rate against a world average of 21%, includes about 16% of world cattle, only producing 6 and 2.6% of global meat and milk, respectively. These low performances have economic and environmental consequences reflecting the necessity for upgrading livestock managing skills in the region. This effort includes various components such as sanitary prophylaxis, reproduction, nutrition, and in particular, substantial increase in livestock yield for human consumption. This will allow for an improved animal and pasture management and soil preservation, enhancing meat production and decreasing methane and nitrogen emissions from enteric fermentation and manure processing. These environmental gains due to increased livestock off-take rates can represent relevant credits in the global Environmental Carbon Market under the United Nations Framework Convention on Climate Change Kyoto protocol. These credits can be used for investments in livestock essential services and marketing facilities leading to improved productivity (Cardoso 2012)

The aim of this review is to participate in the debate on the role of agriculture in the development process as a tool to fight against poverty and to enhance population wellbeing in Mozambique through better access to nutrients. In this country there is still no accurate data on food production, consumption and trade trends in a large sample, although the complexity of the food security concept and the need of a multidimensional definition and approach are recognized. The increase in agricultural productivity is seen as a necessary, but not sufficient to achieve a sustainable food security in Mozambique or indeed in Sub-Saharan Africa. Different views on the relevance of agriculture for growth and development in Africa suggest different policy choices in this continent (Ferrão et al. 2018).

The correlation of established statistical indexes of population welfare with main development parameters was the subject of one of our studies within this area. The statistical behavior of two indexes, Protein Retention Level (NRL) and Energy Ingestion Level (EIL) were analyzed against nutrition and macroeconomic indicators concerning 66 countries including 80% of world population. It was shown that there is greater

correlation between those indicators with NRL than with EIL, suggesting that the Protein retention level should replace Energy Ingestion Level as a development indicator (Cardoso 1998).

6 Conclusion

Promising perspectives are open with advances in Omics studies regarding animal and plant tolerance to hyperthermia and drought conditions which represent agriculture productivity issues concerning most tropical regions. Genetic selection improvements in terms of targets and methodology are main research aims that we hope to contribute with our work.

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Animal Health, One Health

The Gut Microbiome and Antimicrobial Resistance in Companion Animals

A. Belas, C. Marques, and C. Pomba(✉)

Antibiotic Resistance Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
cpomba@fmv.ulisboa.pt

Abstract. The spread of antimicrobial resistance represents a serious problem to human and animal health. The use and misuse of antimicrobials in therapeutics and agriculture selects for antimicrobial resistance genes and bacteria that can be directly transferred to animal/human-associated microbiomes by direct human-animal contact. Antimicrobials have a profound impact on the gut microbiome, altering the nutritional landscape of the gut and leading to the expansion of pathogenic bacterial populations. Understanding the effect of antimicrobials in the microbiome dysbioses and resistome is, therefore, a cornerstone of the efforts to limit the spread of resistant and pathogenic bacteria.

Before of the Next Generation Sequencing (NGS) technologies available nowadays, studies to analyze the microbiome (16S rRNA gene sequencing) and resistome were usually conducted by culturomic approaches. Now, the molecular and NGS approaches allow the study of the gut resistome and microbial communities that otherwise would hardly be detected. Studies on companion animals are still limited and the microbiome and resistome overlap between humans and pets is just recently being addressed. This is a major research focus of the Antibiotic Resistance Laboratory. Furthermore, ongoing studies using NGS technologies will unravel the microbiome and resistome overlap between healthy and infected companion animals and their human caretakers.

Keywords: Microbiome · Anti-microbial resistance

1 The Gut Microbiome

As defined by Joshua Lederberg in 2001, the microbiome is "the ecological community of commensal, symbiotic and pathogenic microorganisms that literally share our body space and have been all but ignored as determinants of health" (Lederberg and McCray 2001; Noli 2017). It should be noted, however, that the gut microbiota and the gut microbiome have different meanings. While the gut microbiota is the dynamic community of trillions of microorganisms living in analogy with eukaryotic cells; the gut microbiome is the diverse consortium of bacteria, archaea, fungi, protozoa, viruses, and also their genomes that are present in the gastrointestinal tract (Shreiner *et al.* 2015; Kim *et al.* 2017). The gut microbiome is mainly composed of bacteria including harmless symbionts, commensals

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and pathogens. The gut microbiome is composed by a diverse bacteria population and has an estimated size of $10^{10}-10^{14}$ microbial cells. Furthermore, it has been shown to have an important role in health and disease (Morgan and Huttenhower 2012; Cho and Blaser 2012; Belkaid and Hand 2014; Minamoto *et al.* 2015; Bäumler and Sperandio 2016; Swanson 2016; Bradley and Pollard 2017; Kim *et al.* 2017; Scotti *et al.* 2017).

The gut microbiome is established early in life and varies along the gastrointestinal tract from less than 10^3 bacteria ml^{-1} in the stomach and the duodenum to 10^4–10^7 bacteria ml^{-1} in the jejunum and ileum (Schaik 2015).

2 Methods to Study the Gut Microbiome

Prior to the availability of sequence-based methodologies for microbiome analysis, studies were conducted using classic microbiology that relies on the ability to culture viable organisms outside their natural habitat (Tyler *et al.* 2014). The use of culturing methods presents several difficulties in regard to slow growing microorganism and those that are not viable in *in vitro* conditions (Tyler *et al.* 2014). Despite this limitation, the use of classic microbiology has been very useful to study fast growing microorganisms. In this field, the Antibiotic Resistance Laboratory has focused its efforts in the study of the gut colonization of animals by resistant and pathogenic bacteria (Centeno *et al.* 2010; Fernandes *et al.* 2012; Belas *et al.* 2013; Centeno *et al.* 2013; Pomba *et al.* 2013; Mateus *et al.* 2013; Belas *et al.* 2014; Fernandes *et al.* 2016; Belas *et al.* 2015; Belas *et al.* 2017aa; Belas *et al.* 2017b; Marques *et al.* 2017a; Marques *et al.* 2017b, Belas *et al.* 2018a; Marques *et al.* 2018a; Marques *et al.* 2018b).

In recent years, the use of Next Generation Sequencing (NGS) has revolutionized the gut microbiome research by using bacterial DNA sequences as a proxy to estimate the microorganism's identity, relative abundance and function (Tyler *et al.* 2014; Noli 2017; Rintala *et al.* 2017).

Within the bacterial genome there are some stable genetic markers that can be used for phylogenetic analysis. The 16S ribosomal RNA (rRNA) is a ± 1,550 bp long gene, composed of nine hypervariable regions, that are present in all bacteria and Archaea and that allow its taxonomic classification (Tyler *et al.* 2014). The study of the microbiome by NGS makes use of the 16S rRNA hypervariable regions that are flanked by conserved regions to allow the simultaneous amplification and sequencing of many microorganisms within a sample. The most commonly used segments are the V1-V3, V4 and V4-V5 16S rRNA regions (Tyler *et al.* 2014). The NGS methodologies have the advantage of allowing the identification of fastidious and unculturable microorganisms that otherwise could not be studied (Tyler *et al.* 2014).

Most gut microbiome studies use either the 454 pyrosequencing or the Illumina sequencer (Tyler *et al.* 2014). After data acquisition, a quality filtering is conducted to remove sequences with low base quality scores, short reads and errors related to the sequencing technologies, like chimeras. Trimmed raw sequences are then assigned to taxonomic groups to generate more meaningful information for downstream analyses which includes the direct assignment to phylotypes or to operational taxonomic units (OTU). The OTUs can then be assigned to a taxonomy through the use of reference databases.

Several databases are available to assist the taxonomic assignment, namely the Ribosomal Database project (Cole *et al*. 2009), Greengenes database (DeSantis *et al*. 2006; McDonald *et al*. 2012) and Silva database (Pruess *et al*. 2007). The Greengenes database was introduced in 2006 and is the most used for assigning the taxonomy to Archaea and bacteria (DeSantis *et al*. 2006; McDonald *et al*. 2012). The microbiome sequencing data analysis is somewhat complex. To simplify this process, several software packages can be used that offer the interaction between many essential algorithms for organism identification and analysis such as MOTHUR (Schloss *et al*. 2009), Visualization and Analysis of Microbial Population Structures (VAMPS) (Sogin *et al*. 2009) and Quantitative Insights Into Microbial Ecology (QIIME) (Lozupone *et al*. 2006; Lozupone *et al*. 2007; Caporasso *et al*. 2011; Caporasso *et al*. 2012; Lozupone *et al*. 2012; Navas-Molina *et al*. 2013).

Most gut microbiome studies use traditional ecological indicators, as the alpha and beta diversity, to characterize and compare the bacterial community composition. The alpha diversity is a measure of the diversity within a sample that can be determined by different methods taking into account different factors such as the richness (number of OTUs/species present in a sample) and evenness (relative abundance of different OTUs/species and their distribution). The alpha diversity calculous may include one of the following algorithms: observed species (measures unique OTUs in a sample); Chao1 (estimates the species richness); Shannon´s index (measures both richness and evenness); Simpson´s index (measures both richness and evenness, but less affected by the presence of rare species when compared to Shannon´s index); and phylogenetic distance (PD) (includes PD into the diversity calculation) (Kumar *et al*. 2015). The beta diversity is a measure of the distance or dissimilarity between each sample pair and it is often depicted using a principal coordinate or component analysis (PCoA/PCA) plot. These plots are used to visualize the beta diversity distance matrix as a 2- or 3-dimensional plot, which allows the detection of clustering patterns of samples that may be related to experimental conditions. The distance between samples can be determined by many metric systems such as the Bray-Curtis (non-phylogeny-based method that takes abundance into account), the Weighted UniFrac (uses the abundance information for each OTU alongside with their PD) and the Unweighted UniFrac (considers the presence or absence of OTUs between samples along with their PD) (Lozupone and Knight 2005; Lozupone *et al*. 2006; Tyler *et al*. 2014; Kumar *et al*. 2015).

One of the major advantages of NGS methodologies is the high-throughput capacity leading to faster and more detailed results. For this reason, NGS is slowly replacing culture-based studies of the microbiome (Barko *et al*. 2018). Nevertheless, there is a lack of methodological consensus (e.g. sample processing, sequencing technologies and statistical methods) that limits the comparison between different studies (Rintala *et al*. 2017). Furthermore, NGS analysis of the gut microbiome still has some pitfalls namely:

1) 16S rRNA sequencing does not distinguish between live and dead microorganisms;
2) It is not possible to know if a specific microorganism is transient or resident;
3) Cannot distinguish between antimicrobial resistant/susceptible or commensal/pathogenic strains of the same species.

For these reasons the complement use of classic microbiology is still useful and necessary (Balvočiūtė and Huson 2017; Noli 2017).

3 Gut Microbiome and Antimicrobial Resistance

The first metagenomic analysis of the human gut microbiome was conducted in 2007 by the Human Microbiome Project (HMP), soon followed by other initiatives such Metagenomics of the European Human Intestinal tract (MetaHIT) in 2008 (Qin *et al.* 2010; The Human Microbiome Project Consortium 2012). The first study showing an association between the human gut microbiome and obesity was done in 2009 (Swanson 2016).

The entire microbiome influences several important physiological processes such as the immune system development, the harvesting of energy from food, the ability to process polysaccharides and vitamins, hormone production, etc. (Scotti *et al.* 2017). The gut microbiome is also responsible for the breakdown of dietary fibers, which is essential for the stability and growth of the gut microbiota and for the production of beneficial metabolic end products to the host (Scotti *et al.* 2017; Barko *et al.* 2018).

The human and animal gut microbiome ecosystem contains many microbial species, most of which belonging to the *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and, to a lesser extent, *Proteobacteria* phyla. However, the relative abundances of each phyla vary over time and between individuals (Barko *et al.* 2018; Casals-Pascual *et al.* 2018) (Table 1).

The gut microbiome in humans and animals models has been shown to be affected by many factors such as age, diet, environment, prebiotics, probiotics, pump proton inhibitors (omeprazole), antimicrobial use, etc. (Turnbaugh *et al.* 2007; Cho and Blaser 2012; Garcia-Mazcorro *et al.* 2012; Swanson 2016; Barko *et al.* 2018).

Imbalances of the normal gut microbiome, known as dysbiosis, may adversely affect the health status of an individual and lead to the loss of protection against pathogens (Casals-Pascual et *al.* 2018). Certain diseases, such as gastrointestinal infections, type 2 diabetes, cardiovascular diseases, auto-immune diseases, mental diseases, obesity, allergies, liver disease, colon cancer and inflammatory bowel disease have been associated with changes in the normal microbiome (Swanson 2016; Casals-Pascual *et al.* 2018). Understanding the role of different bacterial communities in heathy and sick individuals may uncover new therapeutic targets in the future. The effects of the antimicrobial use and infection in the microbiome is an expanding field of research in which the Antibiotic Resistance Laboratory is developing work by classic microbiology and NGS (Belas *et al.* 2014; Belas *et al.* 2015; Belas *et al.* 2016; Belas *et al.* 2017a; Belas *et al.* 2017b; Belas *et al.* 2017c; Marques *et al.* 2017a; Marques *et al.* 2017b, Belas *et al.* 2018a; Belas *et al.* 2018b).

Currently there is only a limited number of studies characterizing the gut microbiome of companion animals (Suchodolski *et al.* 2009; Garcia-Mazcorro *et al.* 2012; Suchodolski *et al.* 2012; Handl *et al.* 2013; Honneffer *et al.* 2014; Igarashi *et al.* 2014; Minamoto *et al.* 2015; Blake and Suchodolski 2016; Li *et al.* 2018; Kim *et al.* 2017; Sandri *et al.* 2017; Coelho *et al.* 2018). Much like in humans, the faecal microbiome of companion animals contain all three domains of life – Archaea, Bacteria, and Eukarya - and is predominated by bacteria (around 98%) (Table 1) (Barko *et al.* 2018; Moon *et al.* 2018).

Table 1. Taxonomy and phylogeny of common constituents of the gastrointestinal microbiome (adapted from Barko *et al*. 2018)

Phylum	Class	Order	Family	Genus
Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*
			Ruminococcaceae	*Rumminococcus*
				Faecalibacterium
			Eubacteraceae	*Eubacterium*
	Bacilli	*Lactobacilliales*	*Lactobacillaceae*	*Lactobacillus*
			Streptococcaceae	*Streptococcus*
				Enterococcus
	Erysiphelotrichia	*Erisophelotrichales*	*Erysiphelotrichaceae*	*Turicibacter*
				Catenibaterium
				Coprobacillus
				Allobaculum
	Negativicutes	*Selenomonadales*	*Selemonadaceae*	*Megamonas*
		Veillonellales	*Veillonellaceae*	*Dialister*
				Megasphaera
				Villonella
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*
			Bacteroidaceae	*Bacteroides*
Actinobacteria	*Coriobacteriia*	*Coriobacteriales*	*Coriobacteriaceae*	*Colinsella*
			Atopobiaceae	*Olsenella*
		Eggerthellales	*Eggerthellaceae*	*Slackia*
				Eggerthella
	Actinobacteria	*Bifidobacteriales*	*Bifidobacteriaceae*	*Bifidobacterium*
Fusobacteria	*Fusobacteriia*	*Fusobacteriales*	*Fusobacteriaceae*	*Fusobacterium*
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteraceae*	*Escherichia*
				Shigella
		Aeromonadales	*Succinivibrionaceae*	*Succinivibrio*
				Anaerobiospirillum

The gut microbiome of healthy companion animals commonly carries *Proteobacteria*, which is its most diverse bacterial phylum. *Proteobacteria* play a key role in the maintenance of the homeostasis of the anaerobic environment of the gut; however, this phylum also includes well-known opportunistic pathogens (e.g. *Escherichia coli, Klebsiella pneumoniae, Salmonella* spp. and *Campylobacter*) (Moon *et al*. 2018). Antimicrobial resistance is a significant global public health and Enterobacteriaceae are key drivers of dissemination of important resistance mechanisms (Allcock *et al.* 2017; Pomba *et al.* 2017). For this reason, the role of companion animals as reservoirs of resistant bacteria as long been a concern (Pomba *et al.* 2017).

In a study conducted in the Antibiotic Resistance Laboratory, a high frequency of dogs from shelters were shown to be colonized by ESBL/AmpC-producing *E. coli*. Interestingly, dogs living in shelters and dogs with prior history of antimicrobial use (within last year) were significantly more likely to be colonized by ESBL/AmpC-producing *E. coli* (Belas *et al*. 2014). The use of antimicrobials is also a known factor to increased colonization by non-commensal bacteria and infection by foodborne pathogens (Barza and Travers 2002). The use of antimicrobials may have a profound impact on the gut microbiome altering its nutritional landscape and leading to the possible expansion of pathogenic and resistant bacteria (Bäumler and Sperandio 2016). Acquisition of multidrug-resistant bacteria (MDR) by the gut can result from: 1) exogenous acquisition of MDR bacteria that colonize the gut intestinal epithelium; 2) acquisition of resistant mechanisms by previously susceptible bacteria through antimicrobial selective pressure (Casals-Pascual *et al*. 2018). Understanding the effect of antimicrobials in the microbiome dysbioses and resistome is, therefore, a cornerstone of the efforts to limit the spread of resistant and pathogenic bacteria. In fact, the gut colonization of dogs and cats with pathogenic or resistant bacteria has already been vastly demonstrated worldwide (Sayah *et al*. 2005; Damborg *et al*. 2008; Johnson *et al*. 2008; Belas *et al*. 2014; Damborg *et al*. 2015; Belas *et al*. 2016; Johnson *et al*. 2016; Vangchhia *et al*. 2016; Aslantaş and Yilmaz 2017; Belas et al. 2017b; Belas *et al*. 2018a; Marques *et al*. 2018a, Marques *et al*. 2018b). Just recently dogs from Portugal (Lisbon) were shown to be colonized by *K. pneumoniae* belonging to clonal lineages mainly associated with human infection (Marques *et al*. 2018a; Marques *et al*. 2018b). Furthermore, commensal *E. coli* from dogs belonging to phylogroups B2 and D were associated with important pathogenicity islands markers profiles, namely PAI IV_{536}, PAI II_{536}, PAI II_{J96}, PAI I_{CFT073} and PAI IV_{536}, PAI I_{CFT0T3}, respectively (Belas *et al*. 2017c). These studies highlight the importance of companion animals as reservoirs of pathogenic Enterobacteriaceae. The emergence and spread of MDR and pathogenic Enterobacteriaceae in the natural environment by companion animal fecal contamination is also a concern towards animal and human health (Belas *et al*. 2017a; Belas *et al*. 2017c, Marques *et al*. 2018a; Marques *et al*. 2018b). In a study conducted in healthy dogs submitted to surgical procedures, fecal samples were collected before (BS) and after surgery (AS). The number of dogs with gut colonization by MDR bacteria was significantly higher AS likely due to antimicrobial use. The MDR bacteria fecal burden was also significantly higher AS when compared to the samples collected BS. *E. coli* was the most prevalent MDR-resistant bacteria in this study. These findings highlight the importance of nosocomial colonization, infection control systems and judicious antimicrobial use in order to improve animal health and safeguard Public Health (Belas *et al*. 2016).

In another study, the gut antimicrobial resistance gene content of healthy humans and companion animals living in close contact was tested using a genomic approach. The resistome of companion animals was shown to be similar to those of humans living in close contact, namely regarding $bla_{TEM\text{-}1B}$, *tet*A, *sul*1 and *sul*2. The *sul2* was the gene that more humans and animals shared in the same household. Healthy companion animals carried several antimicrobial resistance genes of clinical importance. Therefore, their role in the dissemination of these antimicrobial resistance determinants through fecal contamination should not be neglected (Belas *et al*. 2017b).

To understand the effect of disease and antimicrobial use in the gut microbiome, it is first essential to characterize the gut microbiome of healthy companion animals and all the factors that may affect its balance. In Handl *et al*. (2011) study, *Firmicutes* was the most copious bacterial phylum in the fecal microbiome of healthy companion animals. Interestingly, the fecal microbiome of cats seemed to be more diverse than the gut microbiome of dogs. *Clostridia* was the most predominant bacterial class and *Clostridium* and *Ruminococcus* its dominating genera. The *Bacilli* class was also frequent and almost solely composed by the *Lactobacillales* order in both dogs and cats. This order was mainly represented by the genera *Streptococcus* and *Lactobacillus* in dogs, and *Enterococcus* and *Lactobacillus* in cats. The *Erysipelotrichi* class was exclusively composed by the *Erysipelotrichales* order, which in turn, consisted of the genera *Turicibacter*, *Catenibacterium*, and *Coprobacillus.* In dogs, the second most representative phylum was *Bacteroidetes*, containing the *Prevotella*, *Bacteroides*, and *Megamonas* genera. In cats, the second most abundant phylum was *Actinobacteria.*

Even though most of the studies conducted in companion animals characterize the fecal microbiome using fresh fecal samples, it is important to notice that the gut microbiome varies according to intestinal segment considered (Ritchie *et al*. 2008; Suchodolski *et al*. 2008). In dogs, there is a gradual increase in the bacterial diversity along the intestinal tract, from the duodenum to the colon. In the duodenum and jejunum, the *Clostridiales* is the most abundant order whereas in the ileum and colon the *Fusobacteriales* and *Bacteroidales* order predominate, respectively. The *Proteobacteria* phylum (including *E. coli* organisms) makes an important part of the duodenal microbial community. The order *Lactobacillales* are well represented in the duodenum, jejunum and colon, but are only present in a small fraction of the ileum (Suchodolski *et al*. 2008). In cats the small intestine is dominated by *the Firmicutes* and *Bacteroides* phylum, while the ileum is mostly composed by *Proteobacteria* and *Actinobacteria* phylum. Lastly, the *Firmicutes*, *Proteobacteria* and *Fusobacteria* are the predominant phylum in the colon of cats (Ritchie *et al*. 2010).

Among the factors affecting the gut microbiome, some studies seem to suggest that related humans have a more similar microbiome composition than unrelated humans and that heritability of the gut microbiome is correlated with specific host gene elements (Goodrich *et al*. 2016; Khachatryan *et al*. 2008; Turnbaugh *et al*. 2009). One-study even states that people living in different regions can be distinguished from one another by comparing their intestinal microbiome's composition and genomic features (Yatsunenko *et al*. 2012). Likewise, gut microbiomes of genetically related dogs seem to be more similar between each other than those of unrelated dogs (Hand *et al.* 2013). These finding highlight the important role of the environment in the gut microbiome.

Considering the previous findings from the Antibiotic Resistance Laboratory where shelter dogs where shown to be more likely colonized by ESBL/AmpC producing bacteria (Belas *et al*. 2014), a recent study was conducted to compare the gut microbiome of healthy dogs and cats living in human households and in shelters using NGS (Belas *et al*. 2018b; Belas *et al*. 2018c). Dogs living in households had different gut microbiome composition than dogs living in shelters, demonstrating that the living environment likely has an important influence in its composition. Furthermore, dogs from shelters had a higher relative abundance of bacteria phyla associated with potentially pathogenic bacteria. The

detection of the Enterobacteriaceae family (*Proteobacteria* phylum) was significantly different between shelter and household dogs. Therefore, shelter dogs may play and important role in the dissemination of this bacterial family (Belas *et al*. 2018b). Interestingly, some of the genera found in shelter dogs has been previously associated with stress in mice (Belas *et al*. 2018c). The data gathered in these studies adds an important value to the field of veterinary microbiome research, which is in its infancy. Nevertheless, further studies are required to understand the specific environment factors that may explain the differences detected, such as stress, hygiene practices, among others. Furthermore, it was possible to observe that the shelter dogs had a more diverse microbiome population when compared to household dogs. This fact is of extreme importance because, in studies conducted in humans, it has been shown that some diseases have been associated with lower microbiome diversity (Morgan and Huttenhower 2012; Minamoto *et al*. 2015).

In the current line of research in the Antibiotic Resistance Laboratory, the changes in the gut microbiome of companion animals before and after antimicrobial pressure and the overlap of human and companion animal microbiome and resistome is being investigated to identify the bacterial communities that can represent a particular risk or, on the contrary, lead to new therapeutic solutions.

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Antimicrobial Resistance Trends in Dogs and Cats with Urinary Tract Infection

C. Marques, A. Belas, and C. Pomba(✉)

Antibiotic Resistance Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
cpomba@fmv.ulisboa.pt

Abstract. Urinary tract infections (UTI) are among the most common infections diagnosed in companion animals and usually require antimicrobial use. The antimicrobials available nowadays for UTI treatment are limited and most of them are considered as critically important to humans by the World Health Organization (WHO). Therefore, awareness of the antimicrobial resistance trends and underlying resistance mechanisms in uropathogenic bacteria from dogs and cats is of importance in a one health perspective.

This has long been one of the main research fields of the Antibiotic Resistance Laboratory Team. A recent and pivotal study from our team has given, for the first time, an European overview of the geographic distribution of antimicrobial resistance in uropathogenic bacteria from dogs and cats. The southern European countries, including Portugal, had significantly higher resistance frequencies than Northern countries, mirroring what happens in human invasive bacteria. Noteworthy, increasing antimicrobial resistance trends over 16 years were also detected in bacteria from companion animals with UTI.

The detection of multidrug-resistant (MDR) extended spectrum beta- lactamases (ESBLs)/AmpC beta- lactamases - producing bacteria in companion animals with UTI is worrisome due to its clinical implications. Furthermore, companion animals UTI may frequently be caused by high-risk clonal lineages to humans like *Escherichia coli* ST131, ST648 and *Klebsiella pneumoniae* ST15 which underlines their public-health relevance.

Keywords: Antimicrobial resistance · Animal infection · Public health risk

1 Urinary Tract Infections

Urinary tract infections (UTI) are among the most frequently diagnosed infections in companion animals (Weese et al. 2011). Is has been estimated that close to 14% of dogs visiting a veterinarian will develop a UTI during their lifetime (Thompson et al. 2011). The frequency of urinary tract infections in cats with lower urinary tract disease is considered to be less than 2% (Gunn-Moore 2003); however, some studies have found higher frequencies, varying between 8% and 25% (Lekcharoensuk et al. 2001; Gerber et al. 2005; Eggertsdóttir et al. 2007; Sævik et al. 2011). Therefore, UTIs are an important

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reason for the need to prescribe antimicrobials in small animal veterinary medicine. Considering its relevance, the study of UTIs and antimicrobial resistance in companion animals has been one major research field of the Antibiotic Resistant Laboratory in the last decades (Féria et al. 2000; Féria et al. 2002; Caniça et al. 2004; Pomba et al. 2009; Pomba et al. 2010; Mateus et al. 2013; Oliveira et al. 2014; Pomba et al. 2014a; Pomba et al. 2014b; Marques et al. 2016; Marques et al. 2018a; b; Belas et al. 2019; Marques et al. 2019).

2 Pathophysiology and Predisposing Factors

UTIs occur as a consequence of the failure of host defence mechanisms with subsequent adherence, multiplication and persistence of virulent bacteria in the urinary tract (Bartges et al. 2004). UTI are usually initiated by the adherence and colonisation of bacteria into the urethra followed by migration to the bladder. Successful bacteria will then multiply and colonise the bladder and eventually ascend to the kidney. Ultimately, bacteria will cross the tubular epithelial barrier into the blood stream, resulting in bacteraemia (Flores-Mireles et al. 2015).

There are several predisposing factors associated with higher frequencies of UTI including: *diabetes mellitus*, chronic kidney disease in cats, hyperthyroidism in cats, hyperadrenocorticism and bladder transitional cell carcinoma in dogs, anatomical abnormalities or diseases promoting urine retention and abnormal micturition, corticoid treatment (Saitoh et al. 1985; Freshman et al. 1989; Forrester et al. 1999; Hess et al. 2000; Seguin et al. 2003; Torres et al. 2005; Bailiff et al. 2006; Stiffler et al. 2006; Graves et al. 2007; Mayer-Roenne et al. 2007; Bubenik and Hosgoof 2008; Eriksson et al. 2010; Hirji et al. 2012; Martinez-Ruzafa et al. 2012; Budreckis et al. 2015). Furthermore, UTI is more frequent in female and older dogs and cats, spayed female dogs and Persian and Abyssinian cat's breeds (Lekcharoensuk et al. 2001; Ling et al. 2001; Cohn et al. 2003; Seguin et al. 2003; Bailiff et al. 2006; Graves et al. 2007; Mayer-Roenne et al. 2007; Bailiff et al. 2008).

3 Classification of Urinary Tract Infections and Diagnosis

In veterinary medicine, the Working Group of the International Society for Companion Animal Infectious Diseases (ISCAID) has developed a dedicated guideline for the antimicrobial treatment of UTI in companion animals (Weese et al. 2011). The first step in the UTI treatment decision making in this guideline relies on the classification of the type of UTI.

Bacteriuria can be detected in patients without the presence of clinical signs, then called asymptomatic bacteriuria (Weese et al. 2011). When present, clinical signs of UTI are not pathognomonic and include dysuria, pollakiuria, stranguria, haematuria, urgency to urinate, fever, abdominal/flank pain, vocalisation, among others (Bartges et al. 2004; Gerber et al. 2005; Weese et al. 2011; Passmore et al. 2008).

According to the location, UTIs are considered upper UTIs or pyelonephritis (kidney) and lower UTIs or cystitis (bladder). Based on the frequency of UTI episodes within the

last 12 months, UTIs are classified as simple (<3 episodes) or recurrent (≥3 episodes) (Weese et al. 2011).

Cystitis are considered as uncomplicated when they are diagnosed in patients that are otherwise healthy (i.e. without comorbidities) and with normal genitourinary tract anatomy and function (Weese et al. 2011). These are always simple UTIs, since according to Weese et al. (2001) recurrence points to the presence of undiagnosed comorbidities. Complicated cystitis occurs in patients with comorbidities (e.g. urinary obstruction, renal failure and *diabetes mellitus*) or predisposing factors for UTI (Weese et al. 2011).

Diagnosis and classification of UTI requires the clinical evaluation of the patient, complete type II urinalysis, the necessary complementary diagnostic workout to diagnose suspected comorbidities and a urine culture (Weese et al. 2011). The presence of bacteriuria and pyuria in urine sediment strongly correlates with the presence of UTI, however it is not diagnostic (Bartges et al. 2004; Mayer-Roenne et al. 2007).

Urine culture should preferably be performed with urine collected by cystocentesis, followed by catheterisation or free-catch (midstream voiding or manual expression) in order to minimize sample contamination. The use of free-catch urine in companion animals is controversial among authors (Bartges et al. 2004; Weese et al. 2011, Sørensen et al. 2016). The quantitative urine culture is the gold standard for the diagnosis of significant bacteriuria (Bartges et al. 2004; Weese et al. 2011) because it accounts for the number of colony forming units per urine volume and true bacteriuria is adjusted to the urine collection method used (Bartges et al. 2004). Ideally, urine culture should be followed by antimicrobial susceptibility testing (AST) of the isolated bacteria to guide or adjust antimicrobial therapeutics and to gather epidemiological data on local UTI aetiology and susceptibility patterns (Weese et al. 2011).

4 Aetiology

UTIs are usually caused by bacteria and more rarely by fungi and viruses (Forrester et al. 1999; Pressler et al. 2003). *Escherichia coli* (uropathogenic *E. coli* - UPEC) is the main bacteria isolated in all types of UTIs, although other Gram-negative and Gram-positive bacteria may also be implicated. The frequency of each bacteria genera varies according to the study likely reflecting different geographical/temporal trends as well as different inclusion criteria. (Bush, 1976; Wooley and Blue 1976; Forrester et al. 1999; Hess et al. 2000; Ling et al. 2001; Cohn et al. 2003; Pressler et al. 2003; Torres et al. 2005; Bailiff et al. 2006; Litster et al. 2007; Mayer-Roenne et al. 2007; Passmore et al. 2008; Martinez-Ruzafa et al. 2012; Dorsch et al. 2015; Moyaert et al. 2017).

Studies conducted in the Antibiotic Resistant Laboratory showed that *E. coli* is indeed the most frequently isolated bacteria from companion animals with UTI in the Lisbon area (Marques et al. 2018b) and corroborated previous suspicions that the UTI aetiology varies significantly between cats and dogs (Marques et al. 2016; Marques et al. 2018a).

After *E. coli*, cats have high frequency of UTIs caused by *Enterococcus* spp. and *Staphylococcus* spp. (Wooley and Blue 1976; Bailiff et al. 2006; Litster et al. 2007; Mayer-Roenne et al. 2007; Bailiff et al. 2008; Passmore et al. 2008; Martinez-Ruzafa et al. 2012; Dorsch et al. 2015; Marques et al. 2016; Moyaert et al. 2017; Marques et al. 2018a; Teichmann-Knorrn et al. 2018). *Enterococcus* spp. are significantly more

common in cats than in dogs (Marques et al. 2016; Marques et al. 2018a). *Enterococcus faecalis* is the most prevalent, followed by *Enterococcus faecium,* which is rarely isolated (Litster et al. 2007, Mayer-Roenne et al. 2007; Marques et al. 2018a). Several *Staphylococcus* species may cause UTI in cats (Litster et al. 2007; Marques et al. 2018a). Litster et al. (2007) highlighted the high frequency of UTIs caused by *Staphylococcus felis* in cats from Australia. The same was observed in cats with UTI from Portugal (Lisbon) (Marques et al. 2018b). Other bacteria causing UTIs in cats include *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Streptococcus* spp., *Pasteurela spp.*, *Klebsiella pneumoniae*, among others (Wooley and Blue, 1976; Lekcharoensuk et al. 2001; Bailiff et al. 2006; Bailiff et al. 2008; Litster et al. 2007; Marques et al. 2016; Marques et al. 2018a).

P. mirabilis is significantly more common in dogs with UTI, usually being the second most frequent isolated Enterobacteriaceae after *E. coli* (Wooley and Blue, 1976; Ling et al. 2001; Cohn et al. 2003; Marques et al. 2016; Moyaert et al. 2017; Marques et al. 2018a). Other bacterial causes of UTI in dogs also include *Staphylococcus* spp., *Enterococcus* spp. and more rarely *Pseudomonas* spp., *Klebsiella* spp., *Streptococcus* spp. (Forrester et al. 1999; Norris et al. 2000; Ling et al. 2001; Prescott et al. 2002; Cohn et al. 2003; Marques et al. 2016; Marques et al. 2018a). Although several *Staphylococcus* species may cause UTI in dogs, *Staphylococcus pseudintermedius* predominates (Prescott et al. 2002; Cohn et al. 2003; Penna et al. 2010; Marques et al. 2018b). Interestingly, dogs with complicated/recurrent UTIs seem to have higher frequencies of less common bacteria such as *K. pneumoniae*, *Enterococcus* spp. and *Pseudomonas* spp. (Forrester et al. 1999; Norris et al. 2000; Torres et al. 2005). Additionally, some studies have suggested that there are some differences according to the dog gender (Norris et al. 2000; Ling et al. 2001; Cohn et al. 2003).

5 Antimicrobials for UTI Treatment in Companion Animals

The increasing antimicrobial resistance trends observed over the last decades in human and veterinary medicine are a worldwide concern that requires a One Health approach (World Health Organization [WHO] 2017b). The antimicrobial resistance selective pressure due to antimicrobial use is believed to be one key contributing factor (Guardabassi et al. 2004; Pomba et al. 2017; World Health Organization [WHO] 2017a). Since antimicrobials are the cornerstone for UTI treatment and are frequently required, correct diagnosis and proper antimicrobial selection is crucial to avoid antimicrobial misuse (Weese et al. 2011). The ISCAID guidelines for UTI treatment in companion animals propose a rational list of antimicrobials that should be used according to the type of UTI (Weese et al. 2011) (Table 1).

The WHO (2017a) groups the antimicrobials according to their importance to human medicine into three categories: important, highly important and critically important antimicrobials (CIA). Furthermore, CIAs may also be divided into high or highest priority antimicrobials based on 3 additional prioritisation criteria (WHO 2017a). It should be noted that several antimicrobials approved for UTI treatment in small animal veterinary medicine are also used in human medicine and belong to CIAs of high and highest priority (Table 1). Therefore, the rational use of antimicrobials in small animal veterinary medicine is of the upmost importance.

Table 1. Antimicrobials used for UTI treatment in dogs and cats

Antimicrobials	WHO (2017a) classification[a]	Companion animals[b]
Beta-lactams		
Amoxicillin	CIA - HP	First-line option for UTI treatment. First-line option for empirical treatment of uncomplicated and complicated UTI
Amoxicillin/clavulanic	CIA - HP	Unknown advantage over amoxicillin alone. If amoxicillin resistance rates are high locally, may be a good first-line option for UTI treatment and empirical treatment of uncomplicated and complicated UTI
Second-generation cefalosporins	HIA	Second-line option for UTI treatment
Third-generation cefalosporins	CIA – HestP	Second-line option for UTI treatment
Carbapenems	CIA - HP	Last-resort antimicrobial. Prescribed off-label
Aminoglycosides	CIA - HP	Not recommended for routine use due to side effects. Although not included in Weese et al. (2011) guidelines, gentamicin has been shown to be useful for UTI treatment (Ling and Ruby, 1979)
Chloramphenicol	HIA	Off-label use. Recommended for multidrug resistant bacteria
Doxycycline	HIA	Not recommended for routine use; nevertheless, its usefulness in UTI treatment has been demonstrated (Wilson et al. 2006)
Fluoroquinolones	CIA - HestP	Second-line option for UTI treatment. Considered a good first-line option for empiric antimicrobial treatment of pyelonephritis

(*continued*)

Table 1. (*continued*)

Antimicrobials	WHO (2017a) classification[a]	Companion animals[b]
Nitrofurantoin	IA	Second-line antimicrobial. Off-label use. Reserved for uncomplicated UTIs caused by multidrug resistant bacteria
Trimethoprim/sulfamethoxazole	HIA	First-line option for UTI treatment. First-line option for empirical treatment of uncomplicated and complicated UTI

Legend: CIA, critically important antimicrobial; HP, high priority antimicrobial; IA, Important antimicrobial; HP, high priority; HestP, Highest priority; [a]as defined by WHO (2017a); [b]Major data according to Weese et al. (2011).

These ISCAID guidelines are general recommendations that need to be properly adjusted considering the specific geographic antimicrobial resistance rates, antimicrobial availability and prescribing regulations (Weese et al. 2011). Given the importance of this subject, the Antibiotic Resistance Laboratory has conducted over the last years important antimicrobial resistance surveillance studies to determine the antimicrobial resistance temporal trends and underling resistance mechanisms of bacteria isolated from companion animals with UTI from Portugal (Lisbon) and Europe (Féria et al. 2000; Féria et al. 2002; Caniça et al. 2004; Pomba et al. 2009; Oliveira et al. 2014; Pomba et al. 2014a; Marques et al. 2016; Marques et al. 2018a; Marques et al. 2018b; Belas et al. 2019; Marques et al. 2019).

6 Antimicrobial Resistance Surveillance in Companion Animals

The European Antimicrobial Resistance Surveillance Network (EARS-Net) actively gathers and reports annual data on antimicrobial resistance in human invasive bacteria from several European countries (European Centre for Disease Prevention and Control [ECDC] 2017). These EARS-Net reports show remarkable geographical differences in antimicrobial resistance frequencies among European countries as well as increasing trends in resistance to CIAs (ECDC 2017).

Such important surveillance programs are lacking in small animal veterinary medicine. There have been only few national antimicrobial resistance surveillance networks in place for companion animals in Germany, Sweden and France (Swedres-Svarm 2016; Moyaert et al. 2017). In 2008, the European Animal Health Study Centre started an initiative (Compath) gathering bacterial isolates from companion animals in Europe and just recently published data regarding UTI isolates from 2008–2010 (Moyaert et al. 2017). Moyaert et al. (2017) reported overall high susceptibility to all tested antimicrobials (e.g. >90% for most antimicrobials in *E. coli*). However, since the antimicrobial resistance frequencies were presented for all countries as a group and temporal trends

were not analysed (Moyaert et al. 2017), it was not possible to perceive any geographical differences. Overall high antimicrobial susceptibility frequencies were also detected in previously published data from Sweden (2009 2014) (Swedres-Svarm 2016; Windahl et al. 2014), Norway (2003–2009) (Lund et al. 2014) and Switzerland (*E. coli*, 1999–2001) (Lanz et al. 2003).

In another European study, Kroemer et al. (2014) found lower antimicrobial susceptibility rates among *E. coli* and *P. mirabilis* from companion animals with UTI isolated in 2002–2009. Again, results were presented for all countries as a group (Kroemer et al. 2014). Notably, *P. mirabilis* showed trimethoprim-sulfamethoxazol resistance of about ~53% (Kroemer et al. 2014). Higher levels of antimicrobial resistance in bacteria from companion animals with UTI have also been reported in Portugal (e.g. *E. coli* 25% to cephalotin, 19% to amoxicillin/clavulanate) (Féria et al. 2002), Brazil (e.g. staphylococci 28–74% to all tested antimicrobials, 2006–2007; *E. coli* 40% to aminoglycosides, 40% to sulfonamides, 16% to fluoroquinolones) (Penna et al. 2010; Osugui et al. 2014), Cornell USA (e.g. *E. coli* ~35% to ampicillin, ~70% to cephalotin, ~20% to enrofloxacin, ~40% to gentamicin) (Cummings et al. 2015); Taiwan (*E. coli* 50% amoxicillin, 39% trimethoprim-sulphamethoxazole, 2010–2011) (Chang et al. 2015), Australia (e.g. *E. coli* 29% to amoxicillin/clavulanate, 5–9% to ceftriaxone, 2013) (Saputra et al. 2017), Switzerland (*E. coli*, 10–35% to third-generation cephalosporin [ESBL-producers], 2012–2016) (Zogg et al. 2018b), Belgium (e.g. *E. coli* 12% to amoxicillin/clavulanate, 17% to enrofloxacin, 2010–2012) (Criel et al. 2015), Virginia USA (*E. coli*, 18% to amoxicillin/clavulanate, 15% to trimethoprim/sulphamethoxazole,1986–1996) (Forrester et al. 1999) and Italy (2013–3015) (Rampacci et al. 2018). Furthermore, changing antimicrobial resistance temporal trends have been reported in bacteria isolated from different companion animal infections (Normand et al. 2000a; Authier et al. 2006; Thompson et al. 2011; Beever et al. 2015; Couto et al. 2016), including in uropathogenic bacteria from California (fluoroquinolones, 1992–2001) (Cohn et al. 2003; Cooke et al. 2002), Canada (fluoroquinolones, 1984–1998; several antimicrobial, 2002–2007) (Prescott et al. 2002; Ball et al. 2008), United Kingdom (enrofloxacin, cephalexin and oxytetracicline, 1999–2009) (Hall et al. 2013) and in New Zealand (amoxicillin clavulanate, cephalotin, enrofloxacin, 2005–2012) (McMeekin et al. 2016).

Despite the apparently significant number of studies published regarding the antimicrobial resistance trends in bacteria isolated from companion animals with UTI, the comparison of published data is frequently difficult. Most of the studies that report antimicrobial resistance frequencies use different inclusion criteria (e.g. diabetic animals, recurrent UTI) (Bailiff et al. 2006), were conducted at different time periods, group results from different infection sites (Normand et al. 2000a; Normand et al. 2000b; Authier et al. 2006; Pedersen et al. 2007; Harada et al. 2012; Beever et al. 2015), combine different bacteria genera (Ball et al. 2008; Hall et al. 2013; Dorsch et al. 2015; Wong et al. 2015; Rampacci et al. 2018; Teichmann-Knorrn et al. 2018) and group data from several countries (Meunier et al. 2004; Kroemer et al. 2014; Moyaert et al. 2017). Knowledge of the geographic distribution of antimicrobial resistance, as obtained by surveillance networks in human medicine, is essential to identify the countries where efforts should be made to improve awareness and implement new strategies.

In a collaboration with 16 veterinary microbiology laboratories from 14 European countries, the Antibiotic Resistance Laboratory team coordinated a multicentric study to determine the European distribution of resistance in bacteria isolated from companion animals with UTI. This study showed striking geographical differences on *E. coli* and *P. mirabilis* antimicrobial resistance between some Northern (Denmark and Sweden) and Southern (Italy, Greece, Portugal and Spain) European countries (Marques et al. 2016). Overall, Southern countries showed higher resistance towards the main antimicrobials used in small animal veterinary medicine, including third generation cephalosporins and fluoroquinolones (Marques et al. 2016) (Fig. 1). One limitation from this study that could have biased these finding to some extent was the use of different antimicrobial testing methods and interpretation criteria in some European veterinary microbiology laboratories. However, the wide differences in antimicrobial resistance detected between some Northern and Southern countries (e.g. 2.88% and 48.15% amoxicillin/clavulanate resistance in Denmark and Portugal, respectively) are likely a result from true geographic differences (Marques et al. 2016). It is interesting to note that the European distribution of *E. coli* resistance from companion animals with UTI resembled that of EARS-Net reports about human invasive isolates (ECDC 2017). Since the samples from most countries were obtained from a single veterinary microbiology laboratory these findings may not represent the entire country. Nevertheless, these results should prompt the Southern countries to further investigate this issue.

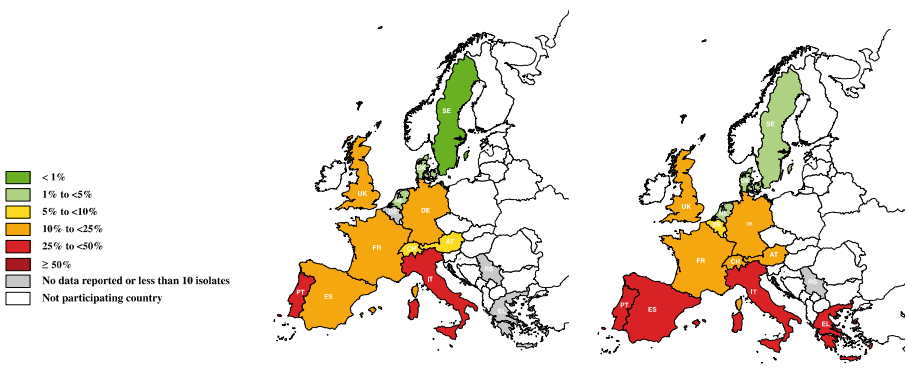

Fig. 1. *Escherichia coli* resistance to third-generation cephalosporin (left) and fluoroquinolone (right) in companion animals with UTI (adapted from Marques et al. 2016)

Prior to this European study, there was little updated information about the antimicrobial resistance of bacteria causing UTIs in companion animals from Portugal (Féria et al. 2000; Féria et al. 2002; Pomba et al. 2008). To better understand the antimicrobial resistance temporal trends in uropathogenic bacteria from companion animals in Portugal (Lisbon), the Antibiotic Resistance Laboratory team conducted a retrospective study over 16 years (Marques et al. 2018a). Notably, a significant increase in Enterobacteriaceae antimicrobial resistance to the main antimicrobials used for UTI treatment

in small animal veterinary medicine was observed in companion animals from Portugal (Lisbon) (Fig. 2). Furthermore, a significant increase in the detection of methicillin resistant *Staphylococcus pseudintermedius* was also detected (Marques et al. 2018a).

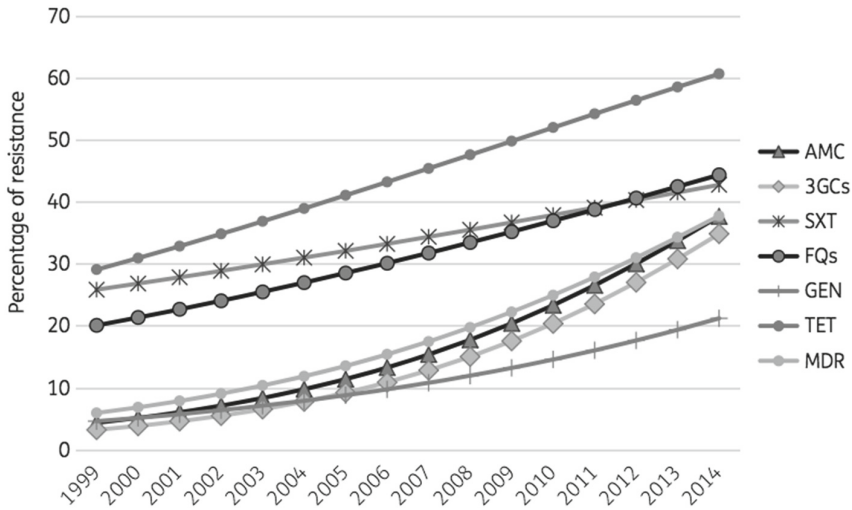

Fig. 2. Trends in antimicrobial resistance in Enterobacteriaceae causing UTI in companion animals from Portugal (Lisbon), 1999–2014 (adapted from Marques et al. 2018b).

The selection of antimicrobial resistance is a complex and multifactorial process (Prescott 2017). Efforts to reduce high antimicrobial resistance frequencies and increasing trends are urgent. The prescription of empirical antimicrobial treatment is sometimes necessary prior to culture to relieve the patient discomfort and prevent systemic infection (Weese et al. 2011). As part of the rational use of antimicrobials, the empirical choice of antimicrobials should rely on first-line antimicrobials and then adjusted (escalation/de-escalation) based on antimicrobial susceptibility data if necessary (Weese et al. 2011). The choice of the appropriate antimicrobial should always be supported on culture and AST (Weese et al. 2011) to avoid the misuse of antimicrobials and therefore contribute to decrease the local antimicrobial selective pressure. In fact, Sørensen et al. (2018) has showed that a high percentage of dogs with suspected UTI were unnecessarily treated regardless of the diagnostic work up conducted prior to culture and that second line antimicrobials were frequently miss prescribed. Interestingly, an European study reported that veterinarians, for instance, in Sweden were 15.64 times more likely to conduct a AST to guide antimicrobial choice than in Spain (de Briyne et al. 2013). All these factors likely contribute to the increase in antimicrobial resistance and reveal the need for the implementation of antimicrobial stewardship programs in small animal veterinary medicine.

Another interesting finding that points to the overuse of antimicrobials in companion animals with UTI is that the recommended treatment duration in companion animals is significantly longer than in humans (e.g. uncomplicated UTI: 3–5 days in humans, 7–14 days in companion animals) (Weese et al. 2011; Smelov et al. 2016). It should be

noted that the treatment duration currently recommended in companion animals are supported by little scientific evidence (Weese et al. 2011). Although some studies have reported short-duration antimicrobial treatment protocols for UTI in companion animals (Westropp et al. 2012; Clare et al. 2014), studies comparing the same antimicrobial regime with differing durations are lacking (Jessen et al. 2015).

7 Multidrug Resistance Bacteria Causing UTIs from Companion Animals

Companion animals with UTI have high bacteria concentration in the urine, thus potentially contributing to its dissemination into the living environment. Therefore, the detection of multidrug resistant (MDR) bacteria in companion animals with UTI creates important therapeutic limitations and also raises public health concerns (Pomba et al. 2017).

MDR bacteria are increasingly being detected in companion animals with UTI and are frequently associated with clinically relevant and mobile resistance mechanisms such as extended spectrum beta-lactamases (ESBLs) and carbapenemases in Enterobacteriaceae and the *mecA* gene in staphylococci (Prescott et al. 2002; Pomba et al. 2008; Pomba et al. 2010; Harada et al. 2012; Pomba et al. 2013; Osugui et al. 2014; Wagner et al. 2014; Windahl et al. 2014; Chang et al. 2015; Thungrat et al. 2015; Wong et al. 2015; Marques et al. 2018a; Zogg et al. 2018b; Belas et al. 2019; Marques et al. 2019).

In the 16-yearlong retrospective study conducted in Portugal (Lisbon) at the Antibiotic Resistance Laboratory, a significant increase in the detection of MDR Enterobacteriaceae from companion animals with UTI was detected (Marques et al. 2018a). Furthermore, in the study conducted in collaboration with the European veterinary microbiology laboratories, Portugal (Lisbon) and the Southern countries were, again, among the geographic locations with higher frequency of MDR *E. coli* and *P. mirabilis* (Marques et al. 2016).

The increase in the detection of MDR *E. coli* and *P. mirabilis* in companion animals from Portugal (Lisbon) was strongly associated with the dissemination of ESBL and AmpC beta-lactamases (Marques et al. 2018a). The production of beta-lactamases is the most common beta-lactam resistance mechanism in clinically relevant Gram-negative bacteria (Bush and Jacoby 2010). Beta-lactamases are frequently plasmid mediated leading to its rapid worldwide dissemination through horizontal transfer and dissemination of high-risk clones (Cantón and Coque 2006; Fernandes et al. 2013). Furthermore, plasmids frequently encode for antimicrobial resistance to different classes of antimicrobials, thus contributing to the dissemination of MDR phenotypes (Cantón and Coque 2006).

The Antibiotic Resistance Laboratory has contributed significantly to the knowledge of the epidemiology of beta-lactamase enzymes in Gram-negative bacteria isolated from companion animals with UTI, including ESBLs and carbapenemases (Féria et al. 2002; Caniça et al. 2004; Pomba et al. 2008; Pomba et al. 2013; Marques et al. 2018a; Marques et al. 2018b; Belas et al. 2019; Marques et al. 2019).

The spread of third-generation cephalosporin resistant Enterobacteriaceae has recently been considered by the WHO as a Priority 1 concern (WHO 2017b). The CTX-M family is endemic worldwide and has become the most frequent ESBL in bacteria

causing health-care and community associated infections in humans (Cantón and Coque 2006; Fernandes et al. 2013; Doi et al. 2017). There is host and geographic variation in the distribution of CTX-M enzymes; nevertheless, some enzymes, such as CTX-M-15, seem to be disseminated worldwide in humans and companion animals (Cantón and Coque 2006; Coque et al. 2008; Nicolas-Chanoine et al. 2008; Pomba et al. 2008; Smet et al. 2010; Ewers et al. 2012; Bevan et al. 2017; Marques et al. 2018a; Marques et al. 2019).

There is a strong association between the *E. coli* O25b:H4-B2-ST131 clonal lineage and the dissemination of CTX-M-15 ESBL (Coque et al. 2008; Nicolas-Chanoine et al. 2008; Doi et al. 2017). This clonal lineage is also relevant because it exhibits a large virulence gene profile and is an important uropathogen in humans (Nicolas-Chanoine et al. 2008; Vimont et al. 2012). This important CTX-M-15-producing *E. coli* clonal lineage (O25b:H4-B2-ST131) has sporadically been reported in companion animals, including from Portugal (Nicolas-Chanoine et al. 2008; Pomba et al. 2009; Ewers et al. 2010; Pomba et al. 2013; Belas et al. 2019). Just recently, some *E. coli* isolated from companion animals with UTI in Portugal were found to belong to the fluoroquinolone resistant CTX-M-15-producing O25b:H4 B2-ST131-H30Rx subclone, representing its first description in companion animals living in Europe. Moreover, these subclones belonged to the virotype D, which confirms their pathogenicity and virulent characteristics (Belas et al. 2019).

Although *K. pneumoniae* is less frequent in companion animals with UTI (Marques et al. 2016; Marques et al. 2018a; b), it is still a major pathogen that is increasingly associated with the dissemination of ESBLs and carbapenemases (Navon-Venezia et al. 2017; Ewers et al. 2014; Stolle et al. 2013; Schmiedel et al. 2014; González-Torralba et al. 2016). Several studies have shown that the ST15-CTX-M-15 clonal lineage predominates in companion animal infections by third-generation cephalosporin *K. pneumoniae* (Ewers et al. 2014; Maeyama et al. 2018; Marques et al. 2019). A high frequency of MDR *K. pneumoniae* ST15-CTX-M-15 was also detected in companion animals with UTI from Portugal (Lisbon) (Marques et al. 2019). Furthermore, other MDR high-risk clonal lineages disseminated in Portuguese Hospitals (Manageiro et al. 2015), such as the ST11 and ST147, were also detected (Marques et al. 2019).

The AmpC cefalosporinases are still regarded as less frequent than ESBLs, with CMY-2 being the most disseminated in humans and companion animals (Smet et al. 2010; Ewers et al. 2012). Interestingly, the studies conducted in Portugal (Lisbon) showed a significant increase in the detection of CMY-2 producing *E. coli* ST648 and *P. mirabilis* in companion animals with UTI (Marques et al. 2018a; Marques et al. 2018b). This CMY-2 increase is worrisome because these enzymes show stronger β-lactamase activity than ESBLS (Jacoby 2009) and may exhibit resistance to carbapenems due to the presence of other resistance mechanisms (e.g. porin deficiency) (Chia et al. 2009). Furthermore, all *E. coli* and *P. mirabilis* from this study (Marques et al. 2018a; Marques et al. 2018b) were MDR which creates great therapeutic limitations and highlights their clinical relevance.

The choice of an appropriate antimicrobial for the treatment of infections caused by MDR bacteria is a true challenge in small animal veterinary medicine. Not rarely, the lack of therapeutic options require the use of off-label of antimicrobials as demonstrated

by Pomba et al. (2010). Unlike AmpC and carbapenemases, ESBL-producing Enterobacteriaceae may be susceptible in vitro to amoxicillin/clavulanic acid (Paterson and Bonomo 2005). Due to limited research data, beta-lactam/beta-lactam inhibitor combinations are not considered as suitable first line options for the treatment of serious infections caused by ESBL-producing bacteria (Paterson and Bonomo 2005). However, the successful treatment of UTIs caused by some fully amoxicillin/clavulanate susceptible ESBL-producing *E. coli* in humans has been reported. Presumably, the high concentration of amoxicillin/clavulanate achieved in urine are responsible for such success (Lagacé-Wiens et al. 2006; Beytur et al. 2015). In a Pilot study conducted in a cat with a MDR ST15-CTX-M-15 producing *K. pneumoniae* UTI infection, the use of amoxicillin/clavulanate was tested (Marques et al. 2017). Although a definite cure was not achieved, the significant decrease in bacteriuria detected was a promising finding. Additional studies are now being conducted in the Antibiotic Resistance Laboratory to fully evaluated this therapeutic approach in small animal veterinary medicine.

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The Public Health Risk of Companion Animal to Human Transmission of Antimicrobial Resistance During Different Types of Animal Infection

C. Pomba(✉), A. Belas, J. Menezes, and C. Marques

Antibiotic Resistance Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
cpomba@fmv.ulisboa.pt

Abstract. Antimicrobial resistance represents a major threat to human health. As a result, we are faced with potential antimicrobial therapeutic failure, thus forcing physicians to use last resort antimicrobials, such as carbapenems, glycopeptides or polypeptides. During the last fifty years, the number of companion animals has substantially increased and there is a growing concern related to the use of antimicrobials in companion animals as a potential source for antimicrobial resistance to humans. Problems related with antimicrobial resistance and infection control in small animal hospitals are mimicking those in human hospitals. Transmission of pathogens or resistance genes such as methicillin-resistant staphylococci, extended spectrum beta-lactamase- or carbapenemase-producing and colistin-resistant Enterobacteriaceae between people and their pets have been documented or suggested. The public health risks associated with the transfer of antimicrobial-resistant bacteria from companion animals were recently reviewed by the European Medicine Agency which warned to the existence of antimicrobial resistance microbiological hazards coming from companion animals to humans. The magnitude to which these occur, and the risks posed by the different animal species is still inadequately studied. This is the main goal of the JPI-EC-AMR JTC 2016 Pet-Risk Consortium (Portugal, Germany, Switzerland, UK, Canada) JPIAMR/0002/2016 under the CIISA Antibiotic Lab Team Leader Coordination.

Keywords: Antimicrobial resistance · Animal infection · Public health risk

1 The Antimicrobial Use in Veterinary Medicine

The increase in antimicrobial resistance represents a major threat to human and animal health (WHO 2017a). As a result, we are now faced with the reduction of treatment options and with potential therapeutic failure leading veterinarians to use antimicrobials off-label and physicians to use last resort antimicrobials.

There is a growing concern related to the use of antimicrobials in food-producing and companion animals as a potential source for antimicrobial resistance to humans (Greko

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et al. 2009; Catry *et al*. 2010; van Duijkeren *et al*. 2014; Pomba *et al*. 2017). In fact, it is known that the use of antimicrobials increases the risk of antimicrobial resistance and the risk of colonization with antimicrobial-resistant bacteria (Barza and Travers 2002; Belas *et al*. 2014).

Since 2010, the European Medicine Agency started reporting data on antimicrobial sales for companion animals (EMA 2017b). Beta-lactams, including potentiated penicillins, were the most frequently sold for companion animals in most European countries, including Portugal (EMA 2017b). Furthermore, fluoroquinolones were the second most sold in Portugal.

It should be noted that the most sold antimicrobials in companion animals worldwide overlap those routinely used in human medicine and are considered as critically important antimicrobials to humans by the World Health Organization (WHO 2016b). Interestingly, the problems of antimicrobial resistance development and infection control in small animal hospitals are mimicking those in human hospitals (ECDC 2017). Furthermore, in contrast to food-producing animals, the prescription of antimicrobials only approved for human use may occur under the cascade principles in companion animals (Pomba *et al*. 2017). This represents an additional antimicrobial resistance selective pressure towards last resorts antimicrobials and warrants the need for a One Health approach to fighting the dissemination of antimicrobial resistance.

2 Risk of Transfer of Antimicrobial-Resistant Bacteria

The number of companion animals has significantly increased over the last 50 years (Guardabassi *et al*. 2004; Pomba *et al*. 2017). The closer contact between owners and companion animals creates opportunities for pathogen interchange through direct and indirect contact (Guardabassi *et al*. 2004; Damborg *et al*. 2016).

The public health risks associated with the transfer of antimicrobial-resistant bacteria from companion animals have been reviewed in the European Medicine Agency and its Antimicrobial Working Party reflection paper (Pomba *et al*. 2017). Pomba *et al*. (2017) alerted for existence of several antimicrobial resistance microbiological hazards coming from companion animals to humans (Table 1).

The concerns surrounding the role of companion animals in the dissemination of resistant bacteria to humans are strengthened by numerous studies reporting the colonization and/or infection of companion animals with bacteria harboring clinically relevant antimicrobial resistance mechanisms or bacteria belonging to high-risk clonal lineages to humans (Guardabassi *et al*. 2004; Damborg *et al*. 2016; Pomba *et al*. 2017).

This has been one of the main focus of the research conducted in the Antibiotic Resistance Laboratory in Enterobacteriaceae, non-Enterobacteriaceae, Staphylococci and Enterococci (Féria *et al*. 2001a; Féria *et al*. 2001b; Caniça *et al*. 2004; Delgado *et al*. 2007; Braga *et al*. 2011; Couto *et al*. 2011; Pinho *et al*. 2013; Catry *et al*. 2015; Couto *et al*. 2015a; Razuaskas *et al*. 2015a; Razuaskas *et al*. 2015b; Couto *et al*. 2016a; Couto *et al*. 2016b; Razuaskas *et al*. 2016; Pomba *et al*. 2017; Costa *et al*. 2018; Marconi *et al*. 2018; Marques *et al*. 2018; Rodrigues *et al*. 2018; Belas *et al*. 2019; Marques *et al*. 2019a; Marques *et al*. 2019b).

Table 1. Microbiological hazards from companion animals to humans identified by EMA (adapted from Pomba *et al*., 2017).

Antimicrobial-resistant bacteria	Type of hazard	Source
MRSA	Direct hazard[a]	Dogs, cats and horses
MRSP	Direct hazard	Dogs, cats and horses
VRE	Indirect hazard[b]	Dogs and horses
ESBL-producing Enterobacteriaceae	Indirect hazard	Dogs, cats and horses
Carbapenem-resistant Gram-negative bacteria	Indirect hazard[b]	Dogs and cats
Colistin-resistant *E. coli*	Indirect hazard	Dogs and cats

Legend:
[a]Low number of cases of human infections originating from companion animals.
[b]No human infections originating from companion animals have been reported. However, regarding carbapenems, co-colonization has been recently reported (Grönthal *et al*. 2018).

3 Antimicrobial Resistance Mechanisms and Bacteria of Concern

3.1 Staphylococci

Portugal is among the European countries with higher frequency of methicillin-resistance in invasive *Staphylococcus aureus* from humans (ECDC 2009). Methicillin-resistant Staphylococcus aureus (MRSA) have been detected in a wide number of animal species (Catry *et al*. 2010; Pomba *et al.* 2017), including in Portugal (Coelho *et al*. 2011; Couto *et al*. 2014b; Beça *et al*. 2015; Couto *et al*. 2015b; Couto *et al*. 2016a; Rodrigues *et al*. 2018).

In companion animals, MRSA has been isolated from skin and soft-tissue infections, post-surgical wound infections, urinary tract infections and pneumonia (Catry *et al.* 2010; Pomba *et al*. 2017). In a study from Portugal, conducted in the Antibiotic Resistance Laboratory, several MRSA were detected in companion animals with skin and urinary tract infections (Couto *et al*. 2016c). Notably the MRSA strains isolated from companion animals belonged to CC5 which is a lineage associated with human infection in Portugal (Couto *et al*. 2015a; Couto *et al*. 2016c).

In fact, the similarity of MRSA clonal lineages isolated from companion animals and humans has been reported worldwide (Weese and Duijkeren 2010; Pomba *et al*. 2017). In another study about the clonal diversity, virulence patterns and antimicrobial and biocide susceptibility among human, animal and environmental MRSA in Portugal, *S. aureus* clonal lineages from companion animals (CC5 and CC22) were associated with specific sets of virulence genes and often with a lower number of resistance genes than isolates belonging to the livestock associated CC398 (Couto *et al*. 2015b). Colonization of companion animals with MRSA has been previously reported ranging from 0% to 7% (Leornardo and Markey 2008; Catry *et al*. 2010; Pomba *et al*. 2017). In one study from Portugal, 1.4% of cats and 0.7% of dogs were reported to be colonized by MRSA (Couto *et al*. 2014b).

The risk of transmission of MRSA between companion animals and humans has been demonstrated highlighting the role of both species in this issue (Damborg *et al*. 2016; Pomba *et al*. 2017). Interestingly, veterinary staff seems to be at higher risk of being colonized by MRSA (Baptiste *et al*. 2005; Loeffler *et al*. 2005; Catry *et al*. 2010, Pomba *et al*. 2017). Besides MRSA, companion animal health care providers from Portugal also had a high frequency of colonization by methicillin resistant *Staphylococcus epidermidis* (MRSE) (Rodrigues *et al*. 2018). MRSAs colonizing humans from this study belonged to the major human healthcare clone in Portugal (ST22-t032-IV), the livestock-associated MRSA (ST398-t108-V) and to the New York-/Japan-related clone (ST105-t002-II) (Rodrigues *et al*. 2018). Furthermore, *S. epidermidi*s is an important nosocomial pathogen responsible for life-threatening infections associated with the use of medical devices and in immunocompromised individuals, whose management is hindered by frequent resistance to antimicrobials (Costa *et al*. 2018).

Most infections in companion animals are caused by *Staphylococcus pseudintermedius*, especially in dogs (Couto *et al*. 2016a; Couto *et al*. 2016b). The detection of multidrug-resistant methicillin-resistant *S. pseudintermedius* (MRSP) is increasingly being reported leading to significant therapeutic limitation in small animal veterinary medicine (Couto *et al*. 2014b; Couto *et al*. 2016a; Couto *et al*. 2016b; Pomba *et al*. 2017). A significant increase in the detection of multidrug-resistant MRSP has been recently noted in companion animals from Portugal (Lisbon) (Couto *et al*. 2016a). Although methicillin-susceptible *S. pseudintermedius* isolates are genetically diverse, a limited number of MRSP clones have spread worldwide resembling the worldwide dissemination of MRSA (Van Duijkeren *et al*. 2011; Pomba *et al*. 2017). Like MRSA, the emergence of MRSP represents a great problem for small animal veterinary medicine since *S. pseudintermedius* is the primary staphylococcal species colonizing healthy dogs and cats. MRSP colonization is more common in dogs than in cats. Furthermore, MRSP can cause many types of infections in companion animals as skin and ear infections, surgical site infections, gingivitis, hepatitis, urinary tract infections, respiratory infections, arthritis, peritonitis and septicaemia (Van Duijkeren *et al*. 2011; Pomba *et al*. 2017). It is important to keep in mind that veterinary hospitals and clinics play an important role in the dissemination control of MRSP (Pomba *et al*. 2017).

In Portugal, the Antibiotic Resistance Laboratory has conducted extensive studies about the *S. pseudintermedius* (MRSP and MSSP) colonization and infection in dogs and cats to characterize their clonality, antimicrobial susceptibility, biocide susceptibility and immunogenic properties (Couto *et al*. 2014a; Couto *et al*. 2015b; Couto *et al*. 2016c; Couto *et al*. 2016b). A worrying finding from these studies was the significant increase in staphylococci resistance, mainly *S. pseudintermedius,* to a large number of antimicrobials over the last 16 years (Couto *et al*. 2016a). Importantly, this included an increase in the detection of multidrug-resistant MRSP and the *mecA* gene (Couto *et al*. 2016b). The increase of MRSP in Portugal was linked to the dissemination of the S. pseudintermedius clonal lineage ST71-II-III, which is also the most disseminated clonal lineage in dogs and cats from Europe (Kadlec *et al*. 2010; Perreten *et al*. 2010).

Colonization of humans with *S. pseudintermedius* seems to be uncommon and transient, however owners and veterinarians in contact with infected companion animals may have a higher risk of being MRSP positive (Pomba *et al*. 2017). There are some

reports of colonization of veterinarians by MRSP that could suggest an occupational risk (Sasaki *et al*. 2007; Ishihara *et al*. 2010; Paul *et al.* 2011; Soedarmanto *et al*. 2011; Gómez-Sanz *et al*. 2013; Chanchaithong *et al*. 2014; Pomba *et al.* 2017). Furthermore, in 2014 a cluster of infections in a tertiary hospital due to MRSP clone ST71 was described in humans (Starlander *et al*. 2014).

While MRSA strains isolated from companion animals are mainly related to different human-associated MRSA clones, the scenario for MRSP is different. Diverse SCC*mec* elements occur among the different MRSP genetic lineages, suggesting that the *mec*A gene has been acquired by different *S. pseudintermedius* strains on multiple occasions. (Pomba *et al.* 2017). Transfer of SCC*mec* elements between different staphylococcal species is possible, which is a concern.

3.2 Enterococci

Enterococci are opportunistic pathogens, that have become an important cause of nosocomial and community-acquired infections, such as septicemia, endocarditis, UTI and diarrhea. Moreover, these bacteria are an important key indicator for several human and veterinary resistance surveillance systems (Torres *et al*. 2018). *Enterococcus faecalis* and *Enterococcus faecium* are the most common species isolated from human and companion animal infections. Enterococci are intrinsically resistant to several antimicrobials which have important therapeutic implications (Torres *et al.* 2018). Therefore, acquired resistance to ampicillin/penicillin and to high-level gentamicin, a classic therapeutic synergetic combination, strongly limits the treatment options against enterococcal infections (Chow, 2000). Such resistance mechanisms have been described in enterococci isolated from companion animals from Portugal (Delgado *et al*. 2007; Marques *et al*. 2018b).

Some studies provide that healthy livestock, wildlife, food-producing animals and companion animals can harbour pathogenic Enterococci that can be transferred via food chain or through close contact with humans. Furthermore, some Enterococci species are able to evolve from being simple commensal bacteria to being pathogenic to humans and animals through the acquisition of virulence factors encoded in mobile genetic elements (Bortolaia and Guardabassi 2015; Pillay *et al*. 2018).

For instance, the *E. faecalis* ST16 clonal lineage is considered a zoonotic pathogen and food and industries seem to have contributed to its dissemination (Torres *et al*. 2018). Furthermore, this clonal lineage is frequent among high-level gentamicin resistant strains harboring the bifunctional enzyme (Ruiz-Garbajosa *et al*. 2006). Other important Enterococci high-risk clonal complexes (CC) associated with nosocomial infections in humans include the *E. faecalis* CC6 (formerly CC2) and the ampicillin-resistant *E. faecium* CC17 (Leavis *et al*. 2006; Kuch *et al*. 2012).

Due to its clinical relevance, the Antibiotic Resistance Laboratory has contributed with epidemiological studies about the antimicrobial resistance and population structure of enterococci isolated in Portugal (Delgado *et al*. 2007; Pomba *et al*. 2010; Braga *et al*. 2011; Braga *et al.* 2013; Marques *et al*. 2018a). In one of these studies, the first report of a biocide resistance mechanism in *E. faecalis* and its dissemination amongst the genus Enterococcus was reported (Braga *et al*. 2011).

Ampicillin-resistance and/or high-level gentamicin resistance in enterococci from companion animals with UTI in Portugal (Lisbon) over 16 years was low when compared

with the resistance frequencies detected in Enterobacteriaceae (Marques *et al*. 2019a). However, many of these isolates belonged to *E. faecalis* ST16, *E. faecalis* CC6 and to the ampicillin-resistant *E. faecium* CC17. Interestingly, a previous study has shown that healthy dogs seem to be reservoirs of ampicillin-resistant *E. faecium* CC17 (Damborg *et al*. 2009).

The acquired resistance to vancomycin due to *van* gene carriage is another resistance mechanism of great importance in human medicine (Pomba *et al*. 2017). Ampicillin-resistance in *E. faecium* from Europe seams to often predict the increase in the rates of vancomycin-resistant enterococci (VRE) within some years (Werner *et al*. 1904). Although, the level of ampicillin-resistant *E. faecium* in companion animals with UTI was low, higher frequencies have been reported in other parts of Europe (Damborg *et al*. 2009). Therefore, active surveillance is imperative.

Healthy dogs and cats may become colonized by VRE. Furthermore, VRE isolated from companion animals may also belong to clonal lineages associated with hospital-acquired infections (Pomba *et al*. 2017).

4 Enterobacteriaceae and Non-Enterobacteriaceae

There are a large number of studies reporting the detection of extended spectrum beta-lactamases (ESBLs) - producing bacteria in companion animal infections and in colonized animals (Ewers *et al*. 2012; Belas *et al*. 2014; Pomba *et al*. 2014a; Damborg *et al*. 2015; Pomba *et al*. 2017).

Detection
of carbapenemase-producing Enterobacteriaceae and non-Enterobacteriaceae are still a rare event; however, reports in healthy and sick animals are increasing by the day, and will likely become a serious problem in the future (Pomba *et al*. 2014b; Chanchaithong *et al*. 2014; Gentilini *et al*. 2018; Grönthal *et al*. 2015; Köck *et al*. 2018).

The Antibiotic Resistance Laboratory has made the first description of an OXA-23-producing ST2 MDR *Acinetobacter baumannii* in a cat with urinary tract infection (UTI) (Pomba *et al*. 2014a). Just recently, the transmission of a canine clinical NDM-5 *Escherichia coli* between an infected dogs and humans was confirmed for the first time (Grönthal *et al*. 2015) giving additional scientific support to the concerns surrounding the close contact of companion animals with humans (Pomba *et al*. 2017).

Although less studied than other Gram-Negative bacteria, *Pseudomonas aeruginosa* is an important pathogen causing otitis and pyoderma in companion animals (Pomba *et al*. 2017). Notably, carbapenem-producing strains have already been detected in dogs (Hyun *et al*. 2018). Also, some infections caused by these bacteria are in association with other pathogens, such as MRSP (Lupo *et al*. 2017).

In an ongoing study conducted in the Antibiotic Resistance laboratory, *P. aeruginosa* causing external otitis in companion animals from Portugal (Lisbon) showed high resistance levels towards fluoroquinolones and aminoglycosides, which are frequently used topically. Furthermore, resistance to imipenem and doripenem was also noted (Marconi *et al*. 2018).

Companion animals have been found to be colonized by *E. coli* and *Klebsiella. pneumoniae* belonging to important clonal lineages to humans (Pomba *et al*. 2014b;

Johnson *et al*. 2016; Pomba *et al*. 2017; Marques *et al*. 2018; Belas *et al*. 2019; Marques *et al*. 2019a). Since many pathogenic bacteria, are thought to make part of the normal gut flora (Podschun and Ullmann 1998; Drzewiecka 2016; Johnson *et al*. 2016; Martin *et al*. 2016), gut colonization of companion animals may also represent an important hazard. Interestingly, pet ownership (dogs, cats and other companion animals) was suggested to be a risk factor for human gut colonization by ESBL-producing *E. coli* (Meyer *et al*. 2012).

Regarding Enterobacteriaceae, companion animals from the same household may be colonized and share the uropathogenic *E. coli* O25B: H4: B2-ST131 clonal lineage (Johnson *et al*. 2009; Johnson *et al*. 2016). More importantly, humans and dogs with UTI have been shown to share the index uropathogenic *E. coli* with household members including the family dogs and cats (Murray *et al*. 2004; Johnson and Clabots 2016). Just recently, in a study conducted by the Antibiotic Resistance Laboratory, companion animals and humans living in close contact were screened for colonization by *K. pneumoniae* and *Proteus mirabilis* (Marques *et al*. 2018a; Marques *et al*. 2019a). Interestingly, some dogs and humans were shown to be colonized in the gut by undistinguishable (by PFGE and MLST) *K. pneumoniae* strains, suggesting the possibility of transmission between dogs and humans (Marques *et al*. 2018a).

Besides beta-lactams, the dissemination of colistin resistance plasmids mcr-1 to 7 has been recently on the spotlight (Yang *et al*. 2018). The dissemination of MDR carbapenemase-producing bacteria in human medicine has led to the need to return to old antimicrobials such as colistin. The recent identification of the colistin resistance gene mcr-1 in food-production animals and companion animals in multiple countries is a concern (Liu *et al*. 2016; Perreten *et al*. 2010; Schwarz *et al*. 2016). In Portugal, mcr-producing Enterobacteriaceae have been identified in retail meat (Figueiredo *et al*. 2016); clinical strains (Campos *et al*. 2011; Mendes *et al*. 2018) and food producing animals (Kieffer *et al*. 2017; Freitas-Silva *et al*. 2018). Moreover, a recent report of a mcr-1-containing *E. coli* in a person and in multiple dogs and cats heightens these concerns (Zhang *et al*. 2016).

5 The Future

The current scientific knowledge seems to support the suspicion that companion animals may act in the dissemination of resistant and pathogenic bacterial clones to humans and vice versa.

However, several questions still remain answered. Skin (including ear) and urinary tract infections are the most frequent infection in companion animals. Previously published data, including from the Antibiotic Resistance Laboratory (Féria *et al*. 2001a; Féria *et al*. 2001b; Caniça *et al*. 2004; Delgado *et al*. 2007; Braga *et al*. 2011; Couto *et al*. 2011; Pinho *et al*. 1099; Catry *et al*. 2015; Couto *et al*. 2015a; Razuaskas *et al*. 2015a; Razuaskas *et al*. 2015b; Couto *et a*l. 2016a; Couto *et al*. 2016b; Razuaskas *et al*. 2016; Pomba *et al*. 2017; Costa *et al*. 2018; Marques *et al*. 2018a; Rodrigues *et al*. 2018; Belas *et al*. 2019; Marques *et al*. 2019a; Marques *et al*. 2019b), have shown that bacteria causing skin infections and UTIs in companion animals are sometimes associated with major resistance mechanisms and bacterial clonal lineages. Furthermore, several studies

support the sharing/transmission of important bacterial clonal lineages between companion animals and humans (Johnson and Clabots 2016; Murray *et al*. 2004; Johnson *et al*. 2009; Johnson *et al*. 2016; Marques *et al*. 2018a; Marques *et al*. 2019b). However, the extent to which such transfer occur is still poorly studied. The main goal of the JPI-EC-AMR JTC 2016 Pet-Risk Consortium (Portugal, Germany, Switzerland, UK, Canada) JPIAMR/0002/2016 under CIISA Antibiotic Lab Team Leader Coordination is to clarify the extent of transmission and whether different types of infections may convey additional risk to humans or vice-versa. This project will stand on edge using Next Generation Sequencing technologies to unequivocally evaluate the transmission of clinically relevant antimicrobial mechanisms and pathogenic bacteria.

As a laboratory devoted to the study of antimicrobial resistance in veterinary medicine, it is its mission to reach out to the society (clinicians and owners) in the pursuit of better antimicrobial use practices. The development of antimicrobial stewardship programs as long started in human medicine and are urgently needed in veterinary medicine (Loyd and Page 2017). Antimicrobial stewardship programs are complex and require the interaction of multidisciplinary teams. Such programs aim at creating strategies to promote the rational use of antimicrobials, improve infection control measures and consequently decrease the spread of pathogenic and resistant bacteria (Loyd and Page 2017).

Evidence based learning is the key to fight antimicrobial resistance in a One health approach and pursuing the 5Rs of antimicrobial stewardship: Responsibility, Reduction, Refinement, Replacement and Review (Weese *et al*. 2013; Loyd and Page 2017). Recently, the European Society of Clinical Microbiology and Infection Diseases Study Group on Veterinary Microbiology started regular post-graduate courses of antimicrobial stewardship in veterinary medicine representing a landmark towards a better future and in which the Antibiotic Resistance Laboratory team leader collaborates as a regular speaker.

The future of antibiotic resistance and pathogenic bacteria is still uncertain, but the Antibiotic Resistance laboratory will continue to focus its efforts in obtaining useful epidemiological data, guide antimicrobial use through the establishment of antimicrobial stewardship programs, and reaching the society to increase awareness and help to improve this worldwide problem.

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Antibacterial Alternatives in the Scope of One Health

M. Oliveira(✉), I. Serrano, and L. Tavares

Microbiology and Immunology Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
moliveira@fmv.ulisboa.pt

Abstract. The use of antibiotics for the treatment of infectious diseases began in the 1940s, with penicillin. In spite of their unquestionable contribution to the decline of morbidity and mortality rates in both human and veterinary medicine, antibiotics administration prompted the emergence and dissemination of multiresistant bacterial strains. This phenomenon represents a major concern for Public and Animal Health worldwide and has instigated the development of new generation antibiotics. However, these new molecules soon became ineffective due to the capability of bacteria to evolve, in particular through mutations or horizontal gene transfer.

In the scope of One Health, the search for antibacterial alternatives is a major priority, aiming at the successful prevention and control of infectious diseases in both humans and animals, especially due to the decline of the investment from the pharmaceutical industry in the development and commercialization of new antibiotics. Several hypotheses are being investigated, including antimicrobial peptides, phage therapy, probiotics and biocides.

Keywords: Antimicrobial peptides · Antimicrobial resistance · Bacteriocins · Bacteriophages · Biocides · Biofilm · Multidrug-resistance · Nisin · One Health

The dissemination of antimicrobial resistant bacterial strains represents a major threat for the safeguarding of One Health, rendering the development of innovative antibacterial strategies a research priority (Bassetti *et al.* 2011). As the current antibiotic pipeline may not be sufficient to solve this worldwide problem, the development and validation of innovative antibacterial alternatives to be applied in both human and veterinary settings are crucial. Among the hypotheses currently being followed, antimicrobial peptides, probiotics, phage therapy and biocides represent promising strategies.

Antimicrobial Peptides (AMP) are produced by the vast majority of all living organisms as part of their innate immune response against infectious diseases (Lewis 2013; Gharsallaoui *et al.* 2016). They represent a vast group of peptides with cationic and amphipathic regions that act by selectively targeting cell membranes with a high percentage of negatively charged phospholipids and no cholesterol, characteristic of bacteria, and thereby promoting minimal damage to human and animal host cells (Liu *et al.* 2007; Baltzer and Brown 2011). AMP include aminoacids with cationic side chains, such as arginine (R), lysine (K) and histidine (H), and also bulky nonpolar side chains,

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such as proline (P), phenylalanine (F) and tryptophan (W). The cationic side chains are involved in the interaction with the bacterial membranes and/or with the lipopolysaccharide from the cell wall (Hou *et al.* 2010), while the nonpolar side chains promote the lipophilic attachment, which is responsible for membrane disruption (Liu *et al.* 2007; Baltzer and Brown 2011).

AMP present several advantages regarding conventional antibiotics, including the fact that they act against a broad spectrum of microorganisms, including Gram-positive and Gram-negative bacteria; they can act as immune modulators (Batoni *et al.* 2016; Santos *et al.* 2016); strains resistant or co-resistant to AMP were rarely reported, as these molecules not only present several peptide sequences but also act by directly damaging the bacterial membranes integrity and impairing bacterial viability and/or inhibiting Quorum sensing mechanisms (Baltzer and Brown 2011); and they can prevent biofilm formation or eliminate pre-formed biofilms (Schierle *et al.* 2009; Tong *et al.* 2015). In fact, short AMP with (RW) n-NH2 (n = 2 to 4) sequences are effective antibacterial agents, with hexameric and octameric peptides being described as effective biofilm inhibitors, and with octameric peptides also known to be effective biofilm eradicators (Liu *et al.* 2007; Hou *et al.* 2010). Besides, short AMP are good candidates for large-scale production (Liu *et al.* 2007), and can be combined with other molecules for an optimal anti-biofilm action, including furanone (Vestby *et al.* 2009), salicylic acid (Rosenberg *et al.* 2008) and cellulase (Loiselle and Anderson 2003).

Bacteriocins are an heterogeneous group of ribosomally synthesized AMP produced by bacteria (Chopra *et al.* 2014). This group included one of the best characterized AMP, nisin, which is a class I bacteriocin classified in the lantibiotics group (Abts *et al.* 2011), being produced by *Lactococcus lactis* and having a broad spectrum of action against Gram-positive bacteria (Schulz *et al.* 2003; Todorov *et al.* 2012; Field *et al.* 2015). Being classified as Generally Recognized As Safe (GRAS) by the US Food and Drug Administration (FDA), nisin has been used as a food preservative for over 60 years, being approved in both the EU (as additive E234) and the USA (Field *et al.* 2015).

Despite the antibacterial properties and advantages of AMP, including nisin, their successful delivery to the target site is still a challenge, since AMP degradation or inactivation may be responsible for their inability to reach the target at therapeutic concentrations (O'Driscoll *et al.* 2013). Due to several of their properties, natural polysaccharides are being evaluated as promising AMP delivery systems: these molecules are non-toxic, biodegradable and biocompatible, being abundant in nature and therefore economic to produce (Reddy *et al.* 2011). In this group of molecules, Guar gum, a polysaccharide extracted from the endosperm of the leguminous crop *Cyamopsis tetragonolobus*, stands out (Thombare *et al.* 2016). It is constituted by galactomannan, a linear polymer of galactose and mannose, with thickening, emulsifying, gelling and binding properties, quick solubility in cold water, wide pH stability and film forming ability; all these properties render Guar gum a good candidate to be used as an innocuous and adaptable system for the topical delivery of antibacterial agents, including AMP (Reddy *et al.* 2011; Thombare *et al.* 2016).

Recently our research team confirmed the antimicrobial activity of nisin against *Staphylococcus aureus* isolates collected from Diabetic Foot Infections from patients

hospitalized in Lisbon Medical Centers (Mendes *et al.* 2012; Mottola *et al.* 2012a; Mottola *et al.* 2012b; Mottola *et al.* 2012c; Matias *et al.* 2018) and evaluated the potential of a Guar gum biogel to be used as a delivery system for the topical application of this AMP (Santos *et al.* 2016). In fact, the work developed by Santos *et al.* (2016) showed that nisin could rapidly diffuse through the Guar gum biogel and eradicate staphylococcal planktonic cells and established biofilms.

This alternative antibacterial strategy may substitute or complement antibiotherapy protocols applied to Diabetic Foot Infections treatment, ultimately contributing to decrease multidrug resistant bacteria dissemination in the hospital and community settings. Also, the use of Guar gum as a delivery system for antimicrobial peptides can lead to the development of novel topical therapeutics for bacterial skin and mucosa infections, especially those prompted by pathogenic bacteria with reduced susceptibility to the available antibiotics. Moreover, this innovative approach has also application in veterinary medicine. In fact, our research team also evaluated the potential of this new experimental approach for the prevention of periodontal disease in dogs, as this is one of the most widespread diseases in these animals, affecting 85% of the dogs with more than 4 years of age (Glickman *et al.* 2011; Wallis *et al.* 2015). Canine oral enterococci from a previous study conducted by our team (Oliveira *et al.* 2015; Semedo-Lemsaddek *et al.* 2016) were used as bacterial models for the evaluation of a PD prevention protocol based on the incorporation of the AMP nisin in a guar gum gel and also in a veterinary toothpaste, two delivery systems that present the advantage of acting directly at the primary site of bacterial dissemination, i.e., the oral cavity (Cunha *et al.* 2018). It was observed that nisin was effective against all the isolates tested. Independently of being or not incorporated in the guar gum gel, its inhibitory activity on biofilms was higher than regarding their planktonic counterparts, presenting Minimum Biofilm Eradication (MBEC) and Inhibitory Concentrations (MBIC) values lower than the Minimum Inhibitory (MIC) and Bactericidal Concentrations (MBC). The nisin supplemented toothpaste was also effective, showing inhibitory activity against 95% of the isolates tested (Cunha *et al.* 2018). These results show the potential of these supplemented vehicles to be applied to the control of Periodontal Disease in dogs.

Among the bacteriocin-producing strains, some are classified as probiotics, which are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO 2001). These bacterial strains are reported to contribute for the reduction and prevention of specific infectious diseases affecting the gastrointestinal tract of humans and animals (Corr *et al.* 2009; Kanmani *et al.* 2013). Probiotics inhibitory action can be achieved through several mechanisms, including: competitive exclusion of binding sites; promotion of the epithelial barrier function; improvement of the immune response by stimulating the production of sIgA and anti-inflammatory cytokines and by regulating the action of proinflammatory cytokines; production of antimicrobial substances with antimicrobial ability, namely antimicrobial peptides such as bacteriocins; production of antiadhesive biosurfactants that may also present antimicrobial activity; and inhibition of pathogenesis of gastrointestinal pathogens, including expression of virulence genes, quorum sensing systems and biofilm formation (Vasiljevic and Shah 2008; Corr *et al.* 2009; Oelschlaeger 2010; Kanmani *et al.* 2013).

Most of the probiotic bacteria reported so far were isolated from the gastrointestinal tract of humans and animals, and also from fermented dairy products (Peres *et al.* 2012; Fontana *et al.* 2013; Dias *et al.* 2014). Nevertheless, new sources of such strains are being investigated, including fermented vegetables (Peres *et al.* 2012; Fontana *et al.* 2013; Touret *et al.* 2018). Our research team has already characterized bacterial isolates obtained from São Jorge and Parmigiano-Reggiano cheeses (Dias *et al.* 2014) and from sauerkraut (Touret *et al.* 2018). The lactic acid bacteria species obtained in both studies presented antimicrobial activity against two important human pathogens, *Shigella dysenteriae* and *Listeria monocytogenes*, respectively, rendering these food products valid candidates for the selection of bacterial strains with probiotic potential. Considering the intrinsic characteristics of these specific food products, strains isolated from ripened cheeses and fermented cabbages may be more resistant to adverse environmental conditions than the ones obtained from traditional probiotic sources, and therefore present an increased biotechnological potential.

Another antibacterial alternative currently under evaluation is phage therapy, based on the application of lytic bacteriophages which are ubiquitous bacterial viruses that specifically act against numerous bacterial genera (O'Flaherty *et al.* 2005; Johnson *et al.* 2008; Ma *et al.* 2008; Mann 2008; Mattey and Spencer 2008; Parisien *et al.* 2008; Donlan 2009; Górski *et al.* 2009). After bonding to specific phage receptors present in the surface of the bacterial cell, phages inject their nucleic acid into the interior of the host bacterium; then, they use the information coded in their genome and the host cell machinery to produce all the proteins required for replication (Johnson *et al.* 2008; Mattey and Spencer 2008; Donlan 2009; O'Flaherty *et al.* 2009). Afterwards, they promote cell lysis through a virolysin-holin system or through a single lytic factor (Parisien *et al.* 2008), allowing the release of progeny phages (Johnson *et al.* 2008; Mann 2008; Parisien *et al.* 2008; Mattey and Spencer 2008; Donlan 2009; O'Flaherty *et al.* 2009).

Phages action mechanism is host specific, rendering them good candidates for eliminating specific pathogenic bacteria (Johnson *et al.* 2008; Ma *et al.* 2008; Mann 2008; Parisien *et al.* 2008; Donlan 2009; Górski *et al.* 2009; O'Flaherty *et al.* 2009), not affecting the host commensal microbiota, which represents a major benefit of phage therapy in comparison with antibiotherapy (Johnson *et al.* 2008; Mattey and Spencer 2008; Parisien *et al.* 2008). This important advantage, combined with the fact that phage production is simple and cost effective (Mattey and Spencer 2008; Parisien *et al.* 2008; Górski *et al.* 2009; O'Flaherty *et al.* 2009), renders phage therapy a promising approach for the prevention and control of infectious diseases promoted by multiresistant bacterial strains, with some phage products already approved as GRAS for use in both human and veterinary medicine (Parisien *et al.* 2008; Sillankorva *et al.* 2008; Górski *et al.* 2009; O'Flaherty *et al.* 2009).

In fact, the application of phage therapy for the control of suppurative infections was initiated soon after the discovery of bacteriophages, including of skin infections promoted by *Staphylococcus aureus* (Bruynoghe and Maisin 1921). However, research on phage therapy decreased significantly after the discovery and application of antibiotics, a tendency that was recently reverted due to the worldwide increase of infections promoted by antibiotic-resistant bacteria (Chopra *et al.* 1997). The use of lytic phages

for topical applications, including for the treatment of Diabetic Foot Infections, is of particular interest. In a study performed with the participation of our research team and of a Portuguese biotech company, Tecnophage (Mendes *et al.* 2014), bacteriophage cocktails aiming at the treatment of Diabetic Foot Infections promoted by *S. aureus*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* were developed and characterized, particularly regarding their ability to eliminate biofilm-encased bacteria. Results were extremely promising, representing an important advance towards the future application of phage therapy in human medicine. In 2014, FDA has authorized Tecnophage to conduct clinical trials in humans using TP-102, one of the developed bacteriophage cocktails.

Phages can also be combined with antibiotics for an increased antimicrobial effect, similarly to what is observed for biocides. These are natural or synthetic chemical agents that have been used to inactivate a broad range of microorganisms for a long time, but which use has become more relevant in response to the antibiotic resistance crisis (McDonnell and Russell 1999). Biocides can be bacteriostatic or bactericidal, and may be classified as antiseptics if used for inhibiting the multiplication of microorganisms present in living tissue; as disinfectants if applied to the decontamination of inanimate objects or surfaces; or as preservatives if used for preventing the multiplication of microorganisms in several products, such as foods and pharmaceutical compounds (McDonnell and Russell 1999; Russell 2003; Lachapelle 2012). Several classes of molecules can be classified as biocides, including alcohols, aldehydes, anilides, biguanides, diamidines, halogen-releasing agents, silver compounds, peroxygens, phenols (including bis-phenols and halophenols) and quaternary ammonium compounds; these molecules present different chemical structures, action mechanisms, efficacy and toxicity rates (McDonnell and Russell 1999; Dvorak 2008).

Due to their broad spectrum activity, biocides have a major role in human and veterinary medicine. They are frequently used for the skin asepsis of patients undergoing surgery, to impair the development of surgical site infections (SSI), which represent frequent medical-related infections in many countries in Europe, and the United States (WHO 2016). The Center for Disease Control and Prevention (CDC) has established guidelines for human medicine, recommending that pre-surgery skin asepsis should be performed using alcohol-based agents (Berríos-Torres *et al.* 2017); also, the World Health Organization (WHO) guidelines suggest the use of alcoholic solutions of chlorhexidine gluconate (WHO 2016).

In veterinary medicine there is no agreement on which biocide is the most adequate for pre-surgery skin asepsis. In a study developed by our research team, the effectiveness of two pre-surgical skin asepsis protocols based on an aqueous solution of 7.5% povidone-iodine and on an alcoholic solution of 2% chlorhexidine was evaluated in dogs undergoing surgery, by comparing bacterial growth from skin swab samples taken at pre- and post-asepsis (Belo *et al.* 2018). No bacterial growth was observed in most of the samples collected at post-asepsis, independently of the asepsis protocol used, indicating that pre-surgical skin asepsis protocols with povidone-iodine or chlorhexidine evaluated are similarly effective in preventing surgical site infections in dogs undergoing surgery (Belo *et al.* 2018).

In conclusion, all these alternative antibacterial approaches, including phage therapy, antimicrobial peptides and bacteriocins, probiotics and biocides, can be applied in both human and veterinary medicine for the prevention and control of bacterial infections. In fact, several therapeutic products based in these antibacterial alternatives are already approved and available for use in both human and veterinary medicine, but others still need to be validated before proceeding to clinical trials. These innovative antibacterial strategies may substitute or complement antibiotherapy for the treatment of infectious diseases, ultimately contributing to decrease multidrug resistant bacteria dissemination in the hospital and community settings, and for the safeguarding of One Health.

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This work is dedicated to Professor Cristina Vilela (1958–2013). Cristina was a Full Professor with Tenure and the Vice-Dean of the Faculty of Veterinary Medicine of the University of Lisbon. A devoted scientist, her main research areas were Clinical Microbiology and Mucosal Immunology, among other subjects related to Veterinary Sciences. Her dedication to science and teaching was exceptional, and she is deeply missed.

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Domestic, Wildlife and Environmental Virology: Molecular Epidemiology and Serological Surveillance

A. Duarte(✉), M. C. Bento, S. Gil, and L. Tavares

Microbiology and Immunology Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
anaduarte@fmv.ulisboa.pt

Abstract. Epidemiological surveillance is crucial, not only to assess pathogen geographic distribution but also to understand the ecology, impact and dynamics of the diseases, allowing a better population management and prophylaxis. Our Microbiology and Immunology Laboratory has been involved in the phylogeographical analysis and molecular detection of viruses (Parvovirus, Canine Distemper Virus, Coronaviruses, Feline Immunodeficiency Virus) affecting wild and domestic carnivores as well as Herpesvirus and Morbillivirus affecting marine animals (marine turtles; cetaceans; sea lions). This work involved the development of specific molecular assays and phylogenetic methodologies to evaluate the pathogen prevalence and geographic distribution as well as the phylogeographic patterns of the viral sequences.

Keywords: Virus in wildlife · Molecular epidemiology · Phylogenetic geography · Animal population management · Environmental health

1 Introduction

Epidemiological surveillance is crucial, not only to assess pathogen geographic distribution but also to understand the ecology, impact and dynamics of the diseases, allowing a better population management and prophylaxis.

Viruses are biochemical machines, recognized as pathogens for a highly diverse host spectrum, with the capacity to induce acute infectious disease but also chronic diseases; they are also linked to tumour development as well as neurological and autoimmune diseases (Dimaio 2014).

Under a conservation medicine perspective, viruses are highly challenging pathogens and their disruptive role in health management of domestic and wild animal population is recognized. In the last years, an increase of emergent diseases has been identified, with a growing awareness of the importance of wildlife as primary reservoirs of pathogens, providing a spill over platform for domestic animals and humans, but also as the pathogen primary target, posing a conservational threat.

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In 1994 a Canine Distemper Virus (CDV) outbreak was identified in the lion population of the Serengeti National Park, Tanzania, inducing fatal neurological and respiratory lesions. The genetic characterization of the CDV virus identified a spillover episode from a previous CDV outbreak recorded in canids from the Serengeti-Mara ecosystem between 1978–1991 (Roelke-Parker *et al.* 1996). In 2012 in Denmark, the occurrence of a CDV outbreak in farmed mink, suggested the existence of a wildlife viral reservoir, providing an epidemiological link of viral transmission between farmed and wild animals (Trebbien *et al.* 2014).

Canine Parvovirus-2 (CPV) emerged from Feline Panleukopenia Virus (FPV) in the 1970s, inducing a pandemic in the new susceptible hosts. The cross-species transfer most probably involved an unidentified carnivore. Presently CPV-2 has evolved to CPV-2a, 2b and 2c variants, which contrary to CPV-2 can infect cats. In 2012 an intermediate viral variant (CPV2/2a) was identified in several species of raccoons in the USA, shedding light on the mechanisms of parvovirus genetic evolution. A wider phylogenetic analysis including different wild carnivore samples identified two main evolutionary branches (FPV; CPV), contradicting the initial assumption of an intermediate host between the cat (FPV) and dog (CPV) (Allison *et al.* 2013; Miranda and Thompson 2016).

Influenza virus (IV) is widely distributed in wild and domestic birds. Wild birds are also reservoirs for highly pathogenic viral strains (HPAIV), and will continue to be (Ma *et al.* 2008; Ramey *et al.* 2018) independently of the surveillance programs undertaken by different countries and health organizations. Due to the genetics of IV there is a realistic concern about the possibility of a recombination event between human, avian and an animal (most probably swine) influenza virus, leading to the emergence of a new virus capable of infecting and spreading in humans. The importance of swine as an intermediate species ("mixing vessel"), due to its susceptibility to avian and human IV is not a certainty and several authors have been questioning it importance. Although different possibilities are under study (reviewed by Ma *et al.* 2008), avian IV and swine IV capable of infecting humans and swine as well as quails, can serve as new virus incubators, making the possibility of a new flu pandemic real. Due to the identification of IV cross species transmission, the surveillance and research efforts, towards a better understanding and identification of risk, should be maintained as a joint venture between human and veterinary health experts and environmental authorities, under a One health perspective.

The importance of virus wildlife reservoirs was highlighted with the identification of three coronavirus (CoV) able to induce disease in humans and animals, whose reservoir is a bat. Bats are the reservoirs of Severe Acute Respiratory Syndrome (SARS) CoV, causing a respiratory infectious disease, identified in 2002 (Zhong *et al.* 2003). The disease spread through the globe but a joint effort coordinated by the World Health Organization (WHO) allowed its control. However, the risk of re-emergence is still a possibility due to the identification in bats of SARS-CoV like capable of infecting humans without prior adaptation to an intermediate host (de Wit *et al.* 2016). In 2012 a new disease was identified, the Middle East Respiratory Syndrome (MERS), also caused by a CoV (MERS-CoV). MERS spread from Saudi Arabia to 27 countries in the next four years (2012–2016) before being contained. The isolation of SARS and MERS like-CoV from bats allowed the identification of the primary virus reservoir. For SARS-CoV

the identified incidental host was the palm civet cat (wild or from breeding and captivity facilities) whereas for MERS-CoV the intermediate host was the dromedary camel (de Wit *et al.* 2016; Omrani *et al.* 2015). These incidental and intermediate hosts allowed the virus spread to humans. In 2016 in China, a new fatal disease was identified in pig farms near the initial location of the SARS pandemic. The disease was characterized by acute diarrhoea and vomit and was caused by a coronavirus (Swine Acute Diarrhoea Syndrome SADS-CoV). The genome of SADS-CoV shared 98.48% identity with a bat CoV isolated from the same horseshoe bat genus (*Rhinolophus*) reported for SARS-CoV (Zhou *et al.* 2018).

The mechanisms of emergence and cross species transmission of viral pathogens are difficult to identify and frequently imply multiples causes, including epidemiological and climate changes, host and viral genetic evolution, globalization including travelling, and commercial trade, among others (Flores *et al.* 2013; Yon *et al.* 2018). Although database analysis and mathematical models are already available to predict the most probable host for the next zoonotic pathogen (Olival *et al.* 2017), the availability of new laboratory tools, including next generation sequencing and metagenomics, contribute to extend our knowledge of virus genomes and their evolution patterns, as well as their distribution in different habitats and hosts.

In Europe, the high density of the human population increased the proximity of wildlife and domestic animal species, making the European epidemiology of infectious diseases rather peculiar (Yon *et al.* 2018). Nevertheless, to construct an efficient epidemiological network, surveillance programs have to be implemented, for the collection and analysis of pathogen data and susceptible hosts.

To correspond to the growing awareness towards wildlife surveillance, under the One Health concept, that recognizes that the health of humans is linked and dependent on the health of animals and the environment, a concerted surveillance effort is being undertaken for different terrestrials and aquatic pathogens (Yon *et al.* 2018).

To intertwine with this trend our group has been involved in the molecular detection and phylogeographical analysis of wild and domestic carnivores viruses (Feline Immunodeficiency Virus, Feline Coronaviruses, Parvovirus, Canine Distemper Virus) as well as marine animals (marine turtles; cetaceans; sea lions) viruses (Herpesvírus, Astrovirus and Morbillivirus).

2 Feline Immunodeficiency Virus

Feline Immunodeficiency Virus (FIV) has been the target of our research in a phylogenetic perspective, as well as in a therapeutic approach. FIV is an important pathogen of the domestic cat, leading to immune dysfunction and immunodeficiency. Non-domestic felids, including the African lion (Panthero leo) and the North American cougar (Puma concolor), are also hosts of a Feline Lentivirus (FIV PLe), similar to FIV. In these animals, FIV is not directly connected to disease, although in the African lion the virus infection may interfere with the immune competence of these animals (O'Brien *et al.* 2012).

The study of the molecular epidemiology of FIV, allowed the identification of six subtypes (A-F) based on the hypervariable region of the env gene (V3-V5) coding the

surface glycoprotein of the viral envelope (Duarte and Tavares 2006; Kakinuma *et al.* 1995; Pecoraro *et al.* 1996; Sodora *et al.* 1994). Subsequent studies on this subject added important information concerning a growing genetic diversity within each assigned subtype (Nakamura *et al.* 2010), and the identification of highly frequent recombination events, suggesting the circulation in the cat population of unassigned viral sequences (Hayward and Rodrigo, 2009). On the other hand FIV-PLe phylogenetic analysis shows a higher genetic diversity, but a lower evolutionary rate, suggesting a longer association with the host (Troyer *et al.* 2011).

The insight on the genetic heterogeneity of FIV, raises concerns about the protection provided by a vaccine with heterologous strains, such as the dual-subtype FIV vaccine (Fel-O-Vax®, Fort Dodge), including subtype A and D, introduced in the United States, Japan, Australia and New Zealand. The administration of a whole virus vaccine, such as Fel-O-Vax®, implies a humoral response of the cat, similar to the one triggered after natural infection, which would interfere with the serological assays presently used for the laboratory diagnosis of FIV. In this case, the diagnostic methods have to be shifted towards molecular assays, based on the detection of viral nucleic acids. As these approaches rely on the hybridization of primers to complementary regions of the targeted gene followed by the exponential amplification of the internal fragment, the efficiency of the system would be influenced by the genetic diversity of local subtypes, justifying the continuing study of the phylogeography of FIV.

Due to the negative impact of FIV infection in the cat, it is important to address the use of efficient therapeutics. FIV infection is characterized by an acquired immunodeficiency syndrome, similar to the one caused by HIV in humans, making FIV an appealing model for pathogenesis studies of lentivirus infection. The infected animals have an increased risk for the development of opportunistic infections, which in high-density populations, as in cat shelters may represent a serious problem. Our aim was to investigate if recombinant feline Interferon ω (rFeIFN ω) recently licensed for use in Veterinary Medicine, would be effective in controlling the excretion patterns of concurrent viral infections in FIV infected stray cats housed in an animal shelter (Gil *et al.* 2013). Feline Panleucopenia Virus (FPV), Feline Coronavirus (FeCoV), Feline Herpesvirus type I (FHV-1) and Feline Calicivirus (FCV) infection are frequent and their excretion may become exacerbated after introduction in animal shelters, increasing the environmental viral load. In animals whose immune response is weakened by FIV infection, exposure to viral pathogens may induce or complicate clinical outcome. The subcutaneous administration of rFeIFN ω resulted in an improvement of the clinical signs and a reduction of concurrent viral excretion (Gil *et al.* 2013). Oral administration of rFeIFN ω, although assayed in symptomatic FIV-infected companion domestic cats, also proved to be useful in improving the clinical status (Gil *et al.* 2014). The impact in the reduction of concurrent viral loads was not evident, due to the absence of viral excretion in these animals.

Type I Interferons (IFN 1), in which class rFeIFN ω is included, are immune modulators produced in virus infected cells, with an anti-inflammatory, anti-viral and anti-proliferative effect (Collado *et al.* 2007); IFN 1 also interferes with the immunological response triggering the expression of citokines related to innate immunity. To further clarity rFeIFN ω action and its usefulness in the treatment of FIV positive cats, three

acute phase proteins (APP): Serum amyloid-A (SAA), C-reactive protein (CRP) and α-1-glycoprotein (AGP) were evaluated. These proteins are positive APP, reinforcing the innate response during inflammation (Petersen *et al.* 2004). Due to the participation of rFeIFN ω in the innate immunological response, the assessment of these APPs provided a good indicator of the mechanisms of action of this molecule. The study from our lab included FIV positive stray cats, housed in an animal shelter and a significant plasmatic increase was recorded in the measured APPs (SAA, CRP, AGP). These results suggest an enhancement of the innate immune response in these animals (Leal *et al.* 2013), confirming the usefulness of positive APP as predictors of the innate response stimulation and the effectiveness of rFeIFN ω in the control of the immunological dysfunction induced by FIV infection.

3 Feline Coronavirus

Feline Coronavírus (FeCoV) is responsible for a puzzling disease of domestic and wild felids, with two alternative presentations: mild enteritis or feline infectious peritonitis (FIP), the later with a frequent fatal outcome. Two known biotypes of FeCoV are described, the Feline Enteric Coronavirus (FECV) responsible for the enteric form and the Feline Infectious Coronavirus (FIPV); the transformation of FECV to FIPV is associated with three identified mutation events, resulting in biotype conversion (reviewed by (Pedersen 2014). The positive selection of the mutated viruses, exerted by the host immune system, allows switch of the cell tropism (enterocyte to monocyte/macrophage), and eventually a different disease outcome.

Coronavirus are prone to mutations, due the extended use of RNA polymerases, during the process of viral replication. Moreover, and contributing for the capacity the Coronavirus have to adapt to new cells and new hosts, as already happened with SARS, MERS and SADS, recombination events are recorded; these may occur within the same host or between different hosts, as already detected between Canine and Feline Coronavirus. The cross recombination between these viruses gave rise to two serotypes: Type I is strictly feline while Type II is antigenic and genetically closer to Canine Coronavirus (CCoV) resulting from a cross recombination event within the S gene, coding the surface glycoprotein of the virus envelope (Hohdatsu *et al.* 1991). To complicate further the pathogenesis of Feline Infectious Peritonitis, Type I as well as Type II are both associated with the disease.

Due to the importance of genetic diversity in the biological properties of FeCoV (including FECV and FIPV; Type I and II) we aimed at characterizing the genetic diversity of Portuguese FeCoV (Duarte *et al.* 2009). A higher proportion of Type I FeCoV was found in healthy and sick (FIP diagnosed) animals (79%); samples positive for Type II FeCoV were detected only in sick animals (3,5%). The phylogenetic analysis revealed a mixed distribution of Type I sequences, with a higher genetic diversity towards the European and Japanese sequences. Type II sequences, although in a much lower number, were genetically homogenous comparing to the available Type II sequences from the UK and Netherlands (Duarte *et al.* 2009). We also detected the presence of viral quasispecies in 17% of the samples, confirming the virus persistence in the host as mixed viral populations whose interaction with the animal immune response may or not result

in severe disease, supporting"The internal mutation theory" already addressed by other authors (Kipar and Meli 2014; Licitra *et al.* 2013; Pedersen 2014).

4 Virus Surveillance

As already stated in the introduction, information addressing risk assessment of viral infectious pathogens to wild animal populations is an important issue in conservation medicine, particularly when reintroduction is planned, which is the case of the Iberian lynx (Lynx pardinus), in Portugal. Since 2014, a concerted effort has been undertaken for the successful introduction of the Iberian lynx the world's most endangered feline species (IUCN, 2007). To potentiate a successful reintroduction, a number of concerns have been addressed, including a thorough screening of pathogens, capable of posing a health threat to the Iberian Lynx but also to its prey the wild rabbit (Oryctolagus cuniculus).

Concerning viral pathogens Feline Leukemia virus (FeLV), Feline Immunodeficiency Virus (FIV), Feline Coronavirus (FeCoV) and Feline Parvovirus (FPV) are important viral pathogens of the domestic cat (Felis catus) but also of several wild felids, including the European wildcat (Felis silvestris silvestris) and the Iberian lynx. Canine distemper virus is an important viral pathogen for members of the Panthera genus and to the Mustelidae family (Roelke *et al.* 2008; Thorne and Williams 1988), and was reported as cause of death in an Iberian Lynx in Spain (Meli *et al.* 2010). To assess the presence of FeLV, FIV, FCoV, FPV and CDV infections in wildcats and feral cats, as potential viral reservoirs we conducted a virological survey on biological samples collected between 1992 and 2007 and stored at the ICNF tissue bank. Positivity was confirmed for FeLV antigen, CDV antibodies and FeCoV and FPV nucleic acid (Duarte *et al.* 2012b). Although samples had been collected several years before the reintroduction program, they provided useful data to support the choice of a realistic vaccination program.

Virological surveys conducted in different locations, contribute for the health surveillance and provide important data concerning the infectious pressure on the animal populations. Under a neutering and health surveillance program carried by the organization "Veterinarians without Frontiers", Portugal (VSF), in Vila do Maio, Maio Island, Cabo Verde, blood and rectal swabs were collected from dogs. The samples were tested for Canine Parvovirus (CPV), Canine Distemper Virus (CDV) and Canine Coronavirus (CCoV), to estimate the viral prevalence on this population, but also to evaluate the role of these animals as virus reservoirs (Castanheira *et al.* 2014). Although no vaccination program was carried out in Ilha do Maio, Cabo Verde, we detected antibodies against CDV and CPV, confirming contact and implying infection, due to the absence of vaccination. CPV DNA and CDV RNA were also detected; CCoV RNA was detected in 1.4% of the samples (Castanheira *et al.* 2014).

Domestic dogs may act as a platform to pathogen spill over to susceptible newcomers' hosts or for resident susceptible new hosts as sympatric carnivores' species. Information regarding pathogen infectious pressure complement the Health programs undertaken by Veterinarians without Frontiers, Portugal in different places.

5 Virus and Marine Mammals

The identification and sanitary monitoring of sentinel species such as marine mammals provide a relevant approach for both population and environmental surveillance (Bossart 2011). A missing topic concerning epidemiological surveillance are studies on infectious disease epidemiology in coastal cetacean species, although recent reports of reemerging or new infectious diseases in marine mammal populations with epizootic and potential zoonotic consequences are already recognized and signalled (Yon *et al.* 2018). Viral diseases are important due to their pathogenic potential but also due to their impact on the animal immunological system rendering the animals more susceptible to opportunistic infections.

Cetacean morbillivirus (CeMV) were responsible for epizootic episodes in bottlenose dolphins in 1987–1988 and in 1993–1994 in the Gulf of Mexico. Disease outbreaks occurred in striped dolphins (Stenella coeruleoalba) in 1990–1992, in 2007 (Bossart 2011) and 2011 in the Spanish Mediterranean coast and in 2013 in the Italian Mediterranean coast (Yon *et al.* 2018). Until 2010, no CeMV had been detected in marine mammals in Portugal or in Northern Spain. However, during and after the CeMV outbreaks in northern Europe (Van Bressem *et al.* 2014), migrating marine mammals probably acted as carriers introducing CeMV into viral-free (or undetected) regional cetacean populations. To further clarify the prevalence of Dolphin Morbillivirus (DMV) in cetacean populations from the eastern Atlantic, but also to investigate the link between eastern Atlantic and Mediterranean DMVs, we conducted a molecular survey and phylogenetic analysis of viral sequences (Bento *et al.* 2016). We found a higher prevalence of DMV in stranded striped dolphins than in stranded common dolphins (Delphinus delphis) from the Atlantic based populations. Since no outbreaks have been reported and positive samples were detected annually since 2007, the virus persists in the striped dolphin populations from Portugal and Galicia, endemically rather than epidemically. The phylogenetic analysis displayed a clear phylogeographic pattern, with the sequences from Portugal and Galicia clustering together, away from the Mediterranian sequences, suggesting that these populations (Atlantic and Mediterranean cetaceans) are relatively isolated from each other, as already suggested by others (Bourret *et al.* 2007). To assess DMV distribution and pathogenic impact in populations of cetaceans from the eastern Atlantic serological data collection should implemented.

Herpesvirus (HV) are connected to subclinical as well as fatal diseases and their hallmark of infection is the ability to induce a persistent infection, with periodic or continuous shedding of the virus (Maclachlan *et al.* 2011). Herpesvirus (HV) infections are recognized in cetaceans since 1980 (Maness *et al.* 2011). Silent and disseminated HV infection of cetaceans were reported along with reactivation of a HV latent infection following immune suppression caused by CeMV or by pollutants. (Arbelo *et al.* 2010; Belliere *et al.* 2010). While information on HV cetaceans infections is available worldwide, including in Spain, in Portugal there were not reports concerning HV infection in cetacean populations. Due to the availability of samples from stranded cetaceans, collected along the Portuguese continental coastline, and stored in the Marine Animal Tissue Bank a comprehensive survey was conducted, to provide missing molecular data on cetaceans HV (Bento *et al.* 2019). Both Alphaherpesvirus and Gammaherpesvirus were detected; the phylogenetic analysis revealed the absence of species specificity, meaning

that both virus subfamilies were detected in a vast array of cetacean species. In addition, within the Alphaherpesvirus cluster, the Portuguese HV sequences were included in three individual branches, outside previously reported virus genera. Although HV genome was found in animals without macro lesions of disease, which is consistent with the epidemiology of HV, our results suggest that their role in cetacean morbidity may be underestimated (Bento *et al.* 2019).

Due to the negative impact that viral diseases have on the immunological system, the evaluation of marine mammals' immune fitness is important and can be achieved by profiling cytokine levels already identified in stress response and chronic infection in wild dolphins (Beineke *et al.* 2007; Hoffman *et al.* 2013). The identification of reference values, allows the detection of immunological shifts caused by pathogen infections or stress. Based on the expression levels of a panel of cytokines involved in the humoral and cellular response, we were able to assess a physiological baseline of cytokines expression patterns. These will be a valuable tool in order to assess the health status of stranded animals (Bento *et al.* 2016) and also to compare with the corresponding patterns in virus positive animals, particularly DMV and HV.

6 Sea Turtles and Fibropapillomatosis

Under the "One Health" concept Ecosystems Conservation and Conservation Medicine are interconnected compelling ecologists/biologists, veterinarians and other professionals to work together in multidisciplinary teams to study the relations between Human, Animal and Ecosystems Health.

Sea turtles are flag species for biodiversity and conservation. There are only seven species in the world and four of them are classified as critically endangered.

Fibropapillomatosis (FP) is a neoplastic disease recognized since 1938, affecting several species of sea turtles, namely green turtle (*Chelonia mydas*), loggerhead (*Caretta caretta*), olive Ridley (*Lepidochelys olivacea*), Kemp's Ridley (*Lepidochelys kempii*), hawksbill (*Eretmochelys imbricata*), leatherback (*Dermochelys coriacea*), and flatback (*Natator depressus*) turtles (Foley *et al.* 2005), contributing for the morbidity and mortality of already endangered species. In the last two decades, the disease prevalence has dramatically increased and is now considered as an emerging pandemic disease endangering the survival of these animals (Jones *et al.* 2016). Fibrovascular tumours with internal and external location characterize FP; these have a direct consequence on the mobility, vision; feeding and organ function and ultimately leading to death. The disease etiology is still undetermined but the involvement of herpes virus, pollutants, blood flukes, marine toxins, ultraviolet light and rising temperature has been suggested. Although Koch postulates have not been fulfilled, the association of Chelonid herpesvirus 5 (ChHV5), subfamily Alphaherpesvirinae, genus Scutavirus (Davison *et al.* 2009) with FP is strongly suggested (reviewed by Jones *et al.* 2016). Still other interactions should be considered in the development of FP, including environmental factors, the host immune response and viral variants, among others (Jones *et al.* 2016).

To further contribute to FP epidemiology we first characterized ChHV5 in FP lesions from *Chelonia mydas* from Principe Island, Gulf of Guinea (Duarte *et al.* 2012a), after FP detection in Gulf of Guinea (Formia *et al.* 2007; Loureiro and Matos 2009), Detection

of ChHV5 DNA in tumor samples from FP-afflicted turtles, strengthened the potential role of the virus in the etiology of FP in West Africa. In addition, the detection of viral sequences in normal skin of un-afflicted turtles, was consistent with herpesvirus hallmark of infection, suggesting viral latency in normal tissues (Duarte *et al.* 2012a).

Herpesvirus phylogenies tend to follow their hosts evolution, suggesting a co-evolutionary pattern (Firth *et al.* 2010). ChHV5 Principe Island sequences were analyzed, together with ChHV5 from different geographic locations in order to infer ChHV5 phylogeography, contributing for the evaluation of the evolution and demographic pattern of the virus (Patricio *et al.* 2012). The resulting phylogenetic tree mirrored the complexity of sea turtles' evolution. Four geographic virus clades were resolved: Eastern Pacific, Western Atlantic/Eastern Caribbean, Atlantic and Mid-West Pacific. Interestingly the Principe Island, Gulf of Guinea (Atlantic) ChHV5 clustered with the Eastern Caribbean ChHV5, suggesting a virus flow between two distant turtle population, most likely in an eastward direction. This virus link might be explained due to the equatorial current from the Gulf of Guinea to Brazil, influencing the dispersal of sea turtles between Central America and the West coast of Africa (Patricio *et al.* 2012), supported by a previously identified genetic linkage between these turtle populations (Velez-Zuazo 2008).

7 Conclusion

The presented work aimed to address the research work, developed by the Virology and Immunology Laboratory in the last 15 years, under a conservation medicine and One Health perspective. It implied the development of specific molecular assays and phylogenetic methodologies to evaluate the pathogen prevalence and geographic distribution as well as the phylogeographic patterns of the viral sequences. The generated sequencing data, concerning viral sequences are deposited in the Nucleotide Data Bank (https://www.ncbi.nlm.nih.gov/nuccore), and available to the scientific community. All the developed molecular assays are currently used as diagnostics tools in the Virology Diagnostic Laboratory of FMV/ULisboa providing a molecular diagnostic service for both intra- and extra-mural clients.

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Leishmaniosis: New Insights in a Changing World

G. Alexandre-Pires[1], M. Santos[1], M. A. Rodrigues[2], M. A. Pereira[2,3], J. Gomes[4], S. A. Diaz[1], L. Gomes[1], A. Basso[4,5], A. Reisinho[4], J. Gomes[1,6], R. Leal[4,5], J. Correia[4,5], A. Bolas[2], J. Meireles[1], G. Santos-Gomes[2], and I. Pereira-da-Fonseca[1](✉)

[1] Parasitology and Parasitic Diseases Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
ifonseca@fmv.ulisboa.pt
[2] Global Health and Tropical Medicine, Institute of Hygiene and Tropical Medicine, New University of Lisbon, Lisbon, Portugal
[3] Agrarian School of the Polytechnic Institute of Viseu, Viseu, Portugal
[4] Faculty of Veterinary Medicine, Veterinary Teaching Hospital, University of Lisbon, Lisbon, Portugal
[5] Pathology Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
[6] National Institute for Agrarian and Veterinary Research, Oeiras, Portugal

Abstract. Canine leishmaniosis caused by *Leishmania infantum* is a zoonotic disease of serious veterinary concern in the Mediterranean basin. In Portugal has been reported in dogs, cats and synanthropic rodents. Epidemiological changes and new hosts may contribute to increase zoonotic risk. A better knowledge on immune response, treatment and diagnosis are at the forefront of research on this disease. Host immune response is multifactorial, reflecting the organ specificity. Macrophages (MØ) are the definitive host cells, although neutrophils (PMN) are the first cells to encounter parasites soon after inoculation in the dermis. The PMN-parasite interaction decreases parasite viability, but PMN-MØ interaction induces nitric oxide production and release of neutrophil extracellular traps that contain parasites, controlling dog infection at early stages. Liver resident Kupffer cells (KC) efficiently phagocyte *Leishmania* by establishing an intimate contact with circulating blood. The impact of meglumine antimoniate (MG) over infected canine KC was investigated. The effect of different treatment protocols in dog's immune response was assessed. MG+miltefosine treatments plus allopurinol restore lymphokine gene expression, pointing through a drug-induced reduction of anti-inflammatory and regulatory cytokines. Furthermore, increasing feline leishmaniosis and the inconsistent results of therapeutic protocols led the team to evaluate their safety and effectiveness in cat.

Keywords: Leishmania · Leishmaniosis · Host-immune-response · Zoonosis · Treatment

G. Alexandre-Pires and M. Santos—These authors contributed equally for this work.

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What is canine leishmaniosis (CanL)? CanL is a chronic and multisystemic disease caused by the intracellular protozoan parasite *Leishmania infantum* transmitted by Phlebotomine sand flies. A wide range of nonspecific clinical signs is displayed with diverse intensities and symptoms which can affect any organ and be influenced by several factors (Santos-Gomes and Pereira da Fonseca 2008). These include parasite strain and virulence, host genetic background, age, gender, breed, coexistent infections, immune competence and nutrition status (Miró *et al*. 2008). CanL clinical diagnosis can be complicated with 50% of the infected dogs not presenting clinical signs for several years. Other dogs present acute clinical signs and pathological abnormalities with severe disease and progression to death (Solano-Gallego *et al.* 2009). Whereas, some dogs exhibit clinical signs within 3 months to numerous years post-infection or even naturally progress to cure (Koutinas *et al.* 2014).

In dogs with CanL we can identify animals whose lesions are limited to only one lymph node or cases with generalized lymphadenopathy. On the other hand, the lymph nodes most affected are the superficial ones, identifying more lesions in the mandibular, cervical, prescapular, axillary and popliteal region. In the early stage of the disease, the lymph nodes present lymphadenomegaly, although they never reach the lymph node size with high-grade malignant lymphoma. On palpation they are painless, with smooth surface, not adherent and of increased consistency. At cut, they are swollen and show a ferruginous coloration due to the accumulation of hematic pigment hemosiderin. With the evolution of the disease, regression of adenopathy occurs. On histopathological examination and in the initial phase of the disease, we can observe follicular lymphoid hyperplasia, with enlargement of the lymphoid follicles due to the presence of abundant B-type blast lymphoid cells (centroblasts) in the germinal centers. In the paracortical zones and medullary cords, a proliferative reaction of macrophages (MØ) is observed, whose cytoplasm is full of *Leishmania* amastigotes, there is an increase in the number of plasmocytes and a decrease in the number of mature lymphocytes. In an advanced or chronic phase, the phenomena of lymphocytolysis at the level of germinal centers (with hyalinosis) and intense plasmacytosis at the medullary level are associated with connective tissue hyperplasia and sometimes sclerosis (Alexandre-Pires and Correia 2008).

Bone marrow changes may be focal and have a more fluid consistency than normal as well as a uniform red color. There are no alterations in marrow adipose tissue (Rebêlo 1988). Histopathological examination shows granulomas rich in epithelioid MØ, granulocytes and T lymphocytes together with parasites internalized by MØ. There is a marked hyperplasia of the plasma cells that can reach 50% of the myelogram cells. Plasma cells are well differentiated and no atypia is present and their presence is linked to polyclonal hypergammaglobulinemia. Several deposits of hemosiderin may also be seen. Usually, involutive and non-regenerative myelopathy develops with depletion of the erythroblastic, leukoblastic and megakaryoblastic cell series (Bourdeau 1988).

Although, the mechanisms that are involved in *Leishmania* resistance or susceptibility in dogs are not known, and a wide range of immune responses and clinical presentations have been reported in CanL: two extreme immune responses have been described associated with disease susceptibility or resistance. Disease susceptibility is generally related to aggravated humoral non-protective immune response and reduced

cell mediated immune response characterised by mixed Th1 and Th2 cytokines production, leading altogether to symptomatology and clinicopathological abnormalities (Alvar *et al.* 2004). On the other hand, disease resistance is associated with $CD4^+$ T cell protective immunity mediated by the production of interferon (IFN)-γ, interleukin (IL)-2 and tumor necrosis factor (TNF)-α, which will be responsible for MØ anti-*Leishmania* activity. During infection MØ constitute antigen presenting cells (APC) by processing the foreign antigens that can bind to class II molecules of the major histocompatibility complex (MHCII) (Kaye *et al.* 1994). These are subsequently recognized by the T cell receptors (TCR) (Kaye *et al.* 1994) which can become tolerant or differentiate into effector cells (Geppert *et al.* 1990).

A study using MØ and lymphocytes derived from dogs of different sexes, breeds and ages has reported an increased expression of MHCII in MØ infected with *L. infantum* promastigotes or when cultured with *L. infantum* antigens and in the presence of lymphocytes (Diaz *et al.* 2012). These findings suggest that the parasite's antigen presentation by MØ in addition to MHCII expression can be maximized by lymphocytes (Diaz *et al.* 2012).

Additionally, the activation of T lymphocytes by MHCII-restricted antigens can induce the production of IFN-γ which can stimulate MHC expression, foreign antigen processing and the presentation of both MHCI and MHCII restricted antigens. Other studies reported, unchanged surface MHCI or MHCII expression upon infection of MØ derived from beagle dogs with *L. infantum* (Pinelli *et al.* 1999a) or up-regulation of MHCII levels and decrease on APC function in *L. donovani* infection (Kaye *et al.* 1994). Furthermore, loss of T cell activity and inactivation of MØ oxidative pathways were associated with lack of co-stimulatory expression and a reduced release of nitric oxide (NO) both in the presence of *L. infantum* parasites or respective antigens (Diaz *et al.* 2012). Thus suggesting a regulation of host immune response by promastigote stage specific molecules without the parasite being present (Diaz *et al.* 2012). In this sense, it is possible that promastigote stage specific molecules are responsible for the suppression of host immune response with consequent *Leishmania* survival, replication and dispersion. Thereby, the identification of the parasite molecules that interfere with the normal activation of the dog immune system and related pathways are critical in the clarification of *Leishmania* survival mechanisms within the host. This information would also greatly contribute to the determination of new targets for vaccine and therapy design.

What about new *Leishmania* vertebrate hosts? The epidemiology of leishmaniosis has been changing with the increasing number of studies focusing in new vertebrate hosts. All the new information about wildlife as possible reservoir hosts of *Leishmania* spp. can contribute to the knowledge of the true zoonotic risk of leishmaniosis. While dogs are considered the main reservoir of *Leishmania infantum* infection in endemic areas in Europe, with apparent prevalence rates ranging from 5% to 30%, the existence of other wild vertebrate reservoirs might be a possible cause of the deficient success of control measures. Different studies, mostly in Spain, Italy and France have been done in an increasing number of species, undoubtedly due to the increased wildlife monitoring programs that enable the identification of infected host species, especially carnivores, but also due to the use of more specific and sensitive molecular techniques.

Serologic or direct evidence of *L. infantum* infection in animals from the Canidae, Felidae, Mustelidae, Viverridae and Herpestidae families have been reported in Europe. More recently, *L. infantum* infected lagomorphs and rodents have also been detected in Europe (Millán *et al*. 2014).

In Europe, the presence of *Leishmania* spp. in red foxes (*Vulpes vulpes*) has for long been studied, but other Canidae have also been detected with the infection, such as the grey wolf (*Canis lupus*) (Beck *et al*. 2008) and the Golden jackal (*Canis aureus*) (Ćirović *et al*. 2014). The red fox (*V. vulpes*) due to its taxonomic relationship with the dog, and because it is the most abundant wild carnivore in Europe has been considered an important host. *Leishmania* spp. infected-foxes were detected in the Arrábida region, Southern Portugal, reaching a prevalence rate of 5.63%, which is probably sufficient to maintain endemicity. Although some foxes did not show clinical signs, it was possible to isolate the parasite. In Portugal, isoenzymatic studies showed that parasites isolated from foxes were identical to others strains isolated from man and dogs (Abranches *et al*. 1983). Serological and molecular studies in free-ranging red foxes from other European countries also detected a considerable number of infected animals. In Liguria, Italy, serology using immunofluorescence assay and enzyme linked immunosorbent assay (ELISA), detected a prevalence of 18% in 50 animals (Mancianti *et al*. 1994). In Guadalajara, Spain, a survey of leishmaniosis and other parasites in 67 foxes revealed a prevalence of 74% *Leishmania* infection using molecular methods (Criado-Fornelio *et al.* 2000). Wolves have also been studied in southern Europe with positive results for *Leishmania* infection. In Asturias, Spain, a region considered non-endemic to *L. infantum*, 102 wolves were studied by molecular methods to detect *Leishmania* DNA. An average prevalence of 33% for wolves was reported, with a widespread presence of the parasite in the region and an apparent increase in its prevalence in wolves during the last decade (Oleaga *et al.* 2018). In another study from Central Portugal and Central and Northern Spain, captive wolves were tested using ELISA and a molecular test and, positive animals were also detected (Sastre *et al.* 2008). The population of Eurasian golden jackal (*C.aureus*) from Southeastern Europe, Asia, the Middle East and the Caucasus is increasing and spreading quickly, and some studies have revealed their potential role as carriers of zoonotic diseases and this species should be taken under consideration when applying surveillance monitoring schemes. Studies from Serbia tested golden jackal for *Leishmania* species by real-time PCR and detected a prevalence of 6.9% in a total of 216 samples collected (Ćirović *et al.* 2014). Expanding populations of jackals can play a significant role in spreading different diseases including *L. infantum.* Some studies confirm that once established, the populations of Eurasian golden jackals constitute natural reservoirs for many canine vector-borne diseases, analogous to the role of the coyotes in North America (Mitková *et al.* 2017). Wild canidae are extremely useful as sentinel species for the detection and field studies of *Leishmania* and confirms the value of wildlife sanitary surveillance programs for the detection and monitoring of zoonotic diseases (Oleaga *et al.* 2018).

Feline leishmaniosis caused by *L. infantum* is frequently reported in endemic areas and is becoming an emerging feline disease. This is due not only to the increased level of feline medical care, but also to the availability of more sensitive diagnostic tools that contributed to increased number of detected cases in cats (Cantacessi *et al.* 2015). *L. infantum* has been detected in cats in several southern European countries such as

Portugal, Spain, Italy, France, Greece and Cyprus but also in other parts of the world. Recently *L. tropica* and *L. major* were confirmed in cats in Turkey (Paşa *et al.* 2015). Prevalence, molecular and serologic studies show a lower prevalence in cats compared to dogs and also the diagnosis of clinical cases in cats is rare (Pennisi *et al.* 2015). Travelling and rehoming cats can result in the detection of clinical cases in non-endemic areas (Rüfenacht *et al.* 2005). Wild Felidae species from Europe have also been screened with detection of a positive wildcat (*Felis silvestris*) (Del Río *et al.* 2014) and one Iberian lynx (*Lynx pardinus*) (Sobrino *et al.* 2008). Some species, such as the Iberian lynx are of high conservation value and this infection could have a serious impact on their morbidity and mortality.

Small carnivore species from Mustelidae and Viverridae families have also been detected as positive for *Leishmania* infection. In Mallorca, Spain there was the first report of infection by *L. infantum* in the pine marten (*Martes martes*) (Millán *et al.* 2011). Stone marten (*M. foina*) and European badger (*Meles meles*) were also detected infected in Spain but none of those three species had visible lesions. Viverridae carnivores such as the common genet (*Genetta genetta*) (Del Río *et al.* 2014) and Herpestidae such as the Egyptian mongoose (*Herpestes ichneumon*) (Sobrino *et al.* 2008) have also been detected as seropositive. While some populations of such carnivores are decreasing in number, other populations such as the Egyptian mongoose are increasing and these animals, if confirmed as reservoir hosts, might contribute to the epidemiology of leishmaniosis.

The natural infection of *L. infantum* in rodents such as mice (*Mus musculus*) and rats (*Rattus norvegicus*) have been recently identified for the first time in Portugal using molecular methods (Helhazar *et al.* 2013) but other species such as Black rats (*Rattus rattus*) were detected as positive in Italy (Zanet *et al.* 2014). Further studies are needed to clarify if these animals have an important role as reservoirs in the parasite life cycle since rats and mice are extremely prolific animals and have a life expectancy that maintains the parasite availability for phlebotominae vectors thus increasing the risk for humans and domestic animals (Helhazar *et al.* 2013). These studies show the need for efficient rodent control measures to prevent transmission of *Leishmania* parasites.

The Iberian hare (*Lepus granatensis*) has recently been recognized as the origin of a leishmaniosis outbreak in humans in Spain and xenodiagnosis showed that this species is also able to infect sand flies (Molina *et al.* 2012). Retrospective studies had shown a high prevalence in this species but also on European hare (*L. europaeus*) from six regions of Spain (Ruiz-Fons *et al.* 2013). A few molecular and serologic studies in the European rabbit (*Oryctolagus cuniculus*) showed prevalences from 0.6% to 45.7%, depending on the method (Chitimia *et al.* 2011).

The role of wildlife in the epidemiology of leishmaniosis is increasingly being studied, particularly the comparison of parasite isolates from different mammal families, humans and dogs. Other vertebrate taxonomic groups will also be included, for instance in transmission studies. Some vertebrate species should be included in surveillance programs as sentinel animals while endangered species with protected status should be monitored for different infections, including leishmaniosis and other that are invasive or considered as pests should be included in population control programs.

How does the host innate immune response work? *Leishmania* promastigotes are deposited in the dermis of the mammalian host through the bite of a sand fly vector.

The local innate immune response constitutes the first line of defense against *Leishmania* parasites. Polymorphonuclear neutrophils (PMN) are the most abundant circulating leukocytes and the first cells to reach the inoculation site, actively guided by chemotactic factors. *In vivo* studies showed that the inoculation of *Leishmania* parasites in hamsters (Wilson *et al*. 1987), mice (Thalhofer *et al.* 2011) and dogs (Santos-Gomes *et al*. 2000) through needle injection induces a rapid dermal infiltration of PMN. Two-photon intravital microscopy studies carried out in C57BL/6 mice-*L. major* infected through sand fly bite confirmed that PMN are the first cells to infiltrate the dermis (Peters *et al*. 2008).

Although tissue damage following sand fly bites or needle injection in the absence of parasites induced PMN recruitment (Peters *et al*. 2008), the contribution of parasite-derived signals in PMN recruitment was studied. *In vitro* studies showed that viable *L. major*, *L. aethiopica* and *L. donovani* promastigotes release chemoattractant factors that induce the migration of human PMN (van Zandbergen *et al.* 2002). Viable *L. infantum* promastigotes and culture supernatants also induce a strong chemotaxis of canine PMN (Pereira *et al*. 2017), indicating that the parasite has the ability to modulate leukocyte recruitment at the early phase of infection.

As described in *L. donovani*-human PMN (Pearson and Steigbigel 1981), the attachment between *L. infantum* promastigotes and canine PMN is non-random. Indeed, promastigotes preferentially adhere to PMN by the flagellum tip (anterior pole) (Pereira *et al*. 2017) (Fig. 1A and B), which probably reflects the concentration of the main adhesion molecules (gp63 and LPG) in specific areas (adhesiotopes) of the parasite membrane (Rittig and Bogdan 2000). The attachment *via* the flagellum tip promotes the protrusion of symmetrical pseudopods that maintain the directional entry of the parasite into the PMN (symmetrical phagocytosis), favoring parasite killing (Hsiao *et al.* 2011). PMN rapidly internalize the parasite at inoculation sites and at visceral organs, becoming the predominant parasitized cells over the first few hours following *L. donovani* and *L. infantum* infection (Wilson *et al*. 1987; Thalhofer *et al*. 2011). Experimental *L. infantum* infection showed that 3 to 4 h after dermal injection, promastigotes had already been internalized by canine PMN, proving the early involvement of these cells in CanL (Santos-Gomes *et al.* 2000). *In vitro* studies revealed that about one third of canine and C57BL/6 mice PMN had internalized the parasite within 3 h (Marques *et al.* 2015; Pereira *et al*. 2017).

In vitro, canine PMN rapidly kills *L. infantum* promastigotes (Pereira *et al*. 2017) andBALB/c PMN destroys the parasite in the spleen (Rousseau *et al*. 2001) using phagocytosis-dependent mechanisms. Other *in vitro* studies showed that *L. donovani* uptake by mouse and canine PMN *via* lytic organelle-dependent pathway leads to large phagosomes formation and to parasite degradation, but the uptake *via* a lytic organelle-independent pathway promotes tight phagosomes formation and parasite survival (Gueirard *et al*. 2008). It was demonstrated that *L. infantum* promastigotes activate canine PMN to release greater amounts of superoxide (Pereira *et al*. 2017). The induction of a strong oxidative burst results in the elimination of *L. donovani* and *L. major* promastigotes by human PMN (Pearson and Steigbigel 1981; Laufs *et al*. 2002).

Granule exocytosis and Neutrophil Extracellular Traps (NETs) release contribute to extracellular parasite killing. *L. infantum* promastigotes stimulates neutrophil elastase (NE) exocytosis by canine (Pereira *et al*. 2017) and by C57BL/6 mouse PMN (Marques

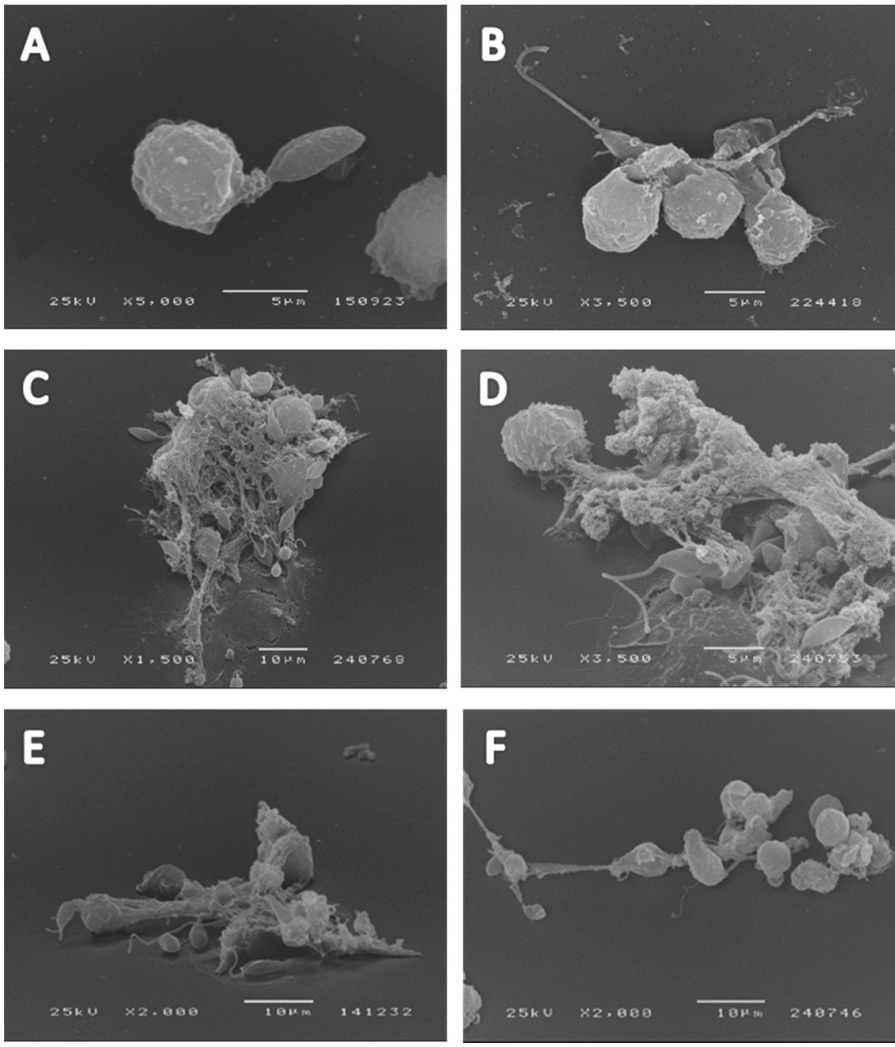

Fig. 1. Attachment and phagocytosis of *L. infantum* by PMNs. Scanning electron microscopy images showing attachment and engulfment of promastigote via their posterior pole (A) and orientated attachment via the flagellum (B). Extracellular interaction between murine PMN and *Leishmania* promastigotes. Scanning electron microscopy images showing filamentous structures entrapping *L. infantum* promastigotes (C), *L. amazonensis* (D), *L. shawi* (E) and *L. guyanensis* (F).

et al. 2015), and *L. braziliensis* stimulates both peritoneal and bone marrow derived BALB/c PMN to release NE (Falcão *et al.* 2015). *L. infantum, L. amazonensis*, *L. shawi*, and *L. guyanensis* promastigotes promoted NETs release by murine PMN (Fig. 1C, D, E and F) (Valério-Bolas *et al*. 2018). However, *L. infantum* seems to reduce NETs

formation by canine PMN, indicating that the parasite modulates negatively this effector mechanism, favoring parasite spreading and survival (Pereira *et al.* 2017).

PMN possess some direct leishmanicidal activity, demonstrated *in vitro* and *in vivo*, capable to reduce parasite burden. However, parasite persistence indicates that promastigote killing is clearly insufficient in controlling the establishment of infection. Indeed, several reports showed that a subset of parasites survives to PMN effector mechanisms (Müller *et al.* 2001). *L. major* viability and capacity to produce infection in naïve mice following *in vivo* phagocytosis by PMN was demonstrated by Peters *et al.* (2008). *In vitro* studies indicated that a considerable proportion of *L. infantum* promastigotes maintain viability and replication capability after canine PMN exposure showing that although dog PMN are competent effector cells able to reduce the parasite burden, some parasites can resist PMN activity (Fig. 2) (Pereira *et al.* 2017). Indeed, it seems that *Leishmania* promastigotes are well equipped to evade PMN killing. For instance, *L. major* blocks the oxidative burst of human PMN (Laufs *et al.* 2002) and *L. donovani* prevents the fusion between parasitophorous vacuole and mouse neutrophilic granules (Gueirard *et al.* 2008). Furthermore, some authors consider that surviving parasites might be transitional forms, better adapted to intramacrophagic life (Ribeiro-Gomes and Sacks 2012).

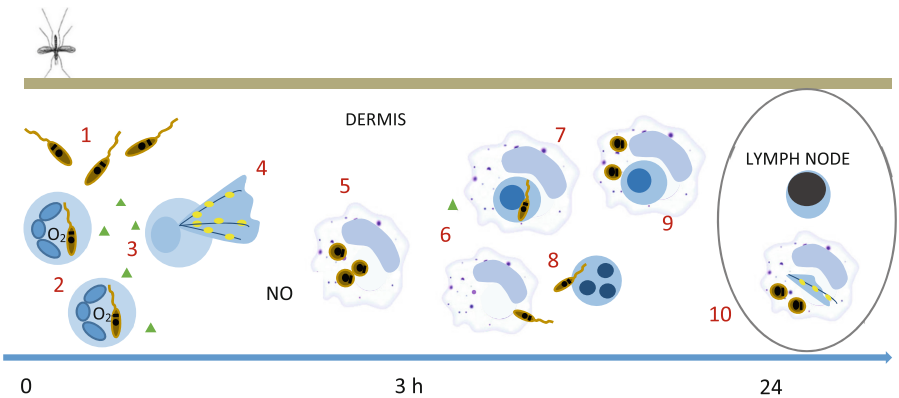

Fig. 2. Interaction between dog PMN and MØ at the early phase of *L. infantum* infection. 1 - PMN are the first cells to reach the inoculation site and rapidly phagocytize the parasite; 2 - The parasite induces the superoxide (O_2^-) production; 3 - The parasite induces the exocytosis of neutrophil elastase (triangles); 4 - PMN release neutrophil extracellular traps (NET), containing DNA (lines) and histones (circles); 5 - MØ produce nitric oxide (NO) in response to *L. infantum* infection; 6 - MØ and eventually infected MØ contact with NE that was released by PMN; 7 - Efferocytosis of infected PMN ensure parasite transference; 8 - MØ internalize parasites that escape from dying PMN; 9 - 24 h after inoculation, parasite dissemination takes place; 10 - Eventually in the regional lymph node, parasitized MØ that had removed NET compounds and contacted with NE kill the parasite and present parasitic antigens to lymphocytes.

Although PMN might serve as temporary host cells for the parasites within the first hours/days after infection (Aga *et al.* 2002), MØ are widely considered the primary host cells of *Leishmania* parasites, ensuring its replication, dissemination and long-term

survival. Thus, the interaction between these two phagocytes seems to be important for the establishment of *Leishmania* infection.

Although, PMN can undergo spontaneous apoptosis at inflamed sites, *Leishmania* modulates PMN apoptosis, prolonging its life span or accelerating its death (Aga *et al.* 2002). The parasite uses apoptotic PMN as "Trojan horses" to gain access to MØ. Interestingly, human infected PMN secrete monocyte-attracting chemotactic factors such as MIP1-β, which participate in the recruitment of monocytes. *Leishmania* internalization by MØ *via* the uptake of infected apoptotic PMN (efferocytosis) prevents the direct interaction with surface receptors, avoiding the activation of MØ effector mechanisms and ensuring parasite survival and replication (van Zandbergen *et al.* 2004). For instance, *L. major* delays the apoptotic death program of human PMN about 24 h (Aga *et al.* 2002). When MØ arrive to the inoculation site, they encounter the parasite inside PMN. *In vitro* studies showed that *L. major* infected apoptotic human PMN are readily phagocytized by MØ (van Zandbergen *et al.* 2004). However, other mechanisms of parasite transference from PMN to MØ have been described. Intra-vital microscopy studies showed viable *L. major* parasites being released from mouse apoptotic PMN in the vicinity of MØ, a mechanism called "Trojan rabbit" (Peters *et al.* 2008).

In vitro studies showed that efferocytosis of *L. major*-infected apoptotic human PMN promotes transforming growth factor (TGF)-β and suppresses TNF-α release, deactivating MØ effector functions and ensuring intramacrophagic parasite viability and replication (van Zandbergen *et al.* 2004). However, the interaction between necrotic PMN and *L. amazonensis*-infected human MØ induces parasite killing *via* TNF-α NE dependent (Afonso *et al.* 2008). Another *in vitro* study demonstrated that infected and non-infected canine co-cultures produce NO, a potent microbicide compound, and release extracellular traps (ETs) (Pereira 2016). In the context of infection, ETs clearance can influence MØ phenotype (Boe *et al.* 2015). Indeed, some studies have shown that the ability of MØ to kill intracellular microorganisms is mediated by the uptake of PMN-derived exogenous proteins. For instance, NE, a NETs component, stimulates *Leishmania*-infected MØ *via* TLR4 and assists parasite elimination (Ribeiro-Gomes *et al.* 2007). Thus, the interaction and cooperation between PMN and MØ seems to be complex and influence the outcome of infection, driving either parasite survival or destruction (Fig. 2).

Do we really know the role of hepatic cells in CanL? The liver is the largest solid internal organ in the mammalian body and it performs a remarkable number of tasks that support the function of other organs and impacts in all physiologic systems. This organ is, likewise, responsible for several immunological functions as the removal of pathogens and exogenous antigens from the systemic circulation. Its anatomic position and distinctive vasculature, contribute to its unique ability to continuously exchange immunological information. In recent years, the liver has been re-discovered and described as a major immunological organ.

In the context of Leishmaniasis, the role of the liver is not yet fully clarified. Few studies on CanL have addressed this question. Most of our current knowledge, on liver's role in disease progression, immune and treatment response is derived from the use of visceral leishmaniosis (VL) murine model and of human VL. The murine model for VL has showed that there is a distinct organ specific pattern of parasite growth during the disease establishment. In humans, dogs and genetically susceptible mice, the liver, the

spleen and the bone marrow are major sites of parasite growth and pathology. Evidences regarding the immune response of target organs against *Leishmania* parasites have been accumulated in recent years, pointing out a tissue specific immunity (Alexandre-Pires *et al.* 2010; Barbosa *et al*. 2011).

Granuloma formation and a Th1 polarized immune environment, appear to be key in the liver immune response. Indeed, granulomas are poorly formed in the immunodeficient murine model and in humans with progressive VL, which do not develop mature granulomas. The livers of asymptomatic dogs showed an effective immunity with well-organized granulomas able to isolate and restrain parasite spreading in an immune environment of activated effector T cells, dendritic cells (DCs) and central memory cells. In contrast, liver of symptomatic dogs showed a non-organized and ineffective infiltrate of T cells and heavily parasitized Kupffer cells (Sanchez *et al*. 2004). Furthermore, the highest proportion of activated effector T cells was also observed in the liver of asymptomatic dogs, correlating with an effective immune response against the parasite. Interestingly, many naive T cells were observed in the liver of symptomatic dogs (Fig. 3). Apparently, central memory T cells sensitized against *L. infantum* may migrate to peripheral tissues, providing protection against these vulnerable sites. In contrast, naive T cells migrate almost exclusively to lymphoid organs, which are designed to receive migrating cells and antigen sampling (Mackay *et al.* 1990, 1992). Rodrigues *et al*. (2017a) endorsed the role of the liver as an important immune memory organ in the context of *L. infantum* infection, using the murine model of VL. The phenotype characterization of liver resident T lymphocytes revealed that *L. infantum* infection generates effector and central memory T cells, but these cells did not expand when recalled, demonstrating a parasite silencing effect. The treatment with a leishmanicidal drug (meglumine antimoniate, MG) increases the levels of memory and effector T cells, eliciting a more robust hepatic immune response. This study evidenced the liver's ability to differentiate resident T cells with memory phenotype, emphasizing the role of the liver as an immunological organ. Hepatic leukocyte populations differ from those of other tissues in several interesting ways. Phenotypically, nearly 50% of lymphocytes express the T cell receptor (TCR) and there is an enrichment of $CD8^+$ T cells in the liver. Typically, in the blood, $CD4^+$ T cells outnumber $CD8^+$ T cells, but in the liver this ratio is reversed. The liver also possesses a unique natural killer T (NKT) cell population. These are important and potent immunomodulatory cell population residing in the liver (Sun *et al*. 2009). After activation, NKT cells release cytotoxic granules containing perforin and granzyme in a cell directed way. In response to stimulation, these cells also release large amounts of cytokines, such as IFN-γ, and by doing so, shape and direct the immune response and also modulate MHC expression of hepatocytes and hepatic stellate cells (Crispe 2009). As a result, NKT cells have great potential to shape the host immune response, together with additional characteristics of these cells, demonstrate the critical importance of this population for the immune surveillance.

Hepatocytes constitute the majority of the hepatic cells and although the primary roles of these cells are of metabolic nature, hepatocytes express innate immune receptors and, in many cases, have been demonstrated that these cells recognize pathogen associated ligands and display an innate immune response.

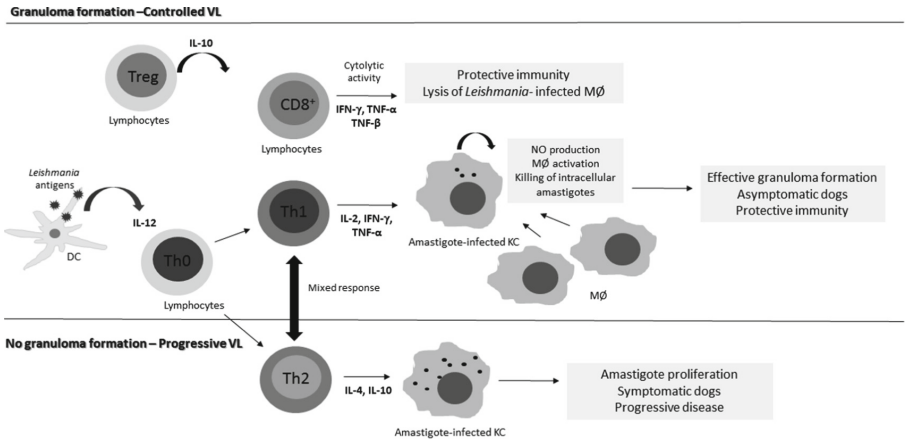

Fig. 3. Granuloma formation in a Th1 polarized immune environment is crucial for a protective liver immune response against *Leishmania* infection. The liver response to *Leishmania* infection may lead to the formation of a granuloma, that results in control of parasite growth and dissemination. This response is initiated by IL-12 secreted by activated dendritic cells and results in the activation of lymphocytes and secretion of IL-2, IFN- γ and TNF-α, which will recruit more lymphocytes and lead to the activation of Kupffer cells and recruitment of macrophages. These well-organized granulomas contribute to an effective immunity. In contrast, liver of symptomatic dogs showed a non-organized and ineffective infiltrate of T cells and heavily parasitized Kupffer cells. DC - Dendritic cells; MØ – Macrophages; KC – Kupffer cell; Treg – regulatory T cell; Th1- T helper cell 1; Th2 –T helper cell 2; Th0- T helper cell 0 (naïve T cell); NO – nitric oxide; VL- visceral leishmaniasis; IL- interleukin; IFN - interferon; TNF- tumor necrosis factor.

Several studies recently conducted also helped to clarify the role of hepatocytes in the orchestration of liver's innate immune response in the context of *L. infantum* infected canine liver. Rodrigues *et al.* (2018) has recently contributed to the elucidation of the immune response generated by dog hepatocytes when exposed to *L. infantum.* These parasites presented a high tropism to hepatocytes, establishing strong membrane interactions with these cells. The possibility of *L. infantum* internalization by hepatocytes was raised, although not confirmed. Hepatocytes were able to recognize parasite presence, inducing pattern-recognition receptor (NOD1, NOD2, and TLR2) gene expression and generating a mixed pro- and anti-inflammatory cytokine response. Reduction of cytochrome P (CYPs) 450 s enzyme activity was also observed concomitant with the inflammatory response. The addition of leishmanicidal drug, mimicking treatment, increased NOD2, TLR4 and IL-10 gene expression, indicating immune modulation of liver microenvironment. There is evidence for the presence of *L. donovani* amastigotes within hepatocytes in liver biopsies from VL patients having undergone through successful therapy (Duarte *et al.* 1989). Gangneux *et al.* (2005) demonstrated *in vitro* that murine, rat and human primary hepatocytes were permissive to *L. donovani* promastigote infection, but parasites did not massively proliferate. Nevertheless, these findings bring into question a possible role for hepatocytes as a parasite reservoir, during host latent infection, redefining the role of hepatocytes in CanL and, consequently questioning their importance in the epidemiology of zoonotic visceral leishmaniasis (ZVL). Hepatocytes seem to have a

major role in coordinating liver's innate immune response against *L. infantum* infection, activating inflammatory mechanisms, but always balancing the inflammatory response in order to avoid cell damage.

Although hepatocytes seem to have a non-negligible role, the main target of *Leishmania* infection in the liver are Kupffer cells (KCs). These cells are the resident MØ population in the liver, located in the vasculature adherent to liver sinusoidal endothelial cells and directly exposed to the contents of blood circulating through the liver tissue. KCs express an array of scavenger receptors, TLR, complement receptors and antibody receptors, molecules that allow these cells to detect, bind and internalize pathogens. Expressing MHCI, MHCII and co-stimulatory molecules needed for T cell activation, KC are important APC. Furthermore, these receptors in part drive the activation of KC, which leads to production of cytokines and chemokines and allows KC to function as immune sentinels, alerting other components of the immune system to the presence of harmful microbes (Bilzer *et al*. 2006). KCs are also extremely effective in activating the invariant NKT (iNKT) cells that live and patrol the sinusoids of the liver, quickly controlling a potential infection (Jenne and Kubes 2013). Rodrigues *et al*. (2017b), investigated how canine KCs sense and react to the presence of *L. infantum* promastigotes and amastigotes by evaluating the gene expression of specific innate immune cell receptors and cytokines, as well as the induction of NO and urea production. In addition, the authors also assessed the impact of MG in infected KCs. These cells revealed to be susceptible to both parasite forms and no major differences were found in the immune response generated. *L. infantum* parasites seem to interact with KCs innate immune receptors and induce an anergic state, promoting immune tolerance and parasite survival. MG addition to infected KCs breaks the parasite-imposed silence and increases gene expression of TLR2 and TLR4, possibly activating downstream pathways. Understanding how KCs, sense and react to parasite presence, could bring new insights into the control or even elimination of CanL.

The delicate balance between immunity and tolerance in the liver, results directly from the complex interactions between the various resident immune cells and peripheral leukocyte populations. Under basal conditions, many liver resident cells (LSEC, KCs and DC) have a critical role, maintaining a state of immune unresponsiveness, accomplished, in part, by the low expression of MHC and the absence of co-stimulatory molecules. However, given an appropriate stimulation a robust immune response can be generated in the liver. The anatomical features, blood supply, diverse network of cells and the broad array of receptors enable the liver to act as a frontline immune sentinel. The role of the liver as an important innate immune organ in the context of ZVL and CanL has been growing, accumulating evidences that this organ is key in controlling parasite growth and dissemination to other organs. The liver may function as a safe harbor for *Leishmania* parasites to growth, due to its tolerant immune environment which may have a significant epidemic impact, not only in diagnosis, but also in treatment response and in possible relapses.

What do we know about the immune response of the dog submitted to CanL treatment? CanL classic treatments improve the dog's clinical condition, reducing parasite load on the skin and consequently the risk of transmission, but do not eliminate the

pathogen (João *et al*. 2006). The common relapses that occur when therapy is discontinued (Manna *et al*. 2009) justify the need to improve the efficiency of treatment protocols used for CanL. Some of those protocols include leishmanicidal drugs like MG (N-methylglucamine antimoniate) and miltefosine (1-O-hexadecylphosphocholine, MT), and leishmaniostatic drugs like allopurinol (Ap) (Frézard *et al*. 2009). MG is a pentavalent antimonial-based drug whose precise mechanism of action is not well understood, being considered a multifactorial drug with probable activity on the molecular processes of the parasite and influence in MØ parasiticidal activity (Frézard *et al*. 2009). MT is an alkylphosphocholine compound able to induce apoptosis by mechanisms still not entirely clear (Dorlo *et al*. 2012). Ap is a purine analog of adenosine nucleotide which blocks RNA synthesis, inhibiting *L. infantum* growth (Denerolle *et al*. 1999). MG in combination with Ap was considered the first line of treatment in Europe and MT plus Ap constituted the second line of treatment (Solano-Gallego *et al*. 2009). Nevertheless, with the rising of more reports of drug resistance that lead to either therapeutic failure, unresponsiveness or relapse, a reassessment of the usual therapies is imperative (Pérez-Victoria *et al*. 2006). Dog clinical signs tend to present type-2 T-helper (Th2) responses associated with the expression of IL-4, IL-5 and IL-6 along with higher levels of specific antibodies (Pinelli *et al*. 1999b; Santos-Gomes *et al*. 2002). On contrast, protective immunity is thought to depend upon a strong type-1 T-helper (Th1) response characterized by IL-2, IL-12, TNF-α and IFN-γ production. Furthermore, parasites may suppress host immunity by engaging regulatory T-cells (Treg) thus enabling the persistence of infection (Rodrigues *et al*. 2009), with higher expression of regulatory lymphokines (IL-10, TGF-β) (Alves *et al*. 2009). In our lab, we aim to understand how these most common treatments affect dogs' ability to develop a protective immune response or, if they elicit immune suppression of effector helper T cells, responsible for the orchestration of the immune response, and of cytotoxic T cells that cause the lysis of infected host cells.

For this, several studies are ongoing, namely the effect on cytokine mRNA expression and T-cell populations in the blood, lymph node and bone marrow of naturally infected dogs. Published results on cytokine expression (Santos *et al*. 2019) show that dogs under the MT+Ap protocol presented a protective Th1 response in all tissues, with the maintenance of a high expression of IFN-γ in all tissues, IL-2 in lymph node and TNF-α in bone marrow. This protocol was also able to restore the gene expression of most cytokines, recovering Th2 (IL-4 and IL-5) and Treg (IL-10 and TGF-β) cytokines to normal values. The MG+Ap protocol presented also a protective Th1 response, but not as pronounced as the MT+Ap. This protocol was also able to, not only, restore the Th2 and Treg to normal values, but also led to a suppression of Th2 and Treg cytokines in blood and of IL-4 and TGF-β in bone marrow beyond normal values. The results also show that changes in cytokine gene expression caused by *L. infantum* in sick dogs seem to be tissue specific, with different tissues presenting different cytokine profiles. Nevertheless, both treatments were able to normalize the cellular immune response and improve the clinical conditions in all dogs. With regard to T-cell populations, preliminary results show that sick dogs present specific immunophenotypes in the different organs analysed, agreeing with what was observed in cytokine expression. Sick dogs presented a predominant pro-inflammatory profile with increase in $CD8^+$ T cytotoxic cell populations. The administration of the treatments seems to cause a shift between

CD4 and CD8 cells, with a decrease of $CD8^+$ cells and increase in $CD4^+$ T helper cells, which associated with the increase in IFN-γ previously noted, promoted a Th1 protective response.

How the neglected feline leishmaniosis should be treated? Comparing to canine species, information about medical management of feline leishmaniosis is scarce and inconsistent. This is mainly explained by the small number of reported cases in the literature.

The European Advisory Board on Cat Diseases (ABCD) has reported in their guidelines that the medical management of feline leishmaniosis consists of long-term administration of Ap (10–20 mg/kg once or twice daily) (Pennisi *et al.* 2013). This treatment is usually effective. Information regarding the use of other drugs such as MG, domperidone and MT is scarce (Pennisi *et al.* 2015). Despite the fact that Ap is actually considered the first-line therapy in feline leishmaniosis, this compound can lead to an unpredictable and overlong response, and eventually several side effects.

Our group has published two clinical cases of feline leishmaniosis, in which Ap therapy did not allow a good clinical management of the disease, and thus required the use of alternative compounds. The first case described a 2-year-old cat with a cutaneous presentatidn of feline leishmaniosis, diagnosed on skin biopsies (Basso *et al.* 2016). In this case, Ap was firstly started (Zyloric, Allopurinol, 10 mg/kg, *per os*, twice daily, FaesFarma). Two weeks apart, as no improvement has been remarked on the physical exam, MG was added to the therapeutic protocol (Glucantime, 50 mg/kg once daily, subcutaneously, for 30 days, Boehringer Ingelheim Animal Health). This combined therapy, allowed a rapid improvement of the dermatological signs without any side effect reported. No relapse occurred in the following 24 months (date of the last control). The second case reported an unusual presentation of inspiratory dyspnea and stertor in a 12-year-old cat, at which a granulomatous rhinitis secondary to feline leishmaniosis was diagnosed (Leal *et al.* 2018). In opposition to previously reported cases, no cutaneous lesions were detected in this cat prior to diagnosis, which was established by nasal biopsies. Ap was started (10 mg/kg, *per os*, twice daily) but five days later, a cutaneous adverse drug reaction was strongly suspected, leading to a discontinuation of this compound. MG was then prescribed (50 mg/kg once daily subcutaneously) but three weeks apart, the cat developed acute kidney injury, presumably induced by this drug. Considering this side effect, this drug was also discontinued, and the cat was subsequently treated with nucleotides and active hexose correlated compounds (Impromune, 1/2 tablet once daily, Bioiberica). A relapse of granulomatous rhinitis was suspected 4 months after the onset of this alternative therapy and MT was started (Milteforan, 2 mg/kg, *per os*, once daily, Virbac). Although there was a transitory worsening of azotemia, the cat progressively improved showing stable clinical signs with no relapse of feline leishmaniosis, 16 months apart (date of the last control).

Overall, these two cases contributed to increase the number of reported cases of feline leishmaniosis, highlighting the relevance of continuous clinical and laboratory evaluation. These cases also support that individual response is unpredictable and medical standard therapy should be adapted in a case-by-case scenario.

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Vectors and Vector Borne Diseases: Morphological and Molecular Diagnosis, Risk Assessment, Population Genetics and Control Strategies

D. W. Ramilo[1], A. M. Alho[1], J. Gomes[1,2], M. Santos[1], A. S. Santos[3,7], M. Santos-Silva[3,7], G. Alexandre-Pires[1], J. Meireles[1], A. Tomás[4], S. Zúquete[1], A. Amaro[2], S. Belo[5], M. Schnyder[6], P. Deplazes[6], M. T. Rebelo[4], L. Madeira-de-Carvalho[1], and I. Pereira-da-Fonseca[1](✉)

[1] Parasitology and Parasitological Diseases Laboratory (LPDP), Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
ifonseca@fmv.ulisboa.pt
[2] National Institute for Agrarian and Veterinary Research, Oeiras, Portugal
[3] Centre for Vectors and Infectious Disease Research Dr. Francisco Cambournac, National Institute of Health Dr. Ricardo Jorge (CEVDI/INSA), Águas de Moura, Portugal
[4] Entomology Laboratory, Department of Animal Biology, Center for Environmental and Marine Studies (CESAM), Faculty of Sciences of University of Lisbon, Lisbon, Portugal
[5] Global Health and Tropical Medicine, GHTM, Instituto de Higiene e Medicina Tropical, IHMT, Universidade Nova de Lisboa, UNL, Lisbon, Portugal
[6] Institute of Parasitology, Vetsuisse Faculty, University of Zürich, Zurich, Switzerland
[7] Environmental Health Institute (ISAMB), Faculty of Medicine, University of Lisbon, Lisbon, Portugal

Abstract. Vector-borne diseases are transmitted through arthropods and new associations between them and pathogens are continually being described. In the present study, authors briefly address their research areas, reporting multiple collaboration studies and major findings achieved by the CIISA's Parasitology and Parasitic Diseases team and its partners over the past few years. Starting with *Culicoides,* their importance as vectors of animal/human diseases, the description of new species and also species modelling as disease surveillance is discussed. Studies on national lice species in wild birds raise awareness to a so far neglected group, evincing known records and new contributions towards a better knowledge. Tick and tick-borne pathogen studies over the years have contributed to a national portrait of species geographic and seasonal distribution and to pathogen endemic associations, describing most common public and veterinary health threats. In this regard, morphology identification strategies are enounced and molecular markers used considered. Due to a reported rise in prevalence, piroplasms impact is

D. W. Ramilo and A. M. Alho---These authors contributed equally for this work.
M. Santos-Silva---In memoriam.

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particularly focused as a major concern for cattle production industry sector. A national overview concerning *Theileria* and *Babesia* prevalence inferences and diagnosis specificities are reviewed. Ultimately, a national *Dirofilaria immitis* and *Angiostrongylus vasorum* epidemiological update is given while diagnosis and treatment options are debated.

Keywords: VBDs · Diagnosis · Risk-assessment · Control · Portugal

Vector-borne diseases are raising awareness throughout the globe due to their devastating repercussions in human and animal health. Known modern challenges as environment, socio-economy and governmental policies are by themselves changing the distribution and the impact of such diseases. Prevention and disease control are dependent on distribution and abundance as well as on population dynamics knowledge. At CIISA´s Parasitology and Parasitic Diseases Laboratory (LPDP) different studies are integrated in multidisciplinary studies that continuously contribute to a national overview of the most important vectors and pathogens of animal and also human concern.

Culicoides **Midges in Portugal: What's New in the Last Decade?** *Culicoides* (Diptera: Ceratopogonidae) genus contains important vectors of animal and human diseases including Bluetongue Disease (BTD), African Horse Sickness, Schmallenberg and filariosis (Linley *et al.* 1983; Mellor *et al*. 1990; Balenghien *et al*. 2014). The species *Culicoides imicola* is the main vector of BTD in Southern Europe and Africa (Wilson and Mellor 2009). Before 1998, BTD occurred sporadically in Europe (Barros *et al.* 2007; Wilson and Mellor 2009). However, since 1998 the scenario changed drastically, with the disease affecting several Southern European countries (1998–2005) and also Central and Northern Europe between 2006–08 (Wilson and Mellor 2009; Stemberg Lewerin *et al.* 2010). The occurrence of the disease in regions where *Culicoides imicola* was absent as in Central and Northern Europe is explained by the presence of Obsoletus group species and *C. pulicaris*, which gained the capacity to acquire, maintain and transmit Bluetongue virus (BTV) to susceptible hosts (Vanbinst *et al.* 2009).

The first incursion of BTV in mainland Portugal was reported in July 1956 (Barros *et al*. 2007). In 4 years, around 180,000 sheep were killed due to BTV serotype 10 (BTV-10) (Barros *et al.* 2007; Office International des Epizooties 2018). In 1960, the country was declared free of BTV (Barros *et al*. 2007). A new introduction of BTV (BTV-4) in mainland Portugal was registered in November 2004, after a 44-year period of epizootic silence. BTV-1 outbreaks have occurred several times in the last decade in mainland Portugal (2007–12 and 2015–17), as well as BTV-4 (2004–06, 2013 and 2018) (Ramilo *et al.* 2018).

Between 2005–2013, the Portuguese National Authority for Animal Health (DGAV) in partnership with the Faculty of Veterinary Medicine established a National Entomologic Surveillance Program (NESP) for BTD in recognition of the high and continuing threat to the Portuguese livestock sector from *Culicoides*-borne viruses (Ramilo *et al.* 2012). NESP was created to better perceive the distribution of different *Culicoides* species, directly reporting to the DGAV the presence of BTD vectors, allowing them to act in real time and implement the necessary measures to prevent the spread of the disease in case of an outbreak. NESP covered all Portuguese regions, including mainland

Portugal, Azores and Madeira archipelagos (Ramilo *et al*. 2012; Ribeiro *et al*. 2015). The NESP identified for the first time in mainland Portugal and Azores archipelago a total of 23 *Culicoides* species including the description of a new species, *Culicoides paradoxalis* Ramilo and Delécolle 2013 (Ramilo *et al.* 2013, 2018) (Fig. 1). The new species identification, resulted from a collaboration with the Faculty of Sciences of the University of Lisbon (FCUL), the Centre de Coopération Internationale en Recherche Agronomique pour le Développment (CIRAD, France) and the University of Strasbourg, France.

After 2013, several works have been performed with other institutions (CIRAD, University of Strasbourg and FCUL) in order to identify *Culicoides* specimens using morphological and molecular biology techniques and to better understand *Culicoides imicola* distribution and its geographic origin (Jacquet *et al*. 2015, 2016). Furthermore, our group participated in VECTORNET Project – *Culicoides* group (2014–18) capturing

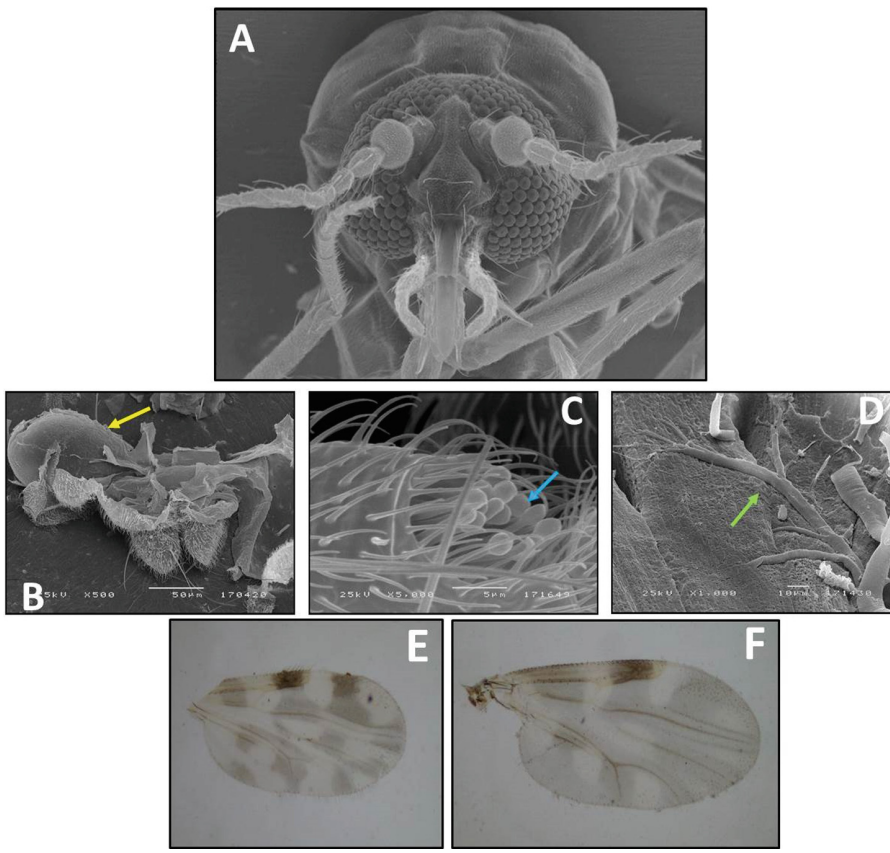

Fig. 1. A - *C. paradoxalis* a new species for science; B – *C. circumscriptus* spermatheca (yellow arrow); C – Sensorial organs from the 3rd palpus segment of *C. obsoletus* (blue arrow); D – Vessels that supply thorax muscles with nutrients (green arrows); E – Wing pattern of *C. imicola*; F – Wing pattern of Obsoletus group species.

Culicoides near horse farms comparing species caught with those captured near domestic ruminants (Ramilo 2016); identifying *Culicoides* species from different European countries (Switzerland, Iceland, Latvia, Denmark and Norway) and providing laboratorial training to international researchers who needed to identify *Culicoides* specimens captured in their country. Such works highlight the importance of this field of study and the international recognition of the work developed at our group by our partners.

In 2016, our group organized the E-SOVE Congress, related with vector and vector-borne diseases. It was held at the Faculty of Veterinary Medicine, University of Lisbon, and brought together several international teams of different research areas, including those working with *Culicoides.* During this congress, a training course concerning *Culicoides* capture and morphological identification was attended by international researchers featuring LPDP team capacity to deliver highly specific training to peers within this research area.

With all the data collected during NESP, our team produced a PhD thesis (Ramilo 2016), a book chapter (Alexandre-Pires *et al.* 2010), a revision article (Ramilo *et al.* 2018), several scientific articles, some of them in collaboration with international teams (Ramilo *et al.* 2012, 2013, 2017; Ribeiro *et al.* 2015; Jacquet *et al.* 2015, 2016), 22 oral and 15 poster presentations. Furthermore, an identification key for *Culicoides* species present in Portugal is also provided online for researchers and students interested in this field of study (http://www.fmv.ulisboa.pt/uploads/2017/11/5a0ac47a19090.pdf).

Presently, several works are being developed at our laboratory concerning biting midges: traps are being placed near wild animals in a zoological context to know *Culicoides* fauna present; identification studies of Obsoletus group specimens with intermediate morphological characteristics (between *C. obsoletus* and *C. scoticus* species) to assert their phylogenetic position; characterizing the distribution of Obsoletus group species in mainland Portugal; capture of live *Culicoides* specimens to evaluate the wing interference pattern of different species; audio recording of *Culicoides* wing movement to evaluate differences between species and identification of *Culicoides* aberrant structures to proceed with correct identification of *Culicoides* species even in the presence of these anatomical modifications.

Overview of Chewing Lice of Wild Birds in Portugal: A Neglected Group of Ectoparasites. Chewing lice or Mallophaga, are the most common ectoparasites found in birds. Avian chewing lice belong to one of two suborders, Amblycera or Ischnocera, being the latter the most representative (Price *et al.* 2003). Morphologically, chewing lice are characterized by a small size (≤0,8–11 mm long), a segmented body dorsoventrally flattened and segmented antennae (3–5 segments). All chewing lice of birds are obligate and permanent ectoparasites, feeding mainly on feathers and skin with the entire life cycle occurring on hosts (Clayton *et al.* 2008). Some species of Amblycera suborder include blood in their diet by scratching or nibbling at the soft skin at the base of the host feathers (Ash 1960). Physical contact between birds is considered the major route of transmission of Mallophaga species. So, being more effective between copulating birds and between parents and their offspring (Hillgarth 1996; Tompkins *et al.* 1996). According to Keirans (1975) ischnoceran species may be transported by phoresis on hippoboscid flies. Generally, chewing lice are highly specific to their hosts at a genus or species level (Clayton *et al.* 2008). However, some mallophagan species

are less specific, and according to Clayton *et al.* (2004) that can be partly explained by the non-host specificity of hippoboscid flies.

In Portugal, the number of louse species documented from wild birds is limited. The first document concerning endemic chewing lice of wild birds was published by Tendeiro (1962), with a monographic review of the genus *Columbicola* Ewing, 1929 collected not only in Portugal, but worldwide. More recently, Literák *et al*. (2015), focusing on chewing lice parasitizing blackcaps (*Sylvia atricapilla*) in the Azores, recorded two new chewing louse species in Portugal: *Brueelia tovornikae* (Balát 1981), currently named as *Guinaraesiella tovornikae* (Balát 1981) and *Myrsidea sylviae* Sychra and Literak, 2008. Tomás *et al.* (2016) published a more complete study of chewing lice in Portugal. These authors sampled wild birds of different genus admitted at the Wildlife Rehabilitation and Investigation Centre of Ria Formosa –ALDEIA Association (RIAS/ALDEIA) and also from mist-nets captured animals during scientific ringing sessions performed at the Ria Formosa Natural Park. This work registered 18 louse species for the first time in Portugal, including a nymph of the genus *Strigiphilus* Mjöberg, 1910 collected from a Eurasian Eagle-Owl (*Bubo bubo*) (Table 1).

When present in small numbers a variety of behavior defenses, with preening as the most important, helps hosts to tolerate infestation with no apparent effects. Large infestations, however can cause dermatitis and pruritus leading to feather and skin damage (Johnson and Clayton 2003; Clayton *et al*. 2008). According to Booth *et al*. (1993), the dramatic increase of lice feeding on feathers reduces the abdominal plumage, increasing thermal conductance and respective metabolic rates required to maintain body temperature. The maintenance of elevated metabolic rates induces birds to use their fat reserves causing a chronic decline in body mass. In the specific case of the louse *Machaerilaemus malleus* (Burm 1838), Kose *et al*. (1999) showed that Barn Swallows (*Hirundo rustica*) males with large white tail spots without parasite damage are selected by females for displaying reliable quality feathers. Furthermore, it is believed that the holes caused by the parasites in the spots may increase feathers breakage and permeability, changing the aerodynamic efficiency of the flight, as occurs with birds parasitized by mites (Bonser 2001).

Chewing lice can have effects at population level, as recorded by Samuel *et al*. (1982). The authors reported severe hemorrhagic ulcerative stomatitis of juvenile American White Pelicans (*Pelecanus erythrorhynchus*), parasitized by amblycera *Piagetiella peralis* (Leidy 1878) and some birds were found dead. However, it is not clear whether lice were the cause of death. Historically, it has been thought that chewing lice fed very occasionally on host blood, i.e., they were ignored as potential vectors or intermediate hosts of other parasites. However, a better knowledge of Mallophaga feeding habits allowed to understand their indirect effects on hosts, as a detailed reviewed by Saxena *et al*. (1985) has shown. For example, the amblyceran *Pseudomenopon pilosum*, common lice of Rallidae birds, already identified in Portugal by Tomás *et al*. (2016), transmits the filarid *Pelecitus fulicaeatrae* (Diesing 1861) to *Fulica americana* (Bartlett and Anderson 1987). The authors found adult worms among tendons at the ankle joint and microfilariae in the skin of the feathered portions of the legs of infected birds, as well as microfilariae, first-, second- and third-stage of this nematode in abdomen of lice, suggesting that *P. pilosum* transmits the filarial *P. fulicaeatrae* when the louse takes

Table 1. Reports of chewing lice species in wild avian hosts from Portugal.

Avian host	Louse species	References
ACCIPITRIFORMES		
Bonelli's Eagle (*Aquila fasciata*)	*Degeeriella fulva* (Giebel 1874)	Tomás *et al.* 2016
Common Buzzard (*Buteo buteo*)	*Degeeriella fulva* (Giebel 1874)	Tomás *et al.* 2016
	Craspedorrhynchus platystomus (Burm. 1838)	Tomás *et al.* 2016
	Laemobothrion (*L.*) *maximum* (Scopoli 1763)	Tomás *et al.* 2016
Eurasian Griffon (*Gyps fulvus*)	*Falcolipeurus quadripustulatus* (Burm. 1838)	Tomás *et al.* 2016
	Laemobothrion (*L.*) *vulturis* (Fabricius [J.C.] 1775)	Tomás *et al.* 2016
	Colpocephalum turbinatum Denny 1842	Tomás *et al.* 2016
	Nosopon casteli Tendeiro 1959	Tomás *et al.* 2016
ANSERIFORMES		
Common Teal (*Anas crecca*)	*Trinoton querquedulae* (L., 1758)	Tomás *et al.* 2016
CHARADRIIFORMES		
Yellow-legged Gull (*Larus michahellis*)	*Actornithophilus piceus lari* (Packard 1870)	Tomás *et al.* 2016
Dunlin (*Calidris alpina*)	*Actornithophilus umbrinus* (Burm. 1838)	Tomás *et al.* 2016
	Lunaceps schismatus Gustafsson and Olsson 2012	Tomás *et al.* 2016
	Austromenopon lutescens (Burm. 1838)	Tomás *et al.* 2016
COLUMBIFORMES		
Common Wood-Pigeon (*Columba palumbus*)	*Columbicola claviformis* (Denny 1842)	Tendeiro 1962
Rock Pigeon (*Columba livia*)	*Columbicola columbae* (L., 1758)	Tendeiro 1962
GRUIFORMES		
Common Coot (*Fulica atra*)	*Pseudomenopon pilosum* (Scopoli 1763)	Tomás *et al.* 2016

(*continued*)

Table 1. (*continued*)

Avian host	Louse species	References
PASSERIFORMES		
Blackcap (*Sylvia atricapilla*)	*Guinaraesiella tovornikae (Balát 1981)*	Literák *et al.* 2015
	Myrsidea sylviae Sychra and Literak, 2008	Literák *et al.* 2015
PHOENICOPTERIFORMES		
Greater Flamingo (*Phoenicopterus roseus*)	*Colpocephalum heterosoma* Piaget 1880	Tomás *et al.* 2016
	Trinoton femoratum Piaget 1880	Tomás *et al.* 2016
STRIGIFORMES		
Eurasian Eagle-Owl (*Bubo bubo*)	*Strigiphilus* sp. Mjöberg 1910	Tomás *et al.* 2016
SULIFORMES		
Northern Gannet (*Morus bassanus*)	*Pectinopygus bassani* (Fabricious [O.] 1780)	Tomás *et al.* 2016
	Eidmanniella pustulosa (Nitzsch [In Giebel] 1866)	Tomás *et al.* 2016

a skin feeding. Mallophagan vectors are not only associated with skin microfilariae. Seegar *et al.* (1976) reported that Tundra swan (*Cygnus columbianus*) is infected by common heartworm, *Sarconema eurycera* Wehr 1939, when amblyceran louse *Trinoton anserium* (Fabricius [J.C.] 1805) takes a bloodmeal. On the other hand, ischnoceran lice were associated with *Eulimdana* species, filarid nematodes of the Charadriiformes neck. According to Bartlett (1993) Mallophaga species, such as *Carduiceps clayae* Timmer 1954 and *Lunaceps numenii numenii* (Denny 1842), act as intermediate host and transmit these worms when they are ingested during bird preening. Bacteria and viruses have also been isolated experimentally from chewing lice as summarized by Saxena *et al.* (1985). For example, the virulent bacteria *Pasteurella multocida*, the pathogen responsible for epidemic outbreaks of fowl cholera, were found in the digestive tract and feces of *Menopon gallinae* (L., 1758) and *Menacanthus stramineus* (Nitzch 1818) (previously named as *Eomenacanthus stramineus*) (Derylo 1970). The author suggested "transmission was thought to be due to direct contamination of a wound with feces or by ingestion of the infected louse" by the Red Junglefowl (*Gallus gallus*). In the case of the role played by mallophagans in viruses transmission, Howitt *et al.* (1948) isolated the Eastern equine Encephalitis virus from fowl lice *Menacanthus stramineus*, suggesting that these and other species of chewing lice, with blood feeding habits, may be important vectors of the virus among birds, especially in the absence of other vectors. However, these authors' assumptions about the lice role in transmission of viruses and bacteria are not totally clear.

Ticks and Tick-Borne Disease with National Relevance. Ticks are ectoparasites that feed exclusively on blood, parasitizing virtually all terrestrial and semi-aquatic vertebrates across the zoogeographic regions of the world. Three living tick families are described: Argasidae, Nutalliellidae and Ixodidae. The latter is the most studied and has the greater number of species with veterinary and medical impact. In Portugal, the last comprehensive revision of the family Ixodidae Murray 1877, dates from 2011 including all the available information gathered on nationwide but also more directed surveys (Santos-Silva *et al*. 2011). Subsequent publications have contributed to further increase the knowledge of the Portuguese tick fauna (Norte *et al*. 2012; Estrada-Peña *et al.* 2014). Presently, it is recognized the occurrence of 21 species of ixodid ticks, distributed by five genera (Table 2). Some of these ticks are host specific, mostly associated to wild animals and generally poorly studied, as is the case of *Haemaphysalis hispanica*, *H. inermis*, *Ixodes acuminatus*, *I. frontalis*, *I. simplex*, *I. vespertilionis* (Santos-Silva *et al.* 2011). Other species were more recently described in Portugal and their impact as pests or vectors is still unknown, such as *Ixodes arboricola* and *I. inopinatus* (Norte *et al.* 2012; Estrada-Peña *et al*. 2014). In any case, the majority of the tick species occurring in our country are commonly found feeding on livestock, companion animals and humans, having a considerable veterinary and public health impact. They can cause anemia due to blood depletion observed in high-rates of tick parasitism, skin secondary infections and injury, anaphylactic and toxic reactions against arthropod´s saliva, but most of all, ticks are responsible for the inoculation of pathogenic agents. During the years, several tick-borne agents have been described in questing ticks collected across the country suggesting a potential vector role for some Portuguese species. Table 2 lists the agents that have been detected in questing ticks in Portugal. Most of these agents are bacteria exclusively vectored by ixodid ticks and belonging to Spotted Fever Group *Rickettsia*, *Borrelia burgdorferi* sensu lato (s.l.), and Anaplasmatacea, although other bacteria, protozoa and virus have also been described. For some of these but also for other tick-borne agents, a medical role was suggested by the detection of hosts harboring infected feeding ticks or presenting specific antibodies that could potentially be linked to agents' exposure (Amaro *et al*. 2017). Moreover, autochthonous human clinical cases were already reported for the tick-borne *Babesia divergens*, *Borrelia burgdorferi* s.l., *Rickettsia conorii*, *R. sibirica* mongolotimonae and *R. slovaca* (Bacellar *et al.* 2003; Centeno-Lima *et al.* 2003; Da Franca *et al.* 2005; De Sousa *et al.* 2003, 2006, 2013; Lopes de Carvalho *et al.* 2006). Regarding companion animal, infections by *Anaplasma platys*, *B. burgdorferi* s.l., *Ehrlichia canis*, *R. conorii*, *Cytauxzoon* sp., *Hepatozoon canis*, *Babesia canis* and *B. vulpes* were detected in symptomatic cases (Alexandre *et al*. 2008; Alexandre *et al*. 2011; Cardoso *et al*. 2008; Santos *et al*. 2009; Simões *et al*. 2011; Maia *et al*. 2015; Alho *et al*. 2016b, 2016f). In livestock industry, the circulation of several tick-borne agents has also been reported, including *Babesia bovis*, *B. bigemina*, *B. divergens*, *Theileria annulata* and *T. buffeli* (Brígido *et al*. 2004; Gomes *et al*. 2013) and LPDP team co-authored detection guidelines for these agents (Lempereur *et al.* 2017; Portillo *et al*. 2017; Silaghi *et al.* 2017).

As for other arthropod vectors, proper species identification is extremely important in the study of ticks. The taxonomic identification has been traditionally supported by

Table 2. List of portuguese ticks species and the agents that have been found in questing ticks collected across the country

Tick	Agents' Order/Species				
Genus/Species	Bunyavirales[a]	Rickettsiales[b]	Legionellales[c]	Spirochaetales[d]	Piroplasmida[e]
Dermacentor					
D. marginatus	*Phlebovirus* sp.	*Anaplasma marginale; Rickettsia slovaca; Rickettsia raoulti*	*Coxiella burnetii*; *Francisella tularensis* subsp. Holarctica (Fth)	*Borrelia lusitaniae*	
D. reticulatus		*R. slovaca*	*F. tularensis* (Fth); *Francisella*-like endosymbiont		
Haemaphysalis					
H. hispanica					
H. inermis					
H. punctata				Relapsing Fever-like *Borrelia* sp.	
Hyalomma					
H. lusitanicum		*Anaplasma platys*; *Candidatus* Midichloria mitochondrii	*C. burnetii*		
H. marginatum				*B. lusitaniae*	
Ixodes					
I. acuminatus					
I. arboricola					

(*continued*)

Table 2. (*continued*)

Tick	Agents' Order/Species				
Genus/Species	Bunyavirales[a]	Rickettsiales[b]	Legionellales[c]	Spirochaetales[d]	Piroplasmida[e]
I. canisuga					
I. frontalis			*C. burnetii*		
I. hexagonus			*Francisella*-like endosymbiont		
I. inopinatus					
I. ricinus		*Anaplasma phagocytophilum; A. marginale; Rickettsia helvetica; Rickettsia monacensis*	*C. burnetii*; *F. tularensis* (Fth)	*Borrelia afzelii; Borrelia garinii; Borrelia burgdorferi* s.s.; *B. lusitaniae; Borrelia miyamotoi*; *Borrelia valaisiana*	
I. simplex					
I. ventalloi		*A. phagocytophilum; A. marginale*; *Candidatus* Neoehrlichia mikurensis-like; *R. helvetica*	*C. burnetii*		*Theileria annulata*
I. vespertilionis					
Rhipicephalus					
R. annulatus					
R. bursa	*Phlebovirus* sp.	*A. marginale*			
R. pusillus		*Rickettsia sibirica mongolitimonae*			

(*continued*)

Table 2. (*continued*)

Tick	Agents' Order/Species				
Genus/Species	Bunyavirales[a]	Rickettsiales[b]	Legionellales[c]	Spirochaetales[d]	Piroplasmida[e]
R. sanguineus s.l.	*Phlebovirus* sp.	*A. marginale; Rickettsia conorii; Rickettsia massiliae*	*F. tularensis* (Fth)	Relapsing Fever-like *Borrelia* sp.	

[a]Pereira *et al*. 2017;
[b]Antunes *et al*. 2016; Bacellar *et al*. 1995a, b; Ferrolho *et al*. 2016; Milhano *et al*. 2010; REVIVE 2014, 2017; Santos *et al*. 2004; Santos-Silva *et al*. 2017; Santos *et al*. 2018;
[c]Lopes de Carvalho *et al*. 2016; Santos-Silva *et al*. 2017; Santos *et al*. 2018;
[d]Núncio *et al*. 1993; Matuschka *et al*. 1998; De Michelis *et al*. 2000; Baptista *et al*. 2004; Nunes *et al*. 2015, 2016;
[e]Antunes *et al*. 2016

the observation of particular morphological features that are regarded as diagnostic characters and constitute the basis of ticks' dichotomous identification keys (examples of these features are the shape and size of the base of the *capituli*, the presence/absence and shape of spurs, *auriculae* and *cornua*). The classic classification should be undertaken through careful morphological features identification before any further study. Recently, in a comparative blind test study performed by several European tick experts, it was possible to ascertain that if the researcher was not familiar with a given species, it might be misidentified (Estrada-Peña *et al*. 2017). This elucidates the need to promote adequate training for experts in tick identification. The molecular analysis done upon stable identification of ticks complement this process and can be based in the study of conserved and moderately conserved genes, as the mitochondrial 12S and 16S rRNA or others such as the second internal transcribed rDNA spacer (ITS2) (Estrada-Peña *et al.* 2017; Santos-Silva *et al*. 2017; Santos *et al.* 2018; Sanches *et al.* 2018). This integrated approach of identification is highlighting a genetic heterogeneity of some tick populations that in some instances is questioning species definition, as in the case of *Rhipicephalus sanguineus/R. turanicus*, *I. ricinus/I. inopinatus* and possibly *I. frontalis* (Santos-Silva *et al*. 2011; Estrada-Peña *et al*. 2014; Santos *et al*. 2018). Differences in vector competence are also discussed for distinct genotypic lineages of a given species, like the case of *R. sanguineus* concerning *E. canis* transmission (Sanches *et al*. 2018). Thus, particular attention is devoted to mega vector *I. ricinus* and to the characterization of its population's genetics and the differences in vector potential of distinct genetic lineages. In this regard, the Portuguese Foundation for Science and Technology has recently financed a project entitled "The *Ixodes ricinus* group of ticks in the western Mediterranean region and North Africa: new insights into their population genetics and microbiome fauna – TickGenoMi (ref PTDC/SAU-PAR/28947/2017)". This project combines the taxonomic issues regarding interspecific diversity and potential gene flow among populations of *I. ricinus/I. inopinatus* with the study of their microbial community.

An Update on Cattle Piroplasmosis. Belonging to phylum Apicomplexa, order Piroplasmida, *Babesia* and *Theileria* parasites are responsible for important diseases with great economic and social impact (Castro 1997; Gubbels 1999). Protozoan parasites of genus *Babesia* are responsible for bovine babesiosis, with *B. bovis* and *B. bigemina* being the most common and studied species that affect cattle worldwide, with *B. bovis* causing more severe disease than *B. bigemina*. *B. divergens* also infects cattle but, unlike the others, it has a zoonotic potential and is one of the species responsible for human babesiosis. Bovine babesiosis has had a huge economic impact, causing losses in beef production due to animal disease and death. There are also other associated costs such as the high cost of tick control, disease detection, prevention and treatment (Yusuf 2017). *Theileria* species are responsible for Bovine theileriosis which is caused by several *Theileria* species such as *T. parva*, responsible for East Coast fever in Africa and *T. annulata*, that causes Tropical Theileriosis, which are the most pathogenic and economically important species. Tropical Theileriosis is widely distributed in the Mediterranean basin, including southern Europe and northern Africa, and also many countries from the Middle East and Asia, such as India and China. Animals that survive the acute disease usually become chronic carriers and play an important role as a reservoir for the maintenance of both parasites' life cycle (Garcia-Sanmartin *et al*. 2006).

Different studies confirmed the occurrence of *Babesia* and *Theileria* parasites in Portugal (Brígido *et al.* 2004; Silva *et al.* 2010). However, for a broader picture of piroplasms' prevalence and distribution in Portugal, an epidemiological survey was conducted to assess the *Theileria* and *Babesia* species infecting bovines in mainland Portugal (Gomes *et al.* 2013). The global prevalence of piroplasm-infected animals was 36.8%, although significant differences were found between the north and south of the country. A higher prevalence was found in the southern regions, with 42.4% infected animals in Lisbon and 51.6% in Alentejo. These differences are certainly related to the distinct vegetation and climatic characteristics of those regions, influencing vector tick distribution and abundance across the country and, as a result, the incidence of piroplasmosis. Different livestock management systems amongst regions can also influence the exposure of animals to vector ticks. *Theileria annulata* was the most frequently found species, with a national prevalence of 21.3%, with a higher regional value in Lisbon (33.5%) and Alentejo (29.2%). The majority of reported clinical cases occur in young calves or imported animals, therefore, a state of endemic stability is presumed to occur. In the northern part of the country, where infected animals and vector ticks are also known to co-exist, the disease is less common and tick challenge is presumed to be relatively lower. *Theileria orientalis* was the second most prevalent species found, with 10.1% infected animals, but there are no reports of clinical cases in Portugal due to this species, supporting the idea that it is non-pathogenic to bovines. Since several *T. orientalis* genotypes are reported to be pathogenic to cattle, this parasite's population should be studied to assess its role in cattle's health (Kamau 2011). A low prevalence was detected for *Babesia* infections (7.9%), with *B. bigemina* being the most frequently found species. *B. bovis*, which is usually considered the most pathogenic bovine *Babesia* was absent. *B. divergens* was detected in one animal from Alentejo region (Gomes *et al.* 2013).

Given the potential threat of cattle piroplasmosis to the livestock industry, an enhanced awareness of the epidemiological traits of all of different species is essential for assessing exposure risks and a better planning of prophylactic and control measures. Therefore, the improvement of diagnostic techniques and development of new methods are fundamental to the control of all tick-borne pathogens. Traditionally, detection of *Theileria* and *Babesia* pathogens in infected animals is accomplished by microscopic examination of stained blood smears, which have low sensitivity for the assessment of carrier animals, in which low numbers of erythrocytes remain infected (Altay et al. 2008). Although serological methods can be employed to diagnose subclinical infections, cross-reactions are common and current infections and previous exposures are generally not distinguished, moreover, antibodies tend to disappear in long-term carriers (Altay *et al.* 2008; García-Sanmartín *et al.* 2006). Different molecular diagnostic-based assays have also been developed, some of which have proven to be effective in detecting piroplasm infections in carrier animals. The reverse line blotting (RLB) assay based on the amplification of the hyper-variable V4 region of the 18S rDNA gene of *Theileria* and *Babesia* parasites and reverse hybridization of the products with species-specific oligonucleotide probes is currently considered to be the most sensitive test for detecting these parasites (Gubbels *et al.* 1999; Georges *et al.* 2001; Bilgic *et al.* 2010). Nevertheless, since RLB is a relatively cumbersome assay, it is not entirely suitable for use in the routine diagnosis

of piroplasm infections. Therefore, different PCR-based assays have also been described for detecting all the diversity of *Theileria* and *Babesia* parasites (d'Oliveira *et al*. 1995; Criado-Fornelio *et al*. 2009). Focusing in the most prevalent species infecting bovines in Portugal, a study was developed to review the *in silico* efficiency of previously described primers, to design new primers for amplifying the *T. annulata* species-specific Tams1-encoding gene and to develop and validate an efficient real-time PCR assay for detecting this protozoan in bovine blood samples (Santos *et al*. 2013). This real-time PCR assay proved to be 100% specific taking the RLB as gold standard.

The studies clearly demonstrated that the reassessment of all primers and probes used in molecular diagnostic assays for targeting pathogen genes should be performed periodically, as novel relevant nucleotide sequences become available from public databases. This was demonstrated by the design of an updated primer set targeting more conserved regions of the Tams1 gene of *T. annulata* (Santos *et al*. 2013). Besides these studies, other methods are being developed such as the Loop mediated isothermal amplification molecular assays. For the specific detection of *T. annulata* in bovine blood samples by targeting the Tams1 gene, a LAMP assay was developed, and a field study was performed where the Tams1-targeted assay was validated using a large set of blood samples collected from cattle in a theileriosis endemic area at southern Portugal and the real-time PCR was used to estimate parasitaemia in naturally infected cattle (Gomes *et al*. 2017). The overall sensitivity of the LAMP assay was estimated as 90%, with negative and positive predictive values of 87% and 100%, respectively, in agreement with the real-time PCR results. The LAMP assay was able to detect *T. annulata* DNA in blood samples with a very low parasitaemia, with a value of 0.00026% considered as the detection limit of this assay. None of the animals in this study showed clinical signs of disease and were considered healthy carriers, which in natural infection usually result in parasitaemias of approximately 0.1 to 0.01% in carrier animals (Aktas *et al*. 2006). When analysing the real-time PCR results and the correlated parasitaemia, the values correspond to a parasitaemia with a mean value of 0.009%, which are difficult to detect by microscopy of blood smears. These studies demonstrate the usefulness of diagnostic methods in the evaluation of different aspects of an important tick-borne disease of cattle.

Theileriosis importance is becoming increasingly recognised in Portugal and, since epidemiological studies have shown a rise in prevalence in the south (Branco *et al*. 2010; Silva *et al*. 2010; Gomes *et al*. 2013), a study was designed to assert *T. annulata* population genetics' with the goal of investigating the genetic diversity of this parasite in Portugal and to perform a comparative analysis with the data available from Tunisia and Turkey (Gomes et al. 2016). This parasite has considerable levels of genetic diversity within its population, as shown by different studies using a variety of serological and molecular methods (Shiels *et al*. 1986; Ben-Miled *et al*. 1994; Weir *et al*. 2007). For the Portuguese cattle population studies, samples were collected from twelve farms at four distinct regions. The panel of micro- and mini-satellites was previously designed to investigate the structure of parasite population in relation to the different geographical regions, but also to evaluate the multiplicity of infection of *T. annulata* genotypes in the cattle hosts (Weir *et al*. 2011). The results show that the parasite population is highly diverse, similar to what happens in other endemic countries and there is some genetic differentiation between geographically separated populations of *T. annulata*. A

moderate level of differentiation was detected between Portugal and Tunisia and between Portugal and Turkey. While Portugal does not experience the same problem of tropical theileriosis in comparison to other Mediterranean countries, it is clear that the extent of genetic diversity of *T. annulata* is similar to countries where the disease represents one of the principal constraints to cattle production. Population genetics' information may provide important baseline data upon which suitable Theileriosis control policies may be developed in the near future.

***Dirofilaria immitis* vs. *Angiostrongylus vasorum* in Portugal: Current Epidemiological Situation and the Status of Co-infections with Important Vector-Borne Pathogens.** *D immitis* and *A. vasorum* are severe and life-threatening cardiopulmonary nematodes of pets, which have increasingly been reported throughout Europe (Traversa *et al.* 2010; Alho *et al.* 2018). Several factors have been suggested for this expansion, namely incremented global transports, demographic and political changes, urbanization, increasing density of vulpine reservoir host populations, climate changes and the availability of better diagnostic tools (Otranto *et al.* 2013). *D. immitis* (Leidy 1856) (Spirurida, Onchocercidae) is a potentially zoonotic vector-borne pathogen, transmitted by multiple species of mosquitoes (Culicidae), and it is the causative agent of canine cardiopulmonary dirofilariosis/heartworm disease. Differently, *A. vasorum* (Baillet 1866) (Metastrongyloidea, Angiostrongylidae) is a non-zoonotic nematode, transmitted by gastropod mollusks and it is the causative agent of canine angiostrongylosis (Guilhon and Cens 1973). Information about the prevalence and distribution of cardiopulmonary parasites is essential for the prevention of animal and potentially associated human diseases, particularly considering their growing incidence and clinical severity. However, in Portugal, accurate data on both illnesses was scarce and limited to a few studies and case reports. Therefore, a multidisciplinary study was designed to characterize and assess the national situation regarding dirofilariosis and angiostrongylosis in canine and red fox populations from Portugal. This study allowed to confirm the occurrence of both diseases in canids at northern, central and southern regions of Portugal, with an overall prevalence of 11.9% dogs and 8.5% foxes positive to *D. immitis,* with higher prevalences registered in Center South and Algarve (Alho *et al.* 2014a, 2016a, 2016b, 2018). *A. vasorum* was throughout the country but with a lower prevalence, 0.7% positive dogs (Alho *et al.* 2016c) and 12.7% positive foxes (Alho *et al.* 2017a). This study showed that although exposure may differ depending on the region of Portugal, the likelihood of both infections is a possibility nationwide. Additionally, the transmission risk period for *Dirofilaria* spp. was estimated in Portugal using a degree-day model, based on the temperatures registered at five meteorological stations in Portugal over a decade. Overall, Madeira Island was found to be the area registering the highest number of days with suitable conditions for *D. immitis* transmission, followed by Faro, Lisbon, Azores and Oporto. As expected, higher average temperatures were observed predominantly in Madeira and in the southern parts of the country. Although risk was found to be markedly seasonal (predominantly in the summer), this study evidenced that in Portugal the transmission period is starting earlier and lasting far beyond the warmest months of the year (Alho *et al.* 2014b). This is in line with the "seasonality paradigm" of vector-borne diseases that shows their occurrence is no longer a seasonal phenomenon (Otranto *et al.* 2013). In parallel and showing once again the southern trend of this disease in

Portugal and the risk for unusual hosts, a high prevalence of *D. immitis* was found in pinnipeds kept at an oceanographic park in the Algarve region. Overall, common seals (*Phoca vitulina*), California sea lions (*Zalophus californianus*) and South African fur seals (*Arctocephalus pusillus pusillus*) were positive for *D. immitis* by real-time PCR, representing an overall prevalence of 43.8% (Alho *et al.* 2017b). This high prevalence detected in a confined area located in a popular summer destination represents a risk interface for zoonotic pathogen transmission, and an example of how a One Health approach is vital to improve early diagnosis and control of zoonotic pathogens in humans and wildlife. The treatment goals of dirofilariosis are to improve the animal's clinical condition and to eliminate all life stages of the parasite with minimal post treatment side effects. This can be achieved through mechanical, surgical or chemotherapeutic approaches. Manual extraction is the preferred method due to its diminished invasiveness, reduced damage to the vascular endothelium and shortened anesthesia duration (Atkins 2010). However, it is an expensive technique that can be highly traumatic when extraction brushes are used. To overcome this issue, a new minimally invasive surgical technique was developed to extract *D. immitis* adult worms from the hearts of dogs through transjugular catheterization with a non-traumatic snare (Alho *et al.* 2016d). A 0.014-inch coronary wire (BMW, Abbott Vascular) was adapted, allowing the successful transvenous extraction of *D. immitis* adult specimens from the pulmonary artery and right ventricle of a severely infected dog. Further surgical interventions need to be performed to improve the efficiency of this technique. Nevertheless, we defend that the possible cost reductions and diminished traumatic damage induced by this snare, will allow heartworm extraction to be more affordable and consequently widespread, thereby promoting the treatment of a larger number of animals, enhancing a specific chemotherapy with higher safety. Furthermore, a questionnaire conducted on Portuguese pet owners at a veterinary hospital in Portugal showed that the majority deworm their dogs at irregular and consequently ineffective intervals. Only 11.8% of the dogs were under the recommended endoparasitic treatment (i.e. quarterly, at least) and only 28.4% were continuously protected throughout the year from vector-borne agents. Moreover, 60% of the owners who kept their dogs outdoors the entire day did not perform adequate ectoparasitic prevention on their animals. Besides, the level of public knowledge in Portugal about parasites and parasitic diseases is still low, i.e. 88% had never heard of "dirofilariosis/heartworm disease" and 85% had never heard of "zoonosis" (Matos *et al.* 2015).

Emerging VBDs and co-infections with other important vector-borne pathogens are being increasingly reported worldwide. That is the case of *Onchocerca lupi* (Rodonaja 1967) (Spirurida, Onchocercidae), a helminth that causes nodular lesions associated with acute or chronic ocular disease in dogs and cats (Grácio *et al.* 2015). Since its first description in 1991, this zoonotic filarioid has been found with increasing frequency in canine and feline species across Europe and the United States. Recently we reported an ectopic location of canine onchocercosis featured by an atypical clinical presentation of dyspnea. An exploratory laryngoscopy revealed a laryngeal nodule causing a severe reduction of the trachea, along with several filiform worms, later morphologically and molecularly identified as *O. lupi* (Alho *et al.* 2016e). This was the first report of an aberrant migration of *O. lupi* in a dog, alerting the veterinary medical community to the occurrence of erratic locations of virulent zoonotic nematodes. Other life-threatening vector-borne parasite is

Cytauxzoon sp., an haemoprotozoan pathogen of the genus *Cytauxzoon* (Theileriidae) that is transmitted by ticks (Lloret *et al.* 2015). Although in literature information is scant, a case of *Cytauxzoon* sp. infection was recently diagnosed for the first time in Portugal in a domestic felid (Alho *et al.* 2016f). The cat was presented with an history of acute lethargy, anorexia, pyrexia and severe anaemia. A molecular screening for the detection of causative agents of infectious anaemia showed a positive result for Piroplasmorida, with DNA sequencing of the 18S rRNA gene revealing 99.9% identity with *Cytauxzoon manul*. Besides being emerging, VBDs agents infect concomitantly the same host and in a recent study, two thirds of the surveyed dogs showed at least one agent and the number of VBDs ranged from 3–4 in the islands and 5–7 in mainland Portugal (Alho *et al.* 2016b). The transmission behavior of these diseases is highly dependent on their vectors and their integrated study should be performed continuously.

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Distribution, Major Epidemiological Features and Control of Equid Gastrointestinal Parasites in Mainland Portugal

A. S. Lopes[1,2], B. Melo-Franco[1,3], T. Nunes[4], S. Sousa[1,5], P. Fabrica[6], B. São-Braz[1,7], and L. Madeira-de-Carvalho[1(✉)]

[1] Parasitology and Parasitological Diseases Laboratory (PPDL), Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon (FMV-ULisboa), Lisbon, Portugal
madeiradecarvalho@fmv.ulisboa.pt
[2] Clínica Veterinária de Lourel, R. Barbosa du Bocage, 1-A, 2710-358 Sintra, Portugal
[3] Tom Walters Equine Vets, Somerford, Cheshire, UK
[4] Infectious Diseases Laboratory, Epidemiology Unit, CIISA, FMV-ULisboa, Lisbon, Portugal
[5] Departamento de Medicina Veterinária, Escola Universitária Vasco Da Gama, Av. José R. Sousa Fernandes 197 Lordemão, 3020-210 Coimbra, Portugal
[6] Boehringer Ingelheim, Sanofi Group, Av. de Pádua, nº 11, 1800-294 Lisbon, Portugal
[7] Clinical Research Laboratory (CRL), CIISA, FMV-ULisboa, Lisbon, Portugal

Abstract. Equids harbour several highly prevalent and pathogenic gastrointestinal (GI) helminths. Since the 1990s research on the major GI equid parasites, in particular strongyles, took place at PPDL-CIISA Laboratory. The first studies in abattoirs, showed that *Strongylus* spp. were prevalent, but research performed in horse stud farms revealed Cyathostominae specimens to be emerging, becoming the most prevalent (100%) and abundant group of strongyles. These studies performed in Ribatejo and Alentejo revealed mares as more susceptible to strongyles, followed by younger horses, with 2–3 Eggs per Gram (EPG) peaks throughout the year. Other parasites with lower prevalence included *Parascaris* spp., Anoplocephalidae and *Oxyuris equi*.

In Portugal, parasite control programmes rely mostly on macrocyclic lactones, but drug characteristics, parasite epidemiology, season or animals' age/husbandry are not considered at the time of deworming. Recently, the survey 'Single Test Of Parasitic Eggs' (STOP) aimed the study of prevalence of equine GI helminths in Portugal, whilst assessing the efficacy of ivermectin (IVM) dewormings. *Cyathostomum* s.l. showed prevalence and abundance greater than 90% and the efficacy of IVM was 100% in horses and donkeys, although 55% of these animals were still dewormed when presenting a negative EPG. Adequate monitoring, including egg counts before and after deworming is paramount to keep parasite control programs effective.

Keywords: Equids · Strongyles · Survey STOP · Ivermectin · Efficacy · Portugal

A. S. Lopes and B. Melo-Franco—Contributed equally for this work.

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1 Introduction

Despite the great advances in equine health, parasitic diseases are still disregarded not only by owners but also by veterinary surgeons (VS). Moreover, the intensive control based on frequent deworming, with no previous evaluation of the parasitic profile and use of "off label" products has increased the prevalence of anthelmintic resistance (AR) by equine parasites. The goal of equine parasite control programs is not to eradicate parasites, something unrealistic, nor to completely avoid the infection. Furthermore, exposure to some parasites leads to the development of acquired immunity, which ultimately, is a more effective strategy rather than the abusive use of dewormers (Reinemeyer 2008). In a perspective of an integrated control of equine parasites, owners and VS should work together on sustainable and evidence-based programs (Kaplan and Nielsen 2010). The strong selection pressure of anthelminthics, due to an abusive and indiscriminate use, based on outdated scientific knowledge, fostered a serious situation of worldwide resistance of equine intestinal parasites to the commercial available anthelmintics, including macrocyclic lactones (Le Jambre *et al.* 1999; von Witzendorff *et al.* 2003; Blanek *et al.* 2006; Milillo *et al.* 2009; Traversa *et al.* 2009; Reinemeyer 2009; Kooyman *et al.* 2016). These practices also have an impact on the environment, a common feature shared both by animals and human beings, a globally esteemed subject highlighted by the concept "One Health – One Medicine": linking human, animal and environmental health.

Nonetheless, some epidemiological data is becoming outdated and, in some aspects, is scant and limited to some geographical areas, namely in southern Europe. Aware of these serious concerns, gathering of epidemiological data on equine parasites prompted further research in Portugal in the last 30 years, concerning their seasonality, implemented control programs and anthelmintic efficacy assessment (Madeira de Carvalho 1991, 2002, 2006a, b, c, 2010; Madeira de Carvalho *et al.* 2009; Madeira de Carvalho *et al.* 2003, 2005, 2007, 2008, 2011, 2014; Cernea *et al.* 2008; Costa 2011; Frouco 2011; Osório 2011; Reis 2011; Medeiros 2012; Lopes *et al.* 2015; Melo-Franco *et al.* 2013, 2014, 2015; Melo-Franco 2014; Couto 2014; Afonso 2016; Ferreira 2016). For all this, it is of the utmost importance to assess the parasitic *status* of Portuguese equids on a more broad geographical area aiming at implementing the best practices of equine parasite control, suitable for each animal, farm and region.

2 Material and Methods

There is a great diversity of approaches regarding the areas involved in the horse parasitological surveys. Lopes (2013) carried out a large study using samples from each of the 18 districts of Portugal mainland, while other studies only assessed samples involving a few districts, namely Viana do Castelo (Medeiros 2012), Vila Real (Couto 2014), Coimbra (Costa 2011), Lisboa (Frouco 2011; Medeiros 2012; Melo-Franco 2014; Afonso 2016), Santarém (Madeira de Carvalho 2002; Frouco 2011; Reis 2011; Ferreira 2016), Portalegre (Osório 2011) and Beja (Melo-Franco 2014).

Although sampling a diverse number of equids, with different ages, in different areas of Portugal, periods of the year and horse management systems over the last 30 years, in each of the different studies aforementioned, faecal samples were analysed by using the

same techniques. These comprised quantitative and qualitative tests, and faecal cultures. Additionally, in some studies (Reis 2011; Melo-Franco 2014), scotch tape technique was also performed to search for eggs and adults of *Oxyuris equi* present in the perineal and perianal areas.

3 Quantitative Technique

McMaster's method adapted from Thienpont *et al.* (1986) and Madeira de Carvalho (2002) was used in order to quantify the number of faecal eggs. According to this technique, 2 grams of faeces are added to 28 ml of sucrose solution at 25%, and stirred until solution becomes homogeneous. The obtained solution is transferred to a glass through a small colander and both compartments of a McMaster slide are then filled in with the filtered solution (Fig. 1).

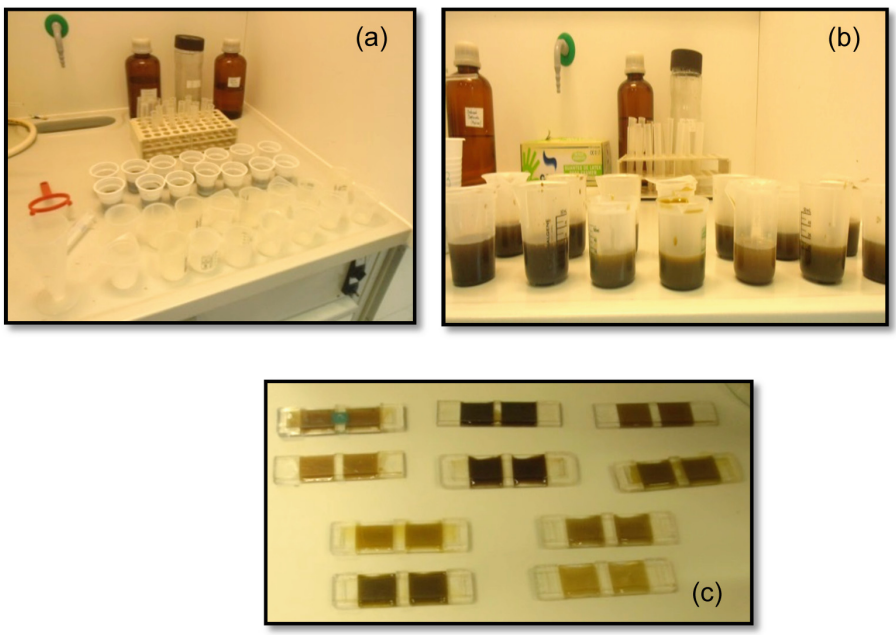


Fig. 1. Coprological techniques in preparation (a), glasses containing the sucrose solution and faeces (b), and respective McMaster slides filled in (c), ready to be examined under light microscopy (Melo-Franco 2014).

The upper part of the McMaster chamber has two grills engraved, with a set area, one over each compartment, within whose limits all the eggs should be quantified. The eggs of nematodes belonging to superfamily Strongyloidea are so similar that they are considered in the whole as being strongyle type eggs, or simply, strongyle eggs (Hummelinck 1946; Bowman *et al.* 2006). *Parascaris* spp. eggs, easily discernible, must also be quantified (Andersen *et al.* 2013; AAEP 2013).

The quantitative method used here has a detection threshold of 50 eggs per gram of faeces (EPG), hence why the number of eggs found has to be multiplied by an adjustment factor of 50. A result is considered negative for Intestinal Strongyles (IS) only when quantitative and qualitative techniques together with faecal cultures of the same sample reveal absence of eggs (Madeira de Carvalho 2002).

In some studies (Lopes 2013; Afonso 2016), EPG values obtained were ranked into mild infection (MI) [0-499], moderate infection (MoI) [500-999] and severe infection (SI) $\geq$ 1000.

4 Qualitative Technique

Using a solution with specific gravity between 1.20 and 1.30 g/cm^3 the lighter eggs can initially be assessed and in a subsequent stage, by modifying the technique, the heavier resting in the sediment (Kaufmann 1996; Foreyt 2001). When transferring the remainder of the solution previously prepared for the McMaster technique to a test tube, the eggs cluster at the top of the solution (Reinemeyer and Nielsen 2013). A microscope coverslip (18 $\times$ 18 mm), is put on the top of the convex meniscus and left for, at least, 15 min, so the eggs have plenty of time to ascend to the surface of the meniscus (Fig. 2). The coverslip is placed over the slide and observed with the microscope.

Fig. 2. Preparation of Willis' flotation technique, which can also allow the search of heavy eggs in the sediment (Melo-Franco 2014).

The method of natural sedimentation is performed straight after the flotation test. That test involves decanting the supernatant from the test tube and, by means of a Pasteur pipette, stir and aspirate 2 to 3 drops of the pellet, and add it on a slide. A drop of Methilene Blue is added and directly stirred on the slide, and a coverslip (24 x 50 mm) is put over. The organic debris show up in blue whilst the intact eggs remain with their original colour, either brownish or golden, once the dye cannot pass through the wall of the eggs (Kaufmann 1996).

An identification key developed by Thienpont *et al.* (1986) and Kassai (1999) was used for egg identification in most of our studies.

5 Faecal Cultures

Eggs of gastrointestinal strongyles are morphologically very similar, reason why it becomes impractical its identification to species and genus level (Lichtenfels *et al.* 2008; AAEP 2013; Reinemeyer and Nielsen 2013). This way, it is essential to perform faecal cultures to obtain third stage infective larvae (L_3), these with distinctive morphological features (Russell 1948; Bevilaqua *et al.* 1993). For this, it is imperative gathering 3 critical conditions for hatching and adequate larvae development, namely, humidity, oxygenation and temperature (Madeira de Carvalho 2002).

Following the methodology implemented by Madeira de Carvalho (2002), a fresh faecal sample is put inside a plastic cup up to, approximately, 75 to 85% of its volume. The content of each plastic cup must not be compressed allowing for the presence of pockets of air and, using a glass rod, a column of air is made in the centre of the sample. This will provide adequate aeration and thus oxygenation. The sample is weighed using a semi-analytical weighing scale and the top of the plastic cup is covered with aluminium foil, perforated subsequently, in order to ensure adequate oxygenation and to avoid marked evaporation. Cold water is poured over the perforated aluminium foil allowing for establishment of an adequate humidity and larvae development. The plastic cups are set on a tray containing cold water. This tray is kept in the oven, at a set temperature of around 27 °C and humidity around 75%, for 14 days. Throughout these two weeks, samples are regularly checked for level of water, which is adjusted accordingly either by adding water to the tray either by pouring some water over the perforated aluminium foil lid of the cups. Ended this period, the plastic cups are taken from the oven, the aluminium foil lid removed and filled in with water to the top and turned upside down over a Petri dish subsequently filled with around 15 – 20 ml of water. Given both their positive hydrotropism and phototropism, strongyle larvae can be collected from the liquid existing on the Petri dish; during this step, a funnel might be helpful for transferring the liquid to a test tube. Faecal culture yield can be maximised by keeping the plastic cups upside down for 24 h. Test tubes are sealed with Parafilm®, aiming at reducing the concentration of oxygen and, consequently, larvae metabolism (Fig. 3) (Madeira de Carvalho 2002).

The larvae to be identified under the microscope are left to deposit in the bottom of the test tubes for at least 24 h, with no need for spinning of the samples. The solution containing the larvae should be kept refrigerated at 4–5 °C for a period never superior to 4 months, to ensure that larvae remain unspoiled and with well-defined morphological features, allowing for correct identification. When the time for larvae identification comes, the solution containing larvae is gently shaken to promote dispersion of L_3 within the supernatant and, by means of a micropipette; an aliquot of 100 μl is collected and put between a slide and a microscope coverslip (24 × 50 mm). A differential count is performed for the first 100 L_3 seen in each sample, when possible (Fig. 4). Identification of L_3 obtained from faecal cultures was based on the identification key developed by Madeira de Carvalho (2002) and Madeira de Carvalho *et al.* (2004), Madeira de Carvalho *et al.* (2007a, 2008a), with new improvements by Cernea *et al.* (2008) and Santos *et al.* (2018). Some of the morphological features considered in this identification key include number and arrangement of the intestinal cells, length of larvae body and tail sheath, as well as the ratio between these two, and other details distinctive of certain types of larvae,

like the differences between L3 of *Cyathostomum* sp. and *Strongylus vulgaris* (Fig. 5). Accurate identification of the different species belonging to subfamily Cyathostominae based on L_3 observation is difficult, not only because there are more than 50 species, but also due to the very subtle differences amongst them. For this reason, discrimination of 8 different larval types (A to H) under the genus *Cyathostomum* s.l., each type concerning several species, was made as proposed and implemented by Madeira de Carvalho (2002) and Madeira de Carvalho *et al.* (2004).

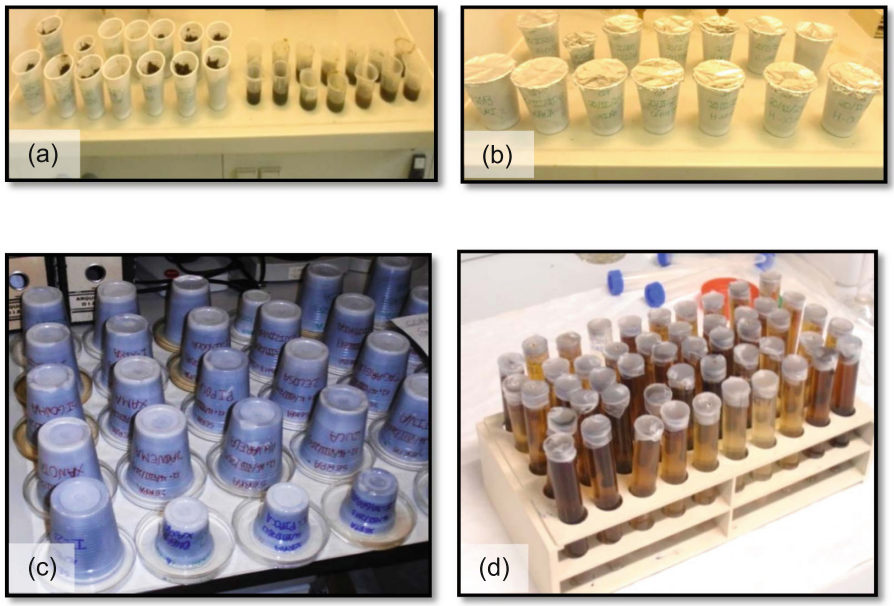


Fig. 3. Preparation of faecal cultures simultaneously with quantitative and qualitative techniques (a), plastic cups containing faecal cultures sealed with aluminium foil (b), plastic cups containing faecal cultures turned upside down on a Petri dish (c) and test tubes containing solution obtained from faecal cultures, sealed with Parafilm® (d) (Melo-Franco 2014).

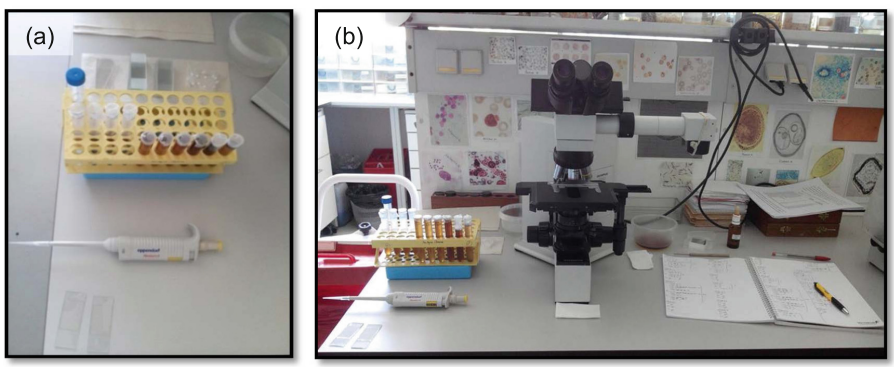


Fig. 4. Detail of test tubes rack over an eutectic pack (blue) (a) and workbench displaying all the material necessary for identification of larvae (b) (Melo-Franco 2014).

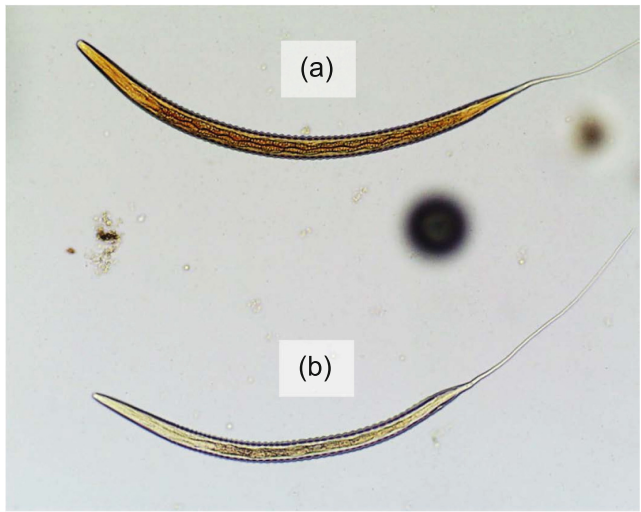


Fig. 5. Infective larval stage of *Strongylus vulgaris* (a) and *Cyathostomum* sp. (b) Note the different total length and number of intestinal cells for each L3 (Madeira de Carvalho 2002).

The prevalence (number of infected individuals/number of examined individuals) of genus/species has also been determined, in accordance with Bush *et al.* (1997) and Madeira de Carvalho (2002). Considering the differential count of L_3, also its percentage of occurrence was determined following the methodologies developed by Bello (1990), Eydal and Gunnarsson (1994) and Madeira de Carvalho (2002).

The number of larvae was also determined and expressed in larvae *per* gram of faeces by using the following formula:

$$\mathrm{LPG} = (\mathrm{N} * \mathrm{F})/\mathrm{W}$$

LPG – Larvae per Gram of Faeces
N – Number of larvae *per* 100 μl
F – Factor of titration, in μl
N * F – Total number of L_3 *per* sample
W – Sample weight in grams

The yield of the faecal culture was calculated as well using the formula:

$$Yield\,(\%) = [LPG/EPG] \times 100$$

The proportional abundance of genera and species (PAgs) was determined for each individual based on the identification of L_3 obtained from faecal cultures, using the following formula:

$$PAgs = (Ngs/N) \times 100$$

Ngs – Total number of L_3 of a specific genus or species
N – Total number of L_3 per sample

6 Scotch Tape Technique

Given the peculiar behaviour of oviposition characteristic of the female specimens of *Oxyuris equi*, eggs are rarely found when using the flotation method, although they may be seen exceptionally if the faecal sample is directly collected from the rectum (Reinemeyer and Nielsen 2013). Nonetheless, the gold standard technique for the diagnosis of this parasitic disease is the scotch tape technique. A strip of scotch tape is patted several times over the skin around the anus, ventral aspect of the base of the tail and perineum, until adhesiveness of the tape has worn off. This strip of tape is then sticked, by means of new tape, onto a slide for observation under the microscope (Fig. 6). Eggs found with this technique were identified using an identification key proposed by Thienpont *et al.* (1986) and Kassai (1999).

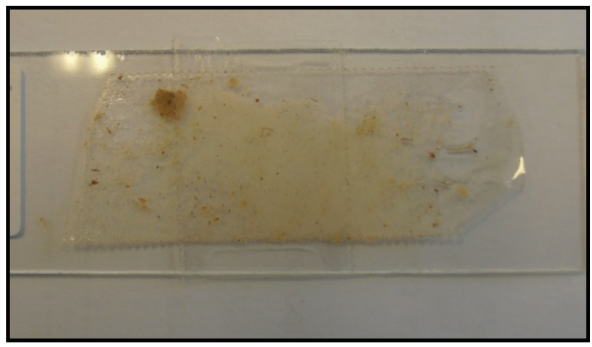


Fig. 6. Scotch tape displaying debris from perianal and perineal areas, sticked onto a slide by means of a new strip of scotch tape (Melo-Franco 2014).

7 Macroscopic Examination

Apart from examination under the microscope, physical examination and assessment of the general demeanour of the animals, as well as, a close macroscopic observation of the faecal samples are important steps in the diagnosis of parasitic diseases. Clinical signs such as loss of body condition, diarrhoea, acute abdomen, pale mucous membranes and purulent nasal discharge, might be indicators of parasitic infection. Individuals infected by pinworms tend to exhibit pruritus around the anus, hence developing alopecic areas and broken hair at the base of the tail. Occasionally, clusters of yellowish eggs and adult specimens of pinworms at the anal and perianal area, tapeworm proglottids, Cyathostominae L_4, adult red worms and bots L3 in faeces and yellowish eggs of bots over the forelimbs might be discernible to the naked eye (Kaufmann 1996; Zajac and Conboy 2012; Reinemeyer and Nielsen 2013).

8 Monitoring the Efficacy of Deworming Protocols

Following the World Association for the Advancement of Veterinary Parasitology (Coles *et al.* 1992, 2006) and the AAEP (2013) recommendations, the Faecal Egg Count Reduction Test (FECRT) was the mathematical tool employed in order to assess the efficacy of deworming protocols. For that, a quantitative evaluation of a faecal sample at day 0 and day 14-post deworming is needed. This is expressed as a percentage and determined for each individual. Each time a population was dewormed with a specific anthelminthic, the average FECRTs was considered based on individual results using the following formula:

$$\textbf{FECRT} = \left[1 - \left({}^{epg14}/_{epg0}\right)\right] \times 100$$

FECRT – Faecal Egg Count Reduction Test (%)
EPG_0 – EPG on day 0 post deworming
EPG_{14} – EPG on day 14 post deworming

In some studies (Reis 2011; Melo-Franco 2014), given logistical constraints inherent to the management within stud farms, livery yards and equestrian centres, it was not possible to respect the 14 days interval between sampling. Although faeces collection at 14 days post deworming had been consistent, some samples were collected within 7 days prior to deworming. Thus, in line with studies carried out by Reis (2011) and Carstensen *et al.* (2013), as it is not expected to occur significant variation of EPG in 7 and 5 days, respectively, that EPG count was considered as being day 0 EPG. Aiming at optimising robustness of this analysis, the Egg Reappearance Period (ERP) was also determined, following the methodology suggested by Borgsteede *et al.* (1993) and adjusted by Madeira de Carvalho (2002). The ERP, in days, gives the time frame between the moment of deworming until EPG values become positive again. This parameter is specific for species of parasites and active ingredient of the dewormer (AAEP 2013).

In 2012, a survey was designed aiming the implementation of effective parasite control programs based on individual parasite status assessment of horses and donkeys in Portugal mainland prior to deworming. This survey, named STOP – Simple Test for Parasitic Eggs – resulted from a partnership between Boehringer Ingelheim and the Centre for Interdisciplinary Research in Animal Health, Faculty of Veterinary Medicine, University of Lisbon (CIISA-FMV-ULisboa) (Lopes 2013; Lopes *et al.* 2015)

In general terms, with the purchase of a dewormer from Boehringer Ingelheim a pack was offered, containing: a cover sheet identifying the project; two information leaflets explaining the project's methodology (one for the owner, the other for the VS); two bags for faeces storage (one for samples taken immediately prior to deworming, the other for samples taken 21 days later) and a Green Mail envelope (for sending of both bags to LPPD, at no charge) (Fig. 7). Purchasers were instructed to keep the samples refrigerated between 2 and 4 °C until they were sent to the laboratory. Those bags had labels to be filled in by owners/VS with the requested and relevant information to the survey. Some of this information included post code of the owner's address, and equid species, sex, age, breed, date of last deworming and dewormer administered, and previous diseases of the sampled animals. The samples were only analysed when proof of purchase of the

dewormer was sent in (batch number of the dewormer, drafted in the cardboard pack). Owners and VS received a report with the coprological results within the following two weeks.

Fig. 7. Example of a kit received at the LPDP (Lopes 2013).

Lopes (2013) and Lopes *et al.* (2015) also assessed the efficacy of Ivermectin, the active ingredient present in both dewormers traded by Boehringer Ingelheim (Eqvalan® and Eqvalan® Duo), by means of the above mentioned FECRT.

Due to the impracticality of quantifying eggs shed by *O. equi*, obtaining FECRT values for this parasite becomes hopeless (Reinemeyer and Nielsen 2014). Thus, Melo-Franco (2014) implemented instead an Infected Animals Reduction Test (IART) as proposed by Vázquez (2010). The formula reads as follows:

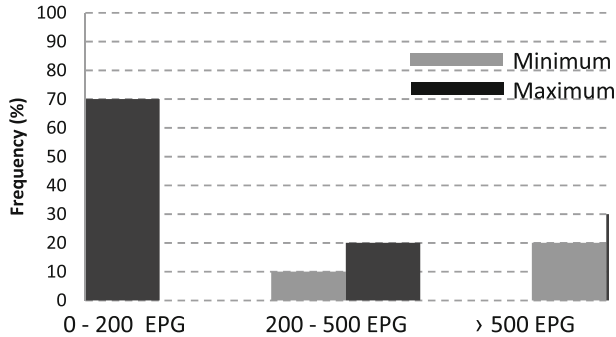
$$\textbf{IART} = \left[1 - \left({}^{nia14}/_{nia0}\right)\right] \times 100$$

IART – Infected Animals Reduction Test (%)
NIA_0 – Number of infected animals at day 0 of deworming
NIA_{14} – Number of infected animals at day 14 post deworming

Melo-Franco (2014) also ranked the two groups of adult horses used in his study into three categories according to their potential for contamination, in other words, to the level of EPG, as suggested by Kaplan and Nielsen (2010) and the AAEP (2013) (Graph I). Although these authors have not validated this EPG ranking in non-adult animals, Melo-Franco (2014) included foals and yearlings under that classification, to obtain a big picture of the whole population of horses studied.

9 Statistical Analysis

In all studies, data was stored and analysed using software Excel© Microsoft Office. Further analyses were done using specific statistical software, chiefly SAS® (SAS Institute, Cary, NC, USA) and R® versão 2.15.1 2012.



Graph I. Classification of an adult population of equine, based on the average levels of EPG of intestinal strongylids.

As pointed out by Becher *et al.* (2010), Spearman test was used in order to determine potential correlations between quantitative variables: EPG *vs.* LPG; EPG *vs.* ERP; EPG *vs.* climatic variables (average temperature, average relative humidity, average rainfall). Wilcoxon, Kruskal-Wallis and Pearson's Chi-squared tests were also performed, namely due to the non-normal distribution of data when dealing with egg counts and parasitic burdens. The significance level for all tests was set at $P < 0.05$.

10 Results and Discussion

10.1 General Epidemiological Data

At the beginning of our research, studies performed at abattoir level showed that *Strongylus* spp. was prevalent, namely *Strongylus edentatus* and *S. vulgaris*, but Cyathostominae arised for the first time as the predominant group of horse strongyles in Portugal. Some years later and at horse farm level, the Cyathostominae emerged and became the most prevalent and abundant group of strongyles, in horses and donkeys of every age and production status, reaching 100% prevalence in most equine premises (Madeira de Carvalho 1991, 2002; Madeira de Carvalho *et al.* 2008, 2014).

The epidemiological studies performed in several horse stud farms in the Ribatejo area, revealed the mares as the group more prone to be infected by GI helminths, followed by the yearlings between 1–3 years and even the foals. Most of this predisposition to GI parasitism depends on the large time spent on the pasture, although in the first studies the stabled animals also showed GI parasites largely. Due to the major influence of pasture in the transmission of the major helminths, the first epidemiological studies showed 2–3 peaks of Eggs per Gram (EPG) and consequent Larvae per Gram (LPG). In dry pastures, these peaks tend to be more visible in spring and autumn/winter, while in irrigated pastures a great shift in EPG and LPG may also occur during the summer. For the first time, type II horse cyathostominosis was diagnosed in Ribatejo, during the winter, being the most prevalent species *Cylicocyclus insigne*, *Cylicocyclus nassatus*, *Cyathostomum catinatum*, *Cylicostephanus longibursatus*, *Cylicocyclus asworthi*, *Cyathostomum pateratum*, *Cylicostephanus calicatus*, *Coronocyclus coronatus*, *Cylicocyclus leptostomum* and *Coronocyclus labiatus*. These species comprised 93.3% of the

total studied strongyles, being *C. nassatus* the most abundant one, contributing to 35% of the total worm count (Madeira de Carvalho 2002; Madeira de Carvalho *et al.* 2007, 2010, 2014).

11 EPG Counts

The remainder studies assessed 584 equids, of which 70 were donkeys and 514 were horses. These animals were screened at least twice during the whole experimental period (e.g., in case of assessing for deworming efficacy only) and, in the majority of studies, were assessed on a monthly basis over several months, or even years. Quantitative assessment of faecal samples in other studies revealed 25.65% of donkeys and 89.79% of horses to have positive EPG counts. Throughout the other studies, the global mean EPG value was 349.13 EPG for donkeys (Couto 2014; Sousa 2016) and 640.87 EPG for horses (Gersão 2010; Costa 2011; Frouco 2011; Osório 2011; Reis 2011; Medeiros 2012; Melo-Franco 2014; Afonso 2016; Ferreira 2016).

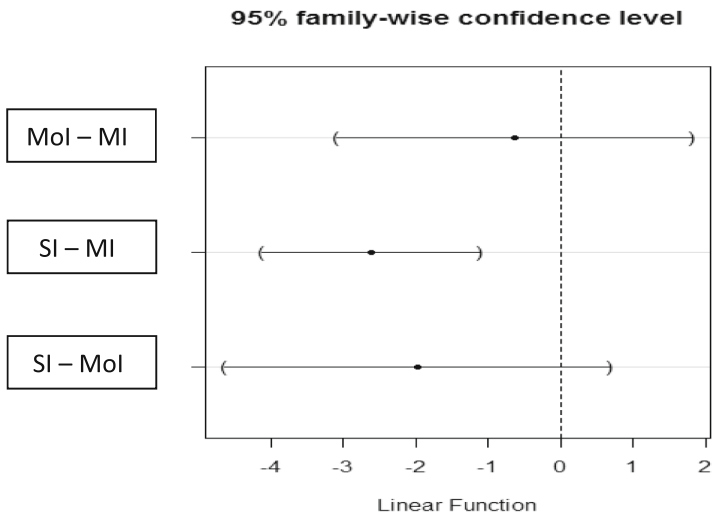
As far as classification of EPG counts into categories of infection is concerned, 68.31% equids (n = 403) were considered to have a mild infection (MI), 7.46% equids (n = 44) to have a moderate infection (MoI) and the remainder 24.23% (n = 143) to have a severe infection (SI). Frouco (2011), Osório (2011), Medeiros (2012), Afonso (2016), and Ferreira (2016) identified a distribution as follows: mild (66.58%), moderate (12.68%) and severe (20.74%). Gersão (2010) found her study population in 2008/2009 to be arranged as follows: in November 2008, more than 62.5% of horses had less than 500 EPG and 95% less than a 1000 EPG; in December 2008, more than 62.5% had less than 1000 EPG and 95% less than 1500 EPG; from January to May 2009, more than 62.5% had less than 200 EPG and 95% had less than 500 EPG.

Throughout the other studies, the global mean EPG value was 349.13 EPG for donkeys (Couto 2014; Sousa 2016) and 640.87 EPG for horses (Gersão 2010; Costa 2011; Frouco 2011; Osório 2011; Reis 2011; Medeiros 2012; Melo-Franco 2014; Afonso 2016; Ferreira 2016).

Whilst adopting the classification proposed by Kaplan and Nielsen (2010), Melo-Franco (2014) obtained the following composition, in adult horses: mild (78.75%), moderate (15%) and severe (6.25%). When considering all adult and non-adult horses together, Melo-Franco (2014) found his population to be ranked as follows: mild (61%), moderate (23.5%) and severe (15.5%).

A significant difference was found between the age of individuals and EPG category ($P = 0.0003$; ANOVA) and it was observed that individuals with severe infection are younger than those with mild infection (Lopes 2013) (Graph II). The same correlation was found by Frouco (2011) ($P = 0.004342$; $R = -0.346739$; Spearman correlation test), Reis (2011) (only amongst foals: $P < 0.0001$; $R = 0.582$; Spearman correlation test), Melo-Franco (2014) ($P < 0.0001$; ANOVA), Ferreira (2016) ($R = -0.582$; Spearman correlation test) and Sousa (2016) ($P = 0.032$; ANOVA).

The EPG level among the different breeds was as follows: crossbred Lusitano (arithmetic mean: 1050.6 EPG; median: 0 EPG), Lusitano (mean: 602.8 EPG, median: 0 EPG), other breeds (mean: 931.9 EPG, median: 0 EPG). There were significant differences in the number of EPG between stabled equids and those living out in the pasture (P



Graph II. Correlation between categories of infection based on EPG counts of samples collected on day of deworming and age of the individuals. *MI* mild infection, *MoI* moderate infection, *SI* severe infection.

= 2.613 and P = 0.001718, respectively, by the Pearson's Chi-squared test). Although animals in mixed systems (stabled and grazing animals) have shown high EPG values, no statistically significant differences existed according to Pearson's Chi-squared test.

Cohabiting with other animals was also another variable taken into consideration and it was reported to be the case with 436 equids. Amongst those cohabitants there were horses (n = 325), dogs (n = 52). No significant correlation between the number of EPG and presence of cohabitants was found (P = 0.9212; Pearson's Chi-squared test).

12 STOP Survey

In a period of four years, from April 2012 until January 2016, 590 kits were sent to the owners or VS and had been received at the LPDP. The districts most represented were Évora (164), Lisboa (92), Setúbal (78) and Beja (64), as shown inside the red line (Fig. 8).

From the 590 samples received only 16 belonged to donkeys. The studied population was comprised of 349 males and 235 females and the age of the animals ranged from 3 months to 33 years old (arithmetic mean: 9.235 years, median: 8 years). The breeds represented in this study included crossbred Lusitano (n = 164), Lusitano (n = 160), Arab (n = 45) and Quarter Horse (n = 11). The mainstay of feeding of the sampled animals is comprised of hay alongside hard feed (various *pellets* and cereals) (n = 346) or exclusive grazing (n = 163). Quantitative assessment of the samples collected on the day of deworming (N = 590) revealed 271 (46%) animals to have positive EPG counts and 319 (54%) to have null EPG counts. The global mean EPG value for all the samples collected on the day of deworming (N = 590) was 966.1 EPG, with horses

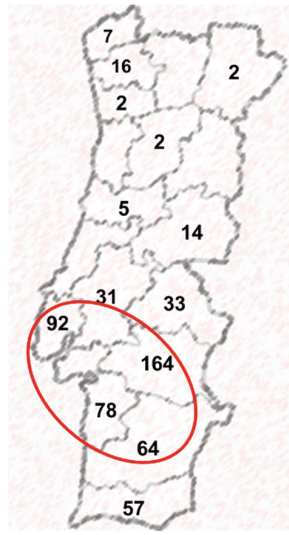
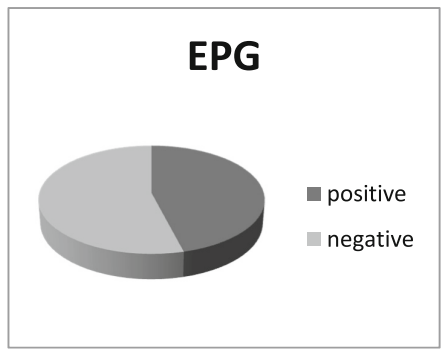


Fig. 8 Distribution of STOP samples analysed across mainland Portugal

contributing with a mean figure of 1007.684 EPG and donkeys a mean figure of 434.375 EPG (Graph III) (Lopes 2013; Lopes *et al.* 2015).



Graph III. Distribution of EPG counts based on assessment of samples collected on the day of deworming (N = 590).

13 Intestinal Strongyles EPG Peaks

In a study involving 67 horses with ages varying from 3 to 17 years old, followed for 11 months in Lisboa and Santarém, Frouco (2011) identified two peaks of IS EPG counts: summer (mean 518 EPG, $\delta = 1344.5536$) and autumn (mean 500 EPG, $\delta = 1272.4309$).

Although accurate determination of mean EPG counts was not attainable, Osório (2011) reported three peaks of IS EPG counts in Portalegre district through a period

of 12 months among 20 horses with ages varying from 4 to 28 years old: May 2010 (spring), August & September 2010 (summer) and April 2011 (spring).

Reis (2011), accompanied 10 mares and respective foals for a period of 13 months in the [Santarém district, describing two main peaks of IS EPG counts: September 2010 (summer) (considering the whole population: max 13600 EPG, mean 3327 EPG) and March 2011 (spring) (considering the whole population: max 11200 EPG, mean 3007 EPG). Amongst mares, the highest peak occurred in mid-summer and, amongst foals occurred in March, although EPG had been increasing since September.

In the population of horses followed in Lisboa (7 horses, age ranging from 2 to 12 years old) in 6 months (January, February, March, July, October, November), Medeiros (2012) reported two main peaks of EPG counts: March (spring) (mean 2692.9 EPG) and July (summer) (mean 1825 EPG).

Melo-Franco (2014) also described some peaks of EPG counts throughout his study in the different groups of horses in two stud farms accompanied over 13 months. In the population in Lisbon, mares showed two peaks: April & May (spring) (mean 90 EPG) and November & December (autumn) (mean 100 & 300 EPG, respectively); foals had shown two peaks: April (spring) (mean 550 EPG) and November (autumn) (mean 679 EPG); yearlings revealed two peaks as well: March (spring) (mean 1036 EPG) and December (autumn) (mean 1060 EPG). In the south population in Beja, mares displayed two peaks: January (winter) (mean 535 EPG) and April (spring) (mean 228 EPG); foals revealed one peak only: May (spring) (mean 888 EPG); yearlings had shown three peaks: March (spring) (mean 635 EPG), June (late spring) (mean 770 EPG) and August (summer) (mean 620 EPG).

In donkeys of Miranda breed in Trás-os-Montes, two recent studies showed two EPG peaks. The annual epidemiological curve of donkey strongyle egg shedding in this region, showed a progressive increase from December to July, when a maximum peak of egg shedding is attained, which then decreases progressively until December (Couto 2014; Sousa 2016).

Aiming at studying two approaches for deworming (selective *versus* strategic) Afonso (2016) used a control group which did not undergo any deworming protocol during the study period (November 2014 to April 2015). Having the lowest EPG counts was another critical criterion for selection of the individuals to become part of the control group. Although being a short period to assess the seasonality of EPG counts, data on this group was deemed relevant once the effect of deworming was absent. Thus, two peaks were identified: March (spring) (mean 1135 EPG) and April (spring) (mean 1715 EPG).

14 Prevalence of Identified Parasites

Over the last two decades, Strongylidae, *Parascaris* spp., *Anoplocephala* spp., *Strongyloides westeri* and *Fasciola hepatica* have been discriminated by quantitative and qualitative techniques, and *Oxyuris equi* identified by the scotch tape technique. *Habronema* spp., *Trichostrongylus axei* and *Gasterophilus* spp. collected after an abattoir study were also added. Table 1 shows information related to the helminths found and their respective prevalence.

Table 1. Prevalence of the various gastrointestinal helminths identified over the last two decades in several researches performed at the LPPD-CIISA, FMV-ULisboa (values are expressed in percentage).

Helminth Authors	*Habronema* spp., *Trichostrongylus axei* and *Gasterophilus* spp.	Strongylidae	*Oxyuris equi*	*Parascaris* spp.	*Strongyloides westeri*	*Anoplocephala* spp.	*Fasciola hepatica*
Madeira de Carvalho (2000)	–	100	–	–	–	5.6	
Madeira de Carvalho (2002)	*T. axei* - 0.1	100	–	–	–	–	–
Costa (2011)	–	80	–	–	–	–	–
Frouco (2011)	–	91	–	–	–	–	–
Osório (2011)	–	100	–	–	–	–	–
Reis (2011)	–	100	90 (*f*)	30	–	–	–
Medeiros (2012)	–	100	–	–	–	–	–
Lopes *et al.* (2013)	–	99.6	–	2.95	–	1.1	–
Melo-Franco (2014)	–	*BS* (100) *LS* (100, except *f* – 80)	*BS* (*m* 90, *f* 70, *y* 50) *LS* (*m* 29, *f* 80, *y* 91)	*BS* (*m* 10, *f* 1, *y* 67) *LS* (*m* 21, *f* 80, *y* 55)	*BS* (*f* 10, *y* 20) *LS* (*m* 14, *y* 9)	*BS* (*m* 3.33) *LS* (*f* 3.33)	–
Fernandes (2014)[a]	–	–	–	–	–	50	–

(*continued*)

Table 1. (*continued*)

Helminth Authors	*Habronema* spp., *Trichostrongylus axei* and *Gasterophilus* spp.	Strongylidae	*Oxyuris equi*	*Parascaris* spp.	*Strongyloides westeri*	*Anoplocephala* spp.	*Fasciola hepatica*
Araújo[a] (2014)	*Habr.*- 69 *T. axei* – 4 *Gasteroph.* - 80	–	–	–	–	–	–
Afonso (2016)	–	*GA*, *GB*, *GC* (100)	–	*GB* (20), *GC* (20)	–	–	–
Ferreira (2016)	–	53.9 (obtained from McMaster)	–	16.7 in individuals < 3 yo; 1.3 in the whole population	–	–	1.32 (in a 6 yo horse)
Couto (2014)[b]	–	100	–	–	–	–	–
Sousa (2016)[b]	*T. axei* - 0.8	35–98	–	0.8	–	–	–

Notes: a - Research performed in horses slaughtered at the Beja abattoir. b – Research performed only with donkeys; BS Beja Studfarm, *LS* Lisbon Studfarm, *m* mare, *f* foal, *y* yearling, *GA* Group A, *GB* Group B, *GC* Group C, *yo* years old.

Intestinal strongyles were predominant in most of these studies, both in horses and donkeys, *Parascaris* sp., *Oxyuris equi* and *Anoplocephala* sp. seem to be the following genera, and *Strongyloides* is rare and *Fasciola hepatica* is very rare to find. Regarding gastric parasites, *Gasterophilus* spp. and *Habronema* spp. seem to be highly prevalent, while *T. axei* showed a very low prevalence.

15 Faecal Cultures

Regarding the main genera and species of strongyles and *Cyathostomum* s.l. morphotypes based on the identification of L3 larval stages found after faecal culture and their prevalence, data is displayed in Tables 2 and 3. The predominant genus in all of them was *Cyathosthomum* sp., followed by other small strongyles genus/species, being *Strongylus* sp. in a descending curve. This last group of strongyles only seems to have higher expression in non-dewormed animals, like in the study of Medeiros (2012) and as previewed by Madeira de Carvalho (1991, 2000, 2002).

Concerning the morphotypes of *Cyathostomum* s.l., type A was the predominant in the vast majority, which probably has to do with very prevalent cyathostomin species producing these larvae, like *C. nassatus*, *C. catinatum* and *C. longibursatus* (Madeira de Carvalho *et al.* 2008, 2008a; Santos *et al.* 2018).

16 Parasite Control

In the last two decades, the first surveys regarding parasite control programs showed that most horse and donkey owners recognize the importance of parasites and consequent clinical signs and although 90% dewormed their animals, 85% did not evaluate its efficacy. This is particularly important, since in the Portuguese horse production system most control programs rely on regular administration of macrocyclic lactones, some of them administered off-label (like injectable ivermectin (IVM), doramectin (DRM) and moxidectin (MOX) or pour on eprinomectin (EPM) for donkeys), and most dewormings are not programmed according to the parasites epidemiology, season and age of the animals. Some field trials showed inefficacies in some horse dewormings, whether using oral administrations (as for pyrantel pamoate (PYRP), levamisol (LEV) and febendazole (FBZ)), injectable (IVM and DRM) and pour on (for EPM in donkeys) (Madeira de Carvalho 2002; Madeira de Carvalho *et al.* 2003, 2009; Martins and Madeira de Carvalho 2005; Martins *et al.* 2007; Melo-Franco 2014).

Concerning intestinal strongyles anthelmintic resistance, for the first time an *in vivo* case regarding pyrantel pamoate (PYRP, Strongid®) (through FECRT) and *in vitro* cases dealing with benzimidazoles (Thiabendazole, TBZ, in an Egg Hatch Assay) were recorded from a Ribatejo horse stud farm. Suspected resistance with off-label use of IVM (Ivomec®) and DRM (Dectomax®) was suspected, due to a reduction of the ERP (Madeira de Carvalho 2002; Madeira de Carvalho *et al.* 2003).

Aiming a different approach on horse deworming, two studies were performed involving Targeted Selective Treatment (TST), meaning that horses were only dewormed if they reached EPG levels higher than 500. Results in both studies show that selective deworming is less costly and a safe alternative to the annual strategic/suppressive

Table 2. Main genera and species of intestinal strongyles L3 found in horses and donkeys during 2010–2016 at the LPPD (prevalence values are expressed in percentage).

Strongyle/Author	ASL	BMF	RC	SS	GF	SG	MF	PM	AA	MC	FO	PR
Cyathostomum spp.	92.9	99.3	74	99.8	97.3	94.7	100	63.4	100	100	54-94	96
Triodontophorus spp.	2.8	0	0	0	0.02	0	6.4	31,1	0	0	0	<4
Triodontophorus serratus	7.6	0.6	0	0	0.03	0	6.4	0	0	0	0	<4
Craterostomum acuticaudatum	6.5	0.2	0	0	0.05	0	2	0	0	0	0	<4
Gyalocephalus capitatus	4.8	0.2	0	0	1.3	0	2	0.1	0	0	0	<4
Oesophagodontus robustum	1.1	0	0	0.02	0.5	0	10.6	0.167	0	0	0	<4
Stongylus edentatus	0	0	0	0	0	0.96	0	2.4	0	0	0	<4
Strongylus vulgaris	3.9	0	3	0.02	0	0	4	37.5	0	0	6-46	<4
Strongylus equinus	0.3	0	0	0	0	1.98	0	0	0	0	0	<4
Trichostrongylus axei	0.3	0	23	0.19	0	0	0	0	0	0	0	<4
Poteriostomum sp.	0	0	0	0	1	2.4	4	0	0	0	0	<4

Notes: ASL (Lopes 2013), BMF (Melo-Franco 2014); RC (Costa 2011); SS (Sousa 2016); GF (Frouco 2011); SG (Gersão 2010); MF (Ferreira 2016); PM (Medeiros 2012); AA (Afonso 2016); MC (Couto 2014); FO (Osório 2011); PR (Reis 2011).

Table 3. Main morphotypes of *Cyathostomum* s.l. found in horses and donkeys during 2010–2016 at the LPPD (prevalence values are expressed in percentage).

ASL		BMF	SS	GF	SG	PM	AA	MC	PR
A	92.4	17.6	91.8	31.9	94.7	59.5	70.2	83.3	60
B	0.3	0.2	0.09	3.5	0	0	0	2.9	0
C	59.2	0.5	1.26	15.3	1.8	0.1	1	8	40
D	67.6	12.6	6.33	58.2	1.2	30.6	7.7	3.9	17.5
E	1.7	0.005	0.02	3.5	0.5	0	0	0	0
F	1.1	0.2	0.03	8.3	0.4	0	0	1.2	0
G	12.1	0.2	0.24	5.5	0.4	0	0	0.6	1
H	0.3	0	0	4.2	0	0	0	0	0

Notes: ASL (Lopes 2013), BMF (Melo-Franco 2014); SS (Sousa 2016); GF (Frouco 2011); SG (Gersão 2010); PM (Medeiros 2012); AA (Afonso 2016); MC (Couto 2014); PR (Reis 2011).

deworming, reducing by half the number of horses dewormed, minimizing the risk of anthelmintic resistance. The efficacy of Ivermectin remained 100% (day 14) in both studies and *Cyathostomum* sensu lato were the predominant population in all coprocultures, although Strongylus spp., namely *S. vulgaris* can increase its prevalence in this type of deworming program (Gersão 2010; Afonso 2016).

In the last decade, some cases of lack of efficacy regarding DRM (Dectomax®), ivermectin plus praziquantel (IVM + PRZ, Equimax®) and FBZ (Panacur®) against Strongylidae, *Parascaris* spp. and *Oxyuris equi* were spotted after field dewormings in Beja and Lisbon districts, namely in mares, foals and yearlings. These cases showed both reduction of efficacy using FECRT (around 90%), reduction of ERP with different timespans according to the parasites and even low IART (especially for *O. equi*), meaning that these ineffective dewormings are becoming more frequent than before. Against horse strongyles, DRM revealed dubious efficacy in mares and inefficacy in yearlings; FBZ showed efficacy in suckling foals, but had inefficacy in yearlings; IVM + PRZ only had full efficacy in suckling foals. Against *Parascaris* spp., DRM had dubious efficacy in yearlings while FBZ and IVM + PRZ had, respectively, full efficacy in yearlings and suckling foals. Against *O. equi*, both DRM and IVM were very ineffective (Melo-Franco 2014; Melo-Franco *et al.* 2015).

The STOP survey, while assessing the efficacy of IVM (Eqvalan®) and IVM + PRZ (Eqvalan Duo®) 2–3 weeks after animal deworming, found that the efficacy of IVM and IVM + PRZ deworming was 100% in horses and donkeys, but 55% of the animals showed to be treated with a negative EPG (Lopes 2013; Lopes *et al.* 2015).

These figures of deworming animals showing low Faecal Egg Counts (FEC), namely EPG ranging 0–500, can reach 94% of the grazing mares. This raises the question if this kind of intervention is needed without a valid justification, namely if animals show no clinical signs and under the scope of TST, once their FEC values were below 500 EPG (Melo-Franco 2014; Melo-Franco *et al.* 2015).

17 Conclusion

The researches performed for the last 30 years at the LPPD showed that the Portuguese equids have the same parasites, although with specific epidemiological patterns, when compared with other horse populations in Europe.

During this time span, Cyathostominae became the most prevalent and abundant horse strongyles, while *Strongylus* spp., namely *Strongylus vulgaris*, decreased their importance. This last strongyle species only remains important in some specific populations, namely non-dewormed horses and donkeys. Therefore, when using TST for parasite control, we should bear in mind their increase if the whole horse population is not dewormed.

Most of the performed studies in dry pastures found two major EPG peaks of horse strongyles, namely on spring and autumn, although in southern horse farms one of the late peaks can also occur in winter. For animals grazing in irrigated paddocks, there can be an additional summer peak, only possible due to the high moisture level of these pastures, associated with high temperatures, enhancing the fast development and migration of L3 to the grass.

These epidemiological features are important, since they will influence the deworming periods and the type of molecule to be used, according to the parasitological scenario, season of the year, horse management system and sex/age of the animals. Although the oldest macrocyclic lactone (ML), IVM seems to be still highly effective against horse strongyles in Portugal, its misuse together with other MLs like DRM and EPM, which are administered to equids as off-label treatments, may induce anthelmintic resistance in a short term for strongyles and even for *Parascaris* spp. and *O. equi.* Therefore, surveillance is needed, even in routine treatments, since regular dewormings with non-suitable products for horses are still frequent.

This drive us to the assumption that a good monitoring program with egg counting before and after deworming, is of the utmost importance to keep horse parasite control programs effective, while only treating animals when they really show significant EPG counts should be more studied and considered as an alternative to strategic deworming. As final remarks, concerning the success of horse parasite control programs in Portugal, we think they must still rely on the following basic ideas: a) a good knowledge of the farm/region/parasite epidemiology; b) faecal tests should be performed before any deworming, addressing the right molecule for the parasite(s) found; c) a good knowledge of the drugs currently used in our country is critical, since their assertive use depends on this and also on their prescription by the veterinary surgeon; d) monitoring of the implemented control scheme is crucial, above all, to assess the reliability of the whole intervention.

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Veterinary Medicine

From Villains to Heroes: Insights into the Antagonizing Functions of Prion like Genes and Proteins

J. Pimenta[1,2(✉)], L. Lopes-da-Costa[1,2], C. C. Marques[2], J. P. Barbas[1,2], M. C. Baptista[2], and R. M. L. N. Pereira[1,2]

[1] Reproduction and Development Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
jorgepimenta7@gmail.com

[2] Genetic Resources and Biotechnology Unit, National Institute of Agrarian and Veterinary Research, Santarem, Portugal

Abstract. During the past decades, prion proteins, the pathological agents causing sporadic, genetic and infectious neurodegenerative disorders, have constituted major concerns for public health authorities. Although the molecular mechanisms of these diseases are not completely understood, the invariably fatal outcome in humans [namely Kuru, Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome, fatal insomnia and new variant CJD (vCJD)] and animals (bovine spongiform encephalopathy and scrapie in sheep) has partly overshadowed the physiological action of prions. In fact, increasing evidence support the essential role of prion and prion-like proteins in reproductive physiology. This review examines the roles played by prion (PrPc), prion-like testis specific (Prt), Shadoo and Doppel genes and proteins, in reproductive physiology, and is supported by the expertise acquired by our group in this domain. This covers spermatogenesis, sperm motility, zona binding, fertilization, 3D NMR structure, as well as the evaluation of the genetic regulation of these genes in granulosa cells, using novel techniques of gene silencing (siRNAs) and epigenetic analysis (methylation). This review will thus appraise key issues concerning the biological characters and physiological functions of prion like family members in reproduction.

Keywords: Prion family genes and proteins · Male and female reproduction · Polymorphisms · Physiology · Pathogenesis

1 Introduction

Prions are the major contributing agents of transmissible spongiform encephalopathies (TSEs) in mammals. A hallmark of prion diseases - whether sporadic, dominantly inherited, or acquired by infection - is the conversion of the normal cellular prion protein (PrPc), encoded by the prion protein gene (*prnp*), into an abnormally folded isoform, designated as PrPsc (prion protein associated with scrapie), which is the major component of infectious prions (Prusiner et al. 1998; Gama et al. 2006; Mesquita et al.

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2010). Prions are the product of a single gene that is highly conserved in mammals (Comincini et al. 2001). Mammalian *prnp* is a housekeeping gene, present in both eutherians and fish (Premzl and Gamulin 2007), that has been characterized in several species, like hamster (Li and Bolton 1997), human, sheep, mouse (Lee et al. 1998) and bovine (Hills et al. 2001). Spontaneous cerebellar neurodegeneration and ataxia syndromes in certain strains of $prnp^{-/-}$ mice led to the discovery of a novel prion-like gene, *prnd*, which encodes a prion-like protein designated as Doppel (also abbreviated as Dpl). *prnd* is located 16–52 kb (depending on the species) downstream from *prnp* (Moore et al. 1999). *prnd* contributes, together with *prnp* and with the recently discovered *prnt* (that encodes prion protein testis specific - Prt) and *sprn* (shadow of prion protein gene that encodes Shadoo) genes, to the so called "prion gene complex". *prnp*, and its homologues, *sprn*, *prnd* and *prnt*, show similar gene organizations, which encompass two or three exons (Premzl and Gamulin 2007). Nonetheless, these genes present distinct expression patterns, suggesting different biological functions (Pimenta et al. 2012c).

2 Prions, the Great Villains

Prions can spread from one organism to another, with oral uptake being the most common natural form of transmission. However, in contrast to viruses and all living organisms, prions lack the canonical information-storage molecules DNA and RNA (Prusiner 1982; Das and Zou 2016). The discovery of the link between a new variant Creutzfeldt-Jakob disease (vCJD) in humans and the outbreak of mad cow disease brought prion diseases to the public eye in the mid-1990s (Das and Zou 2016). In humans, prion diseases can be divided into several categories based on presumed etiologies (Budka et al. 1995; Collinge 2001), namely: sporadic Creutzfeldt-Jakob disease (CJD), iatrogenic CJD associated with injection or grafting of infected tissue (growth hormone, cornea, etc.), variant CJD associated with exposure to bovine spongiform encephalopathy (BSE)-contaminated beef, and genetic/familial prion disease associated with inherited PrP mutations (Race et al. 2018). To date, mutations at 34 different sites in the human prion protein gene are associated with development of genetic prion diseases in an autosomal dominant pattern with heterogeneous phenotypes (Mead et al. 2013). A prion is an illness-inducing misfolded protein. When a prion "collides" with a normal prion protein, the prion protein's shape metamorphoses to the diseased form. Now it, too, can create more prions. The ensuing chain reaction drives a relentless conversion of normal prion proteins into prions (Prusiner 1982). Prions are extremely resistant to disinfection and sterilization methods used so far. The pathogenic prion protein core is resistant to proteolytic enzymes, and even if fixed by desiccation or chemicals may retain infectivity for years. They survive dry heat at 200 °C for 1–2 h, resist to ionizing radiation and are fixed to stainless steel within minutes and remain infectious for long periods (Jung et al. 2003). Also, very little, or nothing, has been done in the majority of world hospitals to prevent iatrogenic prion transmission, and the number of potentially infectious patients is not known. At least 2 cases of prion disease were contracted by people whose implanted depth electrodes had been previously used on a patient with Creutzfeldt-Jakob but were "inadequately" cleaned with benzene and disinfected with 70% alcohol and formaldehyde and sat unused for 2 years prior to implantation (McDonnell and Burke 2003). These reports highlight the need for safe and effective prion decontamination methods. Therefore, patients

undergoing neurosurgery, laryngeal or ophthalmic operations, orthodental treatments and even anaesthetic or endoscopic applications should be classified into risk groups, even if clinically prion disease is unapparent (Jung et al. 2003). Moreover, the described (Haybaeck et al. 2011) airborne pathway of direct prion neuroinvasion without an obligatory replicative phase in lymphoid organs should also warrant re-thinking on current prion-related biosafety guidelines. Polymorphisms within *prnd* prion-like protein (Doppel) gene seem to be associated with the occurrence and age at onset of Alzheimer's disease (AD) (Schroder et al. 2001; Golanska et al. 2004). There are numerous tumours with PrP family protein expression (PrP and/or Doppel), namely: Gastric, Breast, Lung, Colon, and Pancreatic cancer, and also Melanoma, Astrocytoma, Glioblastoma, Osteosarcoma, and Head and Neck Squamous Cell Carcinoma. In fact, overexpressed PrP or Doppel in cancer cells was shown to increase cell proliferation, multiple drug resistance, and angiogenesis, exacerbating tumorigenesis via multiple pathways (Xiaowen Yang et al. 2014; Yang et al. 2017). Moreover, another prion family gene, *sprn*, that encodes Shadoo (Sho, shadow of prion protein) has also been associated with variant Creutzfeldt-Jakob disease (Beck et al. 2008).

3 The Bright Side of Prions

Although associated with numerous neurological diseases, misfolded proteins may also play decisive roles in normal cellular functioning. They can also act as markers or therapeutic targets for oncological and neurological diseases. For instance, targeting PrPc may be considered as a disease-modifying therapy for certain AD-related phenotypes after disease onset (Salazar et al. 2017) and PrPc seems to isplay antimicrobial activity, inhibiting the replication of multiple viruses (Lathe and Darlix 2017). Finally, although prions have a long-standing and well-deserved reputation as calamitous agents, a different side of prions is also coming to light, as key players in basic biological processes. In fact, increasing evidence support the essential role of prion (Gatti et al. 2002) and prion like proteins (namely Doppel, Prt and Shadoo), in male and female fertility, with emphasis in spermatogenesis as well as in sperm motility, zona binding and fertilization processes (Young et al. 2011; Pimenta et al. 2012a; Pimenta et al. 2012b; Pimenta et al. 2013a). These discoveries are driving a new appreciation for prion family genes and proteins as versatile components in the machinery of life.

Taking the ovine Doppel (OvDoppel(1-30)) peptide as a template (Pimenta et al. 2013b), we brought some additional light into the signal recognition particle (SRP) conserved (in all three kingdoms of life) pathway. The universally conserved SRP is essential for the biogenesis of most integral membrane proteins. SRP scans the nascent chains of translating ribosomes, preferentially engaging those with hydrophobic targeting signals, and delivers these ribosome-nascent chain complexes to the membrane (or the endoplasmic reticulum) (Voorhees and Hegde 2015). However, the molecular details of how signal peptides are recognized efficiently by SRP and targeted specifically despite their considerable variation in sequence has been puzzling (Clemons et al. 1999). Using NMR spectroscopy, the OvDoppel(1-30) solution structure was determined in DHPC micelles (Fig. 1), revealing a stable α-helical central region extending from residue Cys^{8} until Ser^{22} (Pimenta et al. 2013). The NMR structure was subsequently included in a

computational docking complex with the mammal SRP subunit (SRP54M), and further compared with the N-terminal structures of mouse Doppel and bovine PrPc proteins. This allowed the determination of (i) common predicted N-terminal/SRP54M polar contacts (Asp^{331}, Gln^{335}, Glu^{365} and Lys^{432}) and (ii) different N-C orientations between prion and Doppel peptides at the SRP54M hydrophobic groove, that are in agreement with each peptide largest positive patch and electrostatic potential. Furthermore, we used a SUMO-Hexa-His tag-type approach to express recombinant OvDoppel(27-153) in the *E.coli* Origami strain, which enhanced significantly its solubility, suggesting that Doppel may, as PrPc, benefit from chaperone-assisted folding. Together, these findings provide new insights into the biosynthesis of prion-like (and other) proteins, raising also new possibilities of achieving soluble mature Doppel protein through the co-expression with the SUMO chaperone.

3.1 Prion-like Genes in the Male Reproductive System

3.1.1 Prnp

In adult testis, *prnp* mRNA is elevated to levels almost as high as in the brain (Makrinou et al. 2002). In mouse testes, the positive signals for *prnp* mRNAs were predominantly observed in spermatogenic cells, but not in somatic cells such as Sertoli cells, Leydig cells and peritubular myoid cells. The signals were observed moderately in spermatogonia, and strongly in spermatocytes and round spermatids, but not in elongate spermatids and spermatozoa, which indicates that *prnp* may have a possible role in germ cell differentiation during mammalian spermatogenesis (Fujisawa et al. 2004). In the reproductive tract of the ram, Gatti et al. (2002) showed that different PrP isoforms are present in the genital tract fluids and are secreted by the epididymal epithelium.

3.1.2 Prnd

Since the discovery of *prnd*, the majority of authors pointed for a physiological function of this gene in male fertility, given that its expression was mainly observed in the male genital tract (Behrens et al. 2002; Peoc'h et al. 2002; Rondena et al. 2005; Espenes et al. 2006; Serres et al. 2006; Kocer et al. 2007; Ferreira et al. 2017). This expression is mainly on testis tissue at adulthood, and takes an important role in maintaining sperm integrity, normal fertility, and motion ability (Guan et al. 2009). With regard to *prnd* (mRNA) expression in the bovine, caprine and ovine reproductive system, it was highest in the testis (Tranulis et al. 2001; Kocer et al. 2007), as also observed in the mouse (Moore et al. 1999; Li et al. 2000; Essalmani et al. 2002) and hamster (Li et al. 2008). Northern blot analyses also revealed a low amount of transcripts in bovine epididymis (Tranulis et al. 2001). Nevertheless, some species specificities may occur demanding a deeper knowledge. In humans, although Doppel is permanently expressed in Sertoli cells, its expression in spermatids is transient and coincides with the acrosome formation, suggesting a role in acrosome biogenesis (Serres et al. 2006). Doppel expression was also detected in bovine ejaculated spermatozoa and in boar epididymal epithelial cells, suggesting also a possible epididymal origin (Rondena et al. 2005; Espenes et al. 2006; Serres et al. 2006; Kocer et al. 2007).

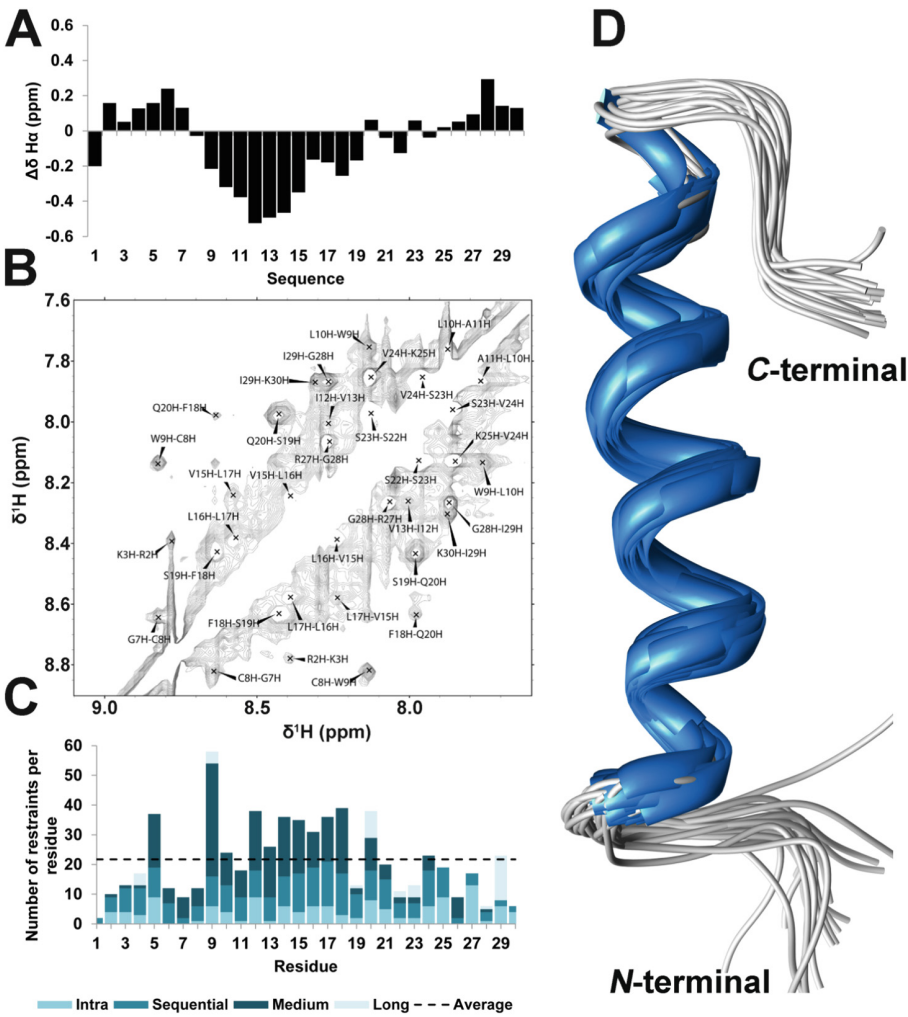

Fig. 1. Conformation of OvDpl(1-30) as determined by NMR spectroscopy. A. Experimental secondary chemical shifts for the 1Hα protons of OvDpl(1-30); **B.** Region of the NOESY spectrum, corresponding to the HN–HN NOE correlations. **C.** Number of restraints per residue. The data corresponds to a solution of 3 mM OvDpl(1-30) in 100 mM DHPC micelles at pH 3.5 and 318 K, prepared in 90% H2O and 10% D2O. D) Representation of the 3D structure of OvDpl(1-30) in solution. Superposition of the minimized 20 best structures (PDB code: 2M1J). The structures are represented as ribbons. The α-helical section is depicted in blue and the random coil is depicted in grey. The letters N and C refer to the amino and carboxyl termini, respectively (Pimenta et al. 2013a).

An association between Doppel gene polymorphisms and ram semen traits/freezability and embryo production was identified by Baptista et al. (2008) and Pereira et al. (2009). Later, Pimenta et al. (2012b) showed that ram sperm supplementation with 190 ngmL^{-1} of recombinant Doppel during *in vitro* capacitation significantly

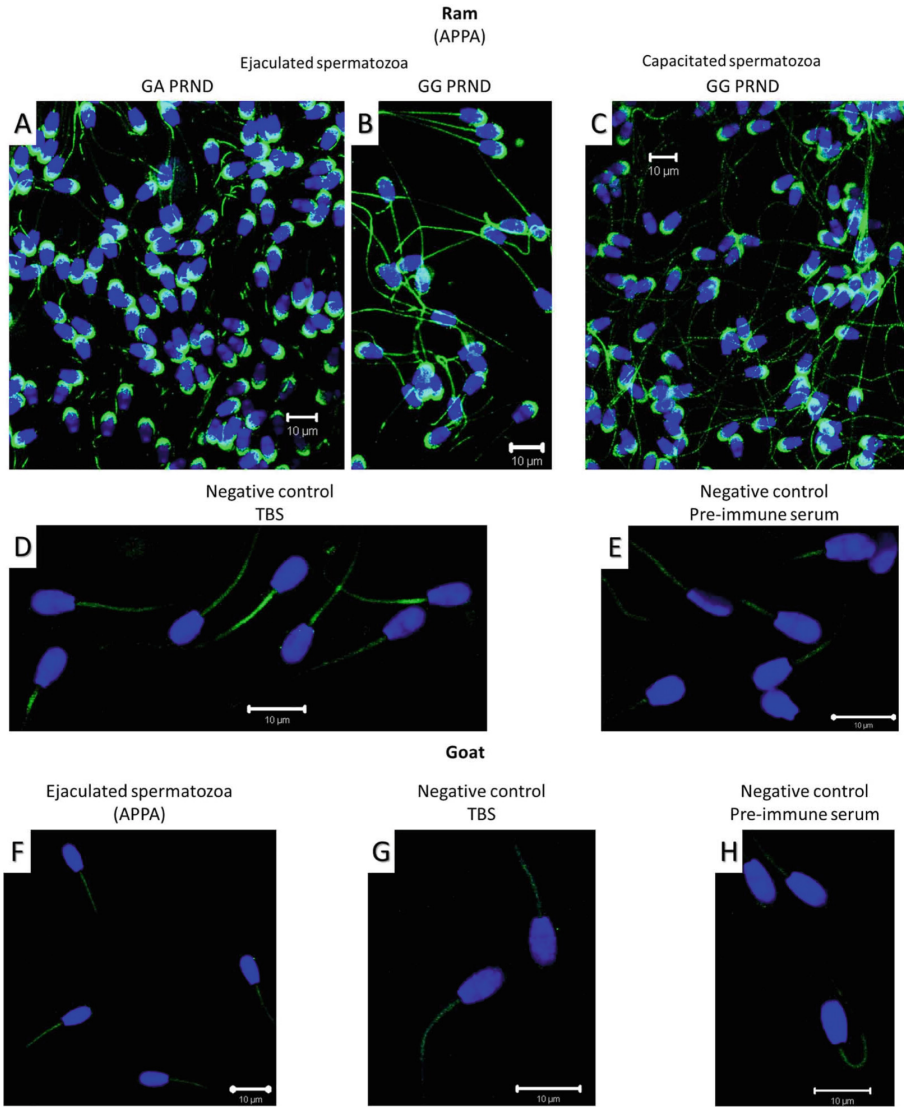

Fig. 2. Immunofluorescence images of Prt location in spermatozoa with anti-ovine Prt polyclonal antibody (APPA). Immunofluorescence images of prion protein testis specific (Prt) location in spermatozoa, using mouse anti-ovine Prt polyclonal antibody (APPA). Ovine Prt (Churra breed) is present on the ram spermatozoa head apical ridge subdomain, corresponding to the membranes over the acrosome, after ejaculation (A and B) and in vitro capacitation (C). Same results were obtained for the Merino breed animals (data not shown). No immunofluorescence staining was detected with APPA on goat (Serrana breed) spermatozoa (F). Negative controls (D, E, G and H) were carried out with samples processed exactly the same way but using either pre-immune serum from the same BALB/c mouse (E and H), or TBS (D and G) as the primary antibody. GG-wild-type genotype; GA-heterozygous [synonymous polymorphism (G78A substitution) in codon 26 of the *prnd* gene]. Anti-mouse FITC antibody (green), nucleus (DAPI/blue), Scale bar: 10 µm (Pimenta et al. 2012a)

improves spermatozoa motility, vigour, viability and fertilization rate. This observation suggests an important function for Doppel during sperm capacitation and the consequent fertilization process, and may argue in favour of putative physiological functions of soluble forms of Doppel. This could be of fundamental importance for biomedical applications of this prion like protein. In a recent study, Ferreira et al. (2017) related different polymorphisms in codon 26 of the ovine PRND gene with sperm capacitation, sperm cryotolerance and *in vitro* embryo production. Results from this study lead to the suggestion that the AA genotype or even carriers of the A allele should be actively selected, by using specific ovine breeding programs, to maximise male fertility. The identification of Doppel protein in ejaculated ovine spermatozoa and its decreased expression after the cryopreservation process further suggested an important physiological role for Doppel in male fertility. However, although greater *prnd* mRNA abundance was detected in frozen–thawed semen of bulls with high sire conception rate (Kasimanickam et al. 2012), we could not demonstrate differences in PRND abundance in ovine spermatozoa.

3.1.3 Prnt

In humans, all three *prnt* isoforms were only detected in adult testis, and were not present in any of the human fetal tissues (including testis) analyzed (11.5–16.5 week gestation) by Makrinou et al. (2002). It was suggested that human *prnt* expression is restricted to cell types found exclusively in testis such as Sertoli, Leydig, and germ cells at various stages of maturation. Absence of *prnt* expression within fetal testis implied that the function of *prnt* is required at a post-pubertal stage, when the above-mentioned cell types are actively involved in the production of male sex hormones and sperm. In caprine, *prnt* is weakly and stochastically expressed in both testes and ovaries at various developmental stages, suggesting either that the expression pattern of this gene differs between ruminant and human or, most probably, that ruminant *prnt* is a pseudogene (Kocer et al. 2007). However, we were able to demonstrate (Pimenta et al. 2012b) for the first time the presence of Prt protein in ruminants, using a new developed anti-ovine Prt polyclonal antibody (named APPA). When tested by indirect immunofluorescence, APPA showed high avidity to the ram sperm head apical ridge subdomain (Fig. 2), before and after induced capacitation. Ovine Prt was also found in the testis when assayed by immunohistochemistry, where spermatogones, spermatocytes, spermatids and spermatozoa, stained positive (Fig. 3). These observations strongly suggest ovine *prnt* to be a translated protein-coding gene, pointing to a role for Prt protein in the ram reproductive physiology. More recently, Li et al. (2018) identified a significant relationship between ovine growth performance and polymorphisms in the PRNT gene. Tested *loci*, were significantly associated with different growth traits, namely: body length index, head length, head depth, and also ewe teat numbers, reinforcing our findings that PRNT gene, at least in sheep, is a functional gene.

Sperm-egg plasma membrane interaction is a critical step in fertilization, which requires a series of proteins from both spermatozoa and oocyte to mediate membrane adhesion and subsequent fusion (Ying et al. 2010). In order to assess the effect of Prt in ovine fertilization, anti-Prt antibody (APPA) was added to the fertilization medium during *in vitro* fertilization (IVF) (Pimenta et al. 2013b). APPA supplemented fertilization medium decreased the number of fertilized oocytes and cleaved embryos. A

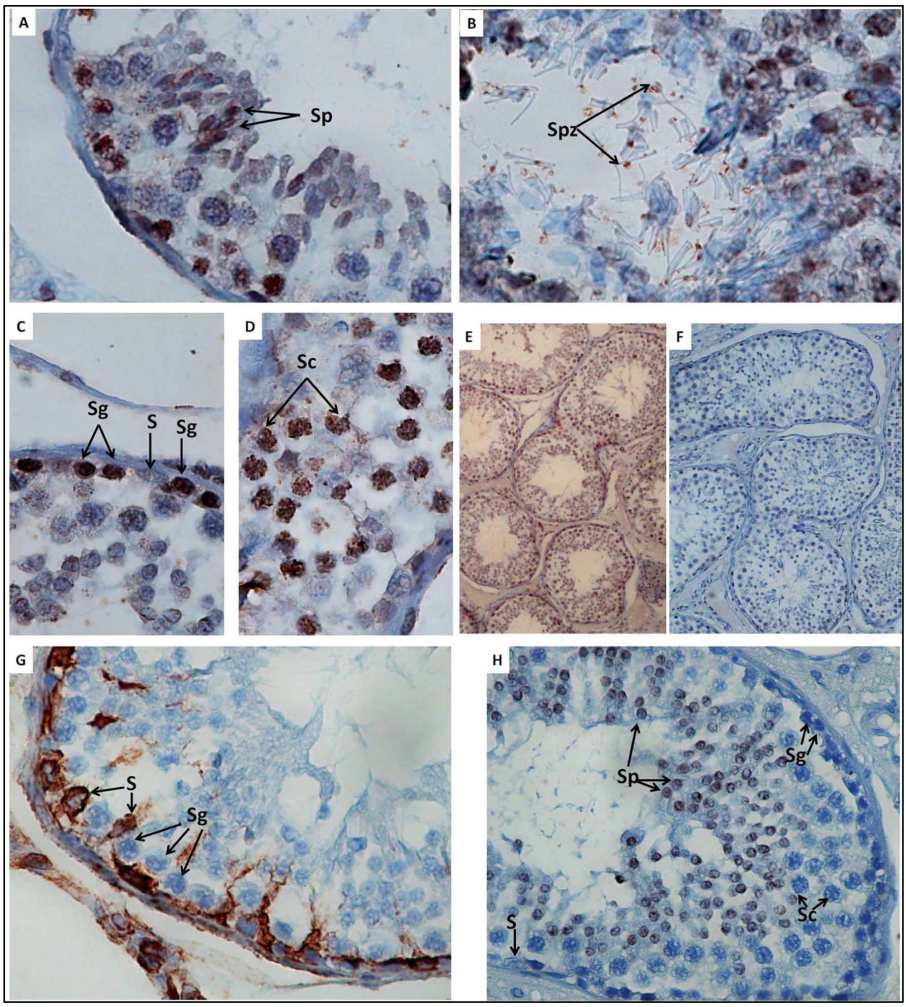

Fig. 3. Immunohistochemistry distribution of Prt in ram testis. Ovine prion protein testis specific (Prt) distribution in ram testis using immunohistochemistry, and produced anti-ovine Prt polyclonal antibody (APPA). APPA labeling of ram germinal cells in seminiferous tubules (E). Positive cells were detected (arrows) in spermatogonia (C), spermatocytes (D), spermatides (A), and spermatozoa (B). Negative control using mouse control serum (pre-immune), as the primary antibody (F). Same results were obtained when incubating sections with the secondary antibody alone (data not shown). Vimentin (G) and Ki-67 antigen (H), immunodetection of Sertoli cells cytoskeletal and spermatides, respectively. Spermatogonia (Sg), spermatocytes (Sc), spermatides (Sp), spermatozoa (Spz), Sertoli cell (S). Magnifications (A, B, C and D) × 1000, (E and F) × 100 (Pimenta et al. 2012b).

computational model was constructed to infer how results could be related to a hypothetical role for Prt in sperm-zona pellucida (ZP) binding. Circular dichroism (CD) analysis reinforced the predicted ovine Prt trend towards an α-helical structure. Predicted protein-protein docking suggests a possible interaction between Prt and ZP, further supporting an important role for Prt in ovine fertilization. Taken together, these results suggest that Prt may exert its main function in the initial steps of the fertilization process, possibly through a structural interaction with the ZP domain proteins. *prnt* gene was also found to be highly polymorphic in Portuguese sheep (Mesquita et al. 2016). The PRNT gene-coding region was analyzed by single-strand conformation polymorphism and sequencing, allowing the identification of the first ovine PRNT polymorphisms, in codons 6, 38, 43 and 48: c.17C > T (p.Ser6Phe, which disrupts a consensus arginine-X-X-serine/threonine motif); c.112G > C (p.Gly38 > Arg); c.129T > C and c.144A > G (synonymous) respectively. O'Flaherty et al. (2004) reported that phosphorylation of the above mentioned X motif (where X represents any amino acid), characteristic of protein kinase A (PKA) substrates, is increased during human sperm capacitation. Furthermore, in human and mouse sperm, PKA was found in both the acrosomal cap and the flagellum (Pariset and Weinman 1994; Visconti et al. 1997), which is in accordance with the detection of Prt in the acrosome region of ejaculated ram spz (Pimenta et al. 2012a). In a recent work, we observed that the c.17C > T (Ser6) variant was the most common, followed by p.Ser6Phe and Phe6 variants (Pereira et al., 2018). These polymorphisms of PRNT coding region seem to interfere on the ram spermatozoa mRNA transcript level, and on sperm freezability and ability to trigger embryo development. Overall, these results may indicate that Prt have a role in the phosphorylation signaling pathway, an important physiological prerequisite for the sperm cell to acrosome react and fertilize the oocyte.

3.1.4 Sprn

Young et al. (2011) generated reporter mice carrying a transgene to study the expression profile of Shadoo. In the testicle, *sprn*-LacZ was expressed in the Leydig cells, the site of testosterone biosynthesis, which is required for the development of the male reproductive system, and the initiation and maintenance of spermatogenesis. In sheep, *sprn* transcription presented high levels in cerebrum and cerebellum, and low levels in testis, lymph node, jejunum, ileum, colon and rectum (Lampo et al. 2007). Also, in *Bos taurus*, northern blot analysis showed that *sprn* is transcribed at high levels in brain and at a less extent in testis and lung (Uboldi et al. 2006). *sprn* transcription suggests a role for Shadoo in fertility and reproduction, that demands further investigation.

3.2 Prion-like Genes in the Female Reproductive System

3.2.1 Prnp

PrP^C expression was associated with ovarian follicle growth and development (Forde et al. 2008). This protein was detected in the ovary, oviduct and uterus of pregnant and cyclic ewes (Moudjou et al. 2001; Tuo et al. 2001) and is highly expressed in human placenta (Alfaidy et al. 2013)

3.2.2 Prnd

A role for Doppel in ovarian differentiation is suggested by the Doppel detection in germ cells of goat fetuses and in bovine granulosa cells and follicular fluid (Rondena et al. 2005; Kocer et al. 2007). This last observation reinforces the idea that Doppel may contribute to regulate fertility, since follicular fluid was shown to influence sperm motility and fertility (Rodriguez et al. 2001; Allais-Bonnet and Pailhoux 2014).

3.2.3 Sprn

To determine the role of PRNT and SPRN in bovine granulosa cells, Pimenta et al. (2018) conducted post-transcriptional gene silencing (siRNAs) of PRNP and SPRN genes in a pooled sample of granulosa cells from slaughterhouse-derived bovine ovaries. The expression of two main steroidogenesis genes was quantified: CYP11A1, which is involved in the conversion of cholesterol to pregnenolone, and CYP19A1, which catalyzes the last steps of estrogen biosynthesis. The *sprn* expression knock-down decreased the expression of the CYP11A1 gene ($p < 0.05$; 2.14-fold; 53.2% reduction), but not that of the CYP19A1 gene. Primed by work from other authors (Premzl and Gamulin 2007; Dalai et al. 2017), we also studied the methylation pattern of the promoters of these two prion family genes. At the studied regions, the SPRN gene promoter exhibits an altogether higher level of methylation, especially in a region of 4 CpGs dinucleotides. These studies conducted to the detection (for the first time, to the best of our knowledge) of expression of the SPRN gene in bovine granulosa cells, and an association between SPRN and CYP11A1 expression. Also, different PRNP and SPRN gene promoter methylation patterns were identified. Transgenic reporter mice for SPRN showed Sprn-LacZ expression in the granulosa cells of the developing follicle. The transgene was consistently expressed only in specific cell types of the testicle (Leydig cells) and ovary (granulosa cells), suggesting a role for Shadoo in fertility and reproduction (Young et al. 2011). The above results suggest an important role for SPRN gene in female reproduction, which prompts for further investigation.

4 Prions Are Forever

Prion diseases or TSEs are neurodegenerative disorders of humans and animals with so far no effective treatments or cure (Lloyd et al. 2011). This has partially overshadowed the most beneficial side of this gene family, which is present in diverse organisms and have probably co-evolved for thousands of years. In fact, although the importance of prion-like genes and proteins in the reproductive field has been highlighted, further studies are necessary to uncover the full extent of the role that normal prions play in the maintenance of cellular homeostasis and body function. Recent groundbreaking research suggests that harmful prions are the "black sheep" of a protein family that actually benefits the body. For instance, prion-like proteins seem to be critical for maintaining long-term memories in mice, and probably in other mammals (Fioriti et al. 2015), help us to distinguish between odours (Le Pichon et al. 2008), prevent brain damage during a stroke (Shyu et al. 2005), aid yeast survival in harsh conditions (Alberti et al. 2009), and act as an adhesive between cells in the embryo (Málaga-Trillo et al. 2009). As

mentioned by Halliez et al. (2014), the general function of PrPc could be to facilitate responses to external stimulus/factors at the cell- and at the organism-scale. By studying prions, we will gain new insight into how organs (namely brain and gonads) function and how its cells communicate with each other. The importance of normal prions and their prion-like relatives are now unanimously recognized. As such, in the light of the foregoing, and in the words of Westaway et al. (2011) when referring to biomedical research with prion-like proteins: "the coming years of molecular exploration should be extremely interesting". We are now in the edge of changing the paradigm of prions being exclusively considered as pathogens.

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Unraveling Notch Signaling in Reproductive Biology

D. Murta[1,2], E. Silva[1], A. Trindade[1], D. Henrique[3], A. Duarte[1], and L. Lopes-da-Costa[1(✉)]

[1] Reproduction and Development Laboratory, CIISA, Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
lcosta@fmv.ulisboa.pt
[2] CBIOS, Faculty of Veterinary Medicine, Lusófona University of Humanities and Technologies, Lisbon, Portugal
[3] Institute for Molecular Medicine, Faculty of Medicine, University of Lisbon, Lisbon, Portugal

Abstract. Studies over last years have consolidated a relevant role for Notch signaling in mammalian reproductive biology and in reproductive pathological scenarios. Notch genes are dynamically transcribed and Notch proteins dynamically expressed in the reproductive tract. Involvement in key reproductive events is mainly related with the regulation of the pace of cell proliferation and differentiation. Physiologically, this occurs in spermatogenesis, along the epithelial seminiferous cycle, during folliculogenesis and luteal development along the estrous/menstrual cycle, as well as during establishment of pregnancy and placentation. Aberrant Notch signaling has also been associated to genital tract disease, infertility and pregnancy failure. Although insights into the mechanistic action of Notch in these several scenarios have been highlighted, the overall picture of Notch is still fragmentary and elusive. Extensive research being presently conducted may reveal even novel and unsuspected branches into the still highly complex modes of action of this signaling pathway.

Keywords: Notch · Spermatogenesis · Estrous cycle · Mouse

1 Introduction

Regulation of mammalian reproductive physiology, at the cellular level, requires a finely tuned intercellular communication coordinated by cell signaling pathways. The Notch cell signaling pathway is a key regulator of cell fate specification (differentiation), cellular proliferation and apoptosis (Artavanis-Tsakonas et al. 1995). These cellular events are the essence of the most relevant embryonic and adult reproductive physiologic and pathologic scenarios. Notch action is both ubiquitous and highly conserved among taxons, being linked to *Drosophila* (López-schier and St Johnston 2001; Ward et al. 2006; Assa-Kunik et al. 2007; Song et al. 2007; Kitadate 2010) and *C. elegans* (Kimble and Crittenden 2007) reproductive biology. This has long prompted the Reproduction and

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Development Lab team to unravel the roles of Notch signaling in mammalian reproductive and developmental physiology, using the mouse model. In this review we address the enrolment of Notch signaling in male and female reproductive biology.

2 The Notch Signaling Pathway

Notch was first identified in *Drosophila* and since then up to humans. In mammals, four receptors (Notch1-4) and five ligands (three delta-like - Dll1, Dll3 and Dll4 - and two serrate-like - Jagged1 and Jagged2) were identified so far (Borggrefe and Oswald 2009). Notch is a ligand-receptor based signaling pathway between adjacent cells, where ligands and receptors are single pass trans-membrane proteins anchored to their respective cell surfaces. Notch receptors are activated by binding with ligands expressed in neighboring cells. This binding promotes two proteolytic cleavages in the receptor, the first catalyzed by ADAM-family metalloproteases and the second by a γ-secretase. The latter releases Notch intracellular domain (NICD), which is translocated to the nucleus. Here, together with co-activators, NICD converts the DNA-binding protein Rbpjk from a transcriptional repressor to an activator of Notch target genes (Borggrefe and Oswald 2009). The more ubiquitous Notch target genes are the *Hes* and *Hey* families of transcription factors (Ohtsuka et al. 1999; Iso et al. 2001; Fischer and Gessler 2007). Besides activation of target genes via Rbpjk, referred to as the canonical pathway, non-canonical forms of Notch activation (Fischer and Gessler 2007) and Notch signaling at a distance, without direct cell contact (Sheldon et al. 2010; Lu et al. 2013; Murta et al. 2016) were also described. Although ligands may show differential affinities to receptors (Benedito et al. 2009), and signaling through different receptors may evoke different results (Shimizu et al. 2002; Yuan et al. 2012), Notch components may be redundant in some scenarios (Zeng et al. 1998; Kitamoto et al. 2005).

Notch regulation of cell fate decisions may act through several modes. Lateral inhibition occurs when, in a type-related group of cells, one cell begins to increase Notch ligand expression. This activates a Notch signaling cascade in neighboring cells, inhibiting their differentiation (Bray 2006). Lateral induction occurs when the Notch activation ligand belongs to a different cell type in close proximity. This determines the cell fate of the Notch receptor cell. In the absence of activation the cell progresses to a different cell fate (Borggrefe and Oswald 2009). In the above ways, Notch signaling can regulate the preservation of the stem cell state and thus, guarantee the stem cell pool maintenance. Alternatively, Notch signaling may drive a terminal differentiation program by inducing cell cycle arrest (Borggrefe and Oswald 2009).

3 Notch Enrolment in Mouse Post-natal Testis Development

Notch is enrolled in testis embryonic (Tang et al. 2008; Hahn et al. 2009; DeFalco et al. 2013) and post-natal (Dirami et al. 2001; Hayashi et al. 2001; Hasegawa et al. 2011; Garcia et al. 2013; Murta et al. 2013) development. In the mouse, only Sertoli cells and spermatogonia are present in seminiferous tubules until post-natal day 8, starting meiosis at post-natal day 10 (Nebel et al. 1961; Bellvi et al. 1977). Table 1 presents

Table 1. Reported transcription and expression of Notch components, and of Notch signaling in mouse post-natal testis development

Post-natal day	Event	Reference
1	*Notch1* transcribed in Sertoli cells and spermatogonia	Hasegawa et al. (2011), Huang et al. (2013)
3	Notch signaling activated in Sertoli cells	Garcia et al. (2013)
4	Notch3 and Dll4 expression in Sertoli cells and spermatogonia	Murta et al. (2013)
5	N1ICD in Sertoli cells *Jagged2* transcribed in spermatogonia	Hasegawa et al. (2011)
6	Notch2 and Jagged1 expression in Sertoli cells Notch1, Notch2, Notch3 and Dll1 expressed in spermatogonia	Dirami et al. (2001)
15	Notch3 expressed in spermatogonia Dll4, Notch1 and Notch2 expressed in germ cells initiating meiosis	Murta et al. (2013)
19	Notch1 and Jagged2 expressed in pachytene spermatocytes and in round and elongated spermatids	Hayashi et al. (2001)
20	Hes1 expressed in Sertoli cells	Hasegawa et al. (2011)
30	Notch2, Notch3 and Dll4 expressed in germ cells Dll1 and Jagged1 specifically expressed in elongated spermatids	Murta et al. (2013)

evidence of Notch transcription and expression, and activation of signaling in post-natal testis development.

Due to the early expression of Notch1 and activation of Notch signaling through Notch1 intracellular domain (N1ICD) in post-natal life, the first mutant mouse designed to evaluate Notch role in reproductive biology had constitutive expression of active Notch1 (Lupien et al. 2006). This resulted in abnormal duct formation and sterility, a phenotype similar to that later observed in mice with deficiency of lunatic fringe (Hahn et al. 2009). Later on, a mutant mouse with the constitutive activation of Notch1 in Sertoli cells evidenced Notch enrollment in germ cells pool maintenance (Garcia et al. 2013).

Notch is also involved in embryonic and post-natal Leydig cells development (Tang et al. 2008; DeFalco et al. 2013; Murta et al. 2013). Receptors Notch2 and Notch3 and ligands Dll1 and Dll4 are expressed in Leydig cells at post-natal days 4 and 15 (Murta et al. 2013). Through the use of TNR-GFP transgenic Notch reporter mouse, an enrollment of Jagged1 in maintenance of Leydig progenitor cells was observed (DeFalco et al. 2013). Notch signaling activation in fetal Leydig cells may be important to development

up to the transition to adult Leydig cells prior to puberty (DeFalco et al. 2013; Murta et al. 2013). However, this might not be required during sexual maturity, as a post-natal decline of Notch signaling in Leydig cells was observed (DeFalco et al. 2013).

4 Notch Enrollment in Spermatogenesis

Notch signaling operates during adult spermatogenesis. Sertoli cells express Notch1 and Notch2 (Sahin et al. 2005; Hasegawa et al. 2011; Murta et al. 2013) and activation of signaling through N1ICD and Hes1 was detected (Hasegawa et al. 2011). This was confirmed through the study using a mouse TNR-GFP transgenic Notch reporter (Garcia et al. 2013). More recently, expression of Jagged1 in Sertoli cells was evidenced by western blotting experiments on different types of primary cells and microdissected tubules, oscillating during the seminiferous cycle (Okada et al. 2016).

Germ cells express most Notch components (Mori et al. 2003; Sahin et al. 2005; Hasegawa et al. 2011; Huang et al. 2013; Murta et al. 2013). Receptors Notch1-4, Jagged2 and NICD (1, 2 and 3) were detected in spermatogonia. Transcription of *Jagged2* and expression of receptors Notch1-4 and Dll4, and presence of NICD (1, 2 and 4) were detected in spermatocytes. Round spermatids express all Notch receptors and Dll4, and the presence of NICD (1, 2 and 4) was also detected. Elongated spermatids express receptors Notch1-3, Dll1, Dll4 and Jagged1, and transcription of *Jagged1* was detected. The expression patterns of Notch components during spermatogenesis are schematically illustrated in Fig. 1.

Notwithstanding the evidential transcription and expression of Notch components in the testis, the role of this cell signaling pathway in spermatogenesis is still controversial. Two studies reported that Notch signaling, although present, was not essential for spermatogenesis (Hasegawa et al. 2011; Batista et al. 2012). These studies used *Pofut1* conditional loss-of-function mutant mice to block Notch signaling. Mutant male mice had normal spermatogenesis and were fertile. Protein Pofut1 is involved in Notch receptors configuration (Wang et al. 2001). However, an unrelated α-glucosidase1 can compensate for Pofut1 in promoting Notch folding and function and thus Pofut1 is not essential for stable cell surface expression of Notch (Stahl et al. 2008). Two other studies reported that receptor Notch1 is not essential for spermatogenesis (Batista et al. 2012; Huang et al. 2013). These studies used spermatogonia specific Notch1 conditional loss-of-function mutant mice. Again, mutant male mice had normal spermatogenesis and were fertile. However, redundancy in Notch receptors operate in most scenarios, as this is crucial to normal signaling, since paralogues exert redundant or additive functions in maintaining the balance (Zeng et al. 1998). Therefore, the above studies cannot entitle for a definite statement on the essential role of Notch signaling in spermatogenesis.

In contrast, several studies associated Notch signaling with physiological spermatogenesis. *In vitro* blockage of Notch1 and Jagged2 suppressed spermatogenesis (Hayashi et al. 2001). Mouse *in vivo* Notch canonical signaling blockade through a γ-secretase inhibitor (DAPT) induced abnormal spermatogenesis (Murta et al. 2014a, b), and a similar approach prompted for a role of Notch signaling in mouse sperm maturation in the epididymis (Murta et al. 2016). $AhR^{-/-}$ mice showed low fertility with degenerative changes in the testes, germ cell apoptosis, and a reduced number of early spermatids.

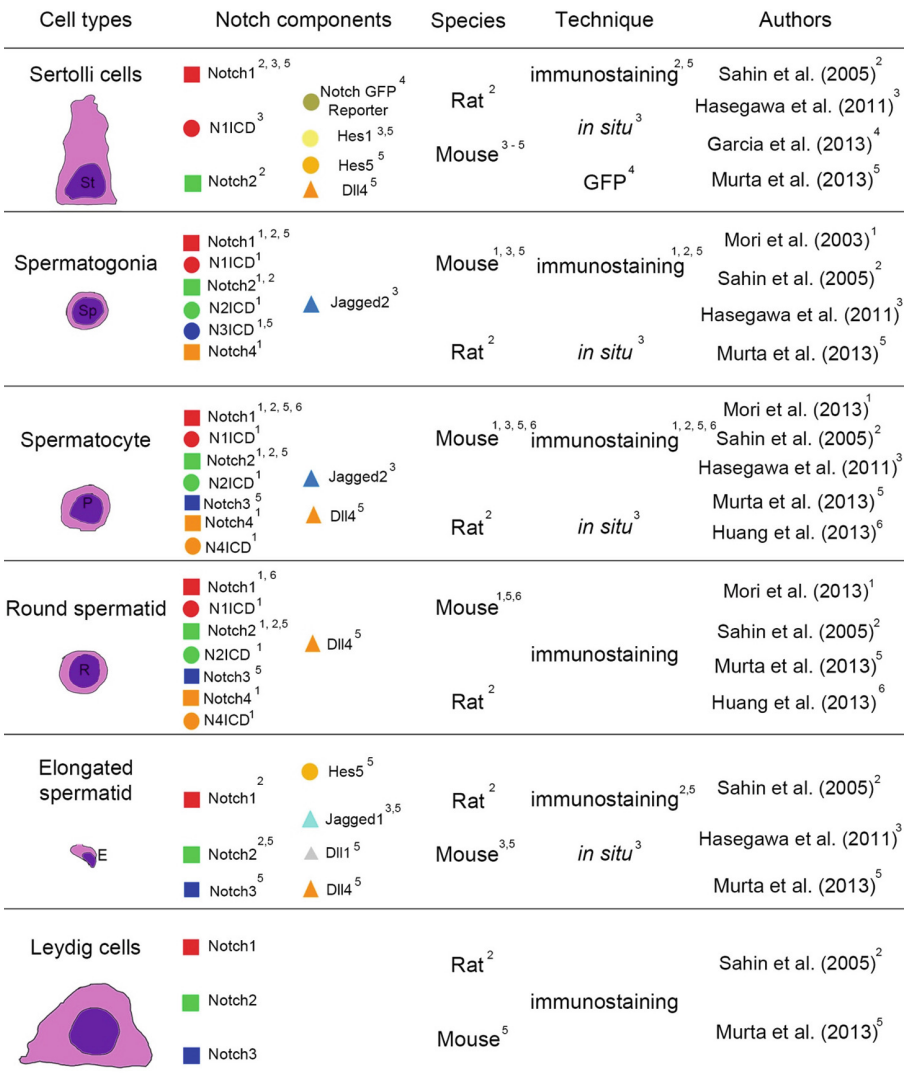


Fig. 1. Cellular localization of gene transcription and expression of Notch components in the mouse and rat testis.

This was associated to reduced expression of Notch1, Notch3 and Hes1, linking Notch signaling to early maturation of spermatocytes and a depletion of primary spermatids (Huanga et al. 2016). The constitutive activation of Notch1 in Sertoli cells evidenced a role in spermatogonia pool maintenance (Garcia et al. 2013), whereas Notch1 gain-of-function mutant mice evidenced a role in the regulation of male germ cells survival and differentiation (Huang et al. 2013). Additionally, through the use of double mutant experiments, Garcia et al. (2017), showed that canonical Hes1 and Hey1 derived Notch

signaling activation in Sertoli cells by Jagged1, induced a decrease of GDNF expression and associated Spermatogonial Stem Cells self-renewal. The participation of Notch signaling in Sertoli cells physiological regulation was also demonstrated in the case of nuclear and membrane androgen receptors (Kaminska et al. 2019). Finally, Notch signaling was shown to be regulated by Nkapl, a germ cell-specific transcriptional suppressor expressed in spermatogonia and early spermatocytes. Nkapl-deleted mice showed complete spermatogenesis arrest at the level of pachytene spermatocytes (Okuda et al. 2015).

Additionally, several studies associated Notch signaling with male dysfunctional spermatogenesis and infertility. Spermatozoa maturation arrest in male human infertility patients was associated to failure of Notch expression in seminiferous tubules (Hayashi et al. 2004a, b). Rat varicocele induced spermatogenesis arrest was related to altered expression patterns of Notch receptors (Sahin et al. 2005). As stated above, the constitutive activation of Notch1 (Lupien et al. 2006) and the deficiency of lunatic fringe (Hahn et al. 2009) resulted in male reproductive tract defects and infertility, although not associated to defects in spermatogenesis. All the above studies evidence now a relevant role for Notch signaling in mammalian male reproductive physiology and fertility.

5 Notch Enrolment in Ovarian Post-natal Development

In the mouse, during the first 4–6 post-natal days, individual oocytes become surrounded by somatic cells to form primordial follicles (Pepling and Spradling 2001). During this process, only one-third of the initial number of oocytes survives to form primordial follicles (Pepling 2012). Perturbations during this critical period of primordial follicle formation can significantly affect the size of the primordial follicle pool and follicular phenotypes (Trombly et al. 2009). In the mouse neonate ovary, Notch2 is expressed in granulosa cells and Jagged1 is expressed in oocytes (Trombly et al. 2009). Notch signaling blockage through a γ-secretase inhibitor resulted in retained germ cell nests and a reduced number of primordial follicles (Trombly et al. 2009). The conditional deletion of Notch2 in granulosa cells evidenced that Notch2-mediated signaling regulates oocyte apoptosis non-cell autonomously, and is essential for the breakdown of germ-cell nests and formation of primordial follicles (Xu and Gridley 2013). These pharmacological and genetic approaches demonstrated the enrollment of Notch signaling in the regulation of germ cells nests breakdown and primordial follicle formation.

6 Notch Enrollment in Ovarian Follicular and Luteal Development

Throughout adult life, just a small proportion of the ovarian pool of primordial follicles escape their arrested state and resume growth and development during estrous/menstrual cycles, the major proportion becoming atretic. Notch receptors were the first components to be detected in the ovary (Uyttendaele et al. 1996; Baker and Spears 1999). Notch2 and effector Hes1 are expressed in granulosa cells of primordial follicles (Trombly et al. 2009), and Notch2, Notch3, Dll1, Dll4, Jagged1 and other Notch effectors are expressed in granulosa cells of primary follicles (Trombly et al. 2009; Zhang et al. 2011; Murta et al. 2014a, b). These Notch expression patterns in granulosa cells are maintained during

primary follicles' progression into secondary follicles, when Jagged2 is also expressed (Johnson et al. 2001; Zhang et al. 2011; Jovanovic et al. 2013; Murta et al. 2014a, b). This pattern is then maintained in antral follicles, where Jagged1 is no longer expressed. All Notch receptors and both Jagged ligands were detected in collected human cumulus cells (Tanriverdi et al. 2013). Notch2, Jagged1 and several Notch effectors are also expressed in oocytes of all follicular stages (Johnson et al. 2001; Trombly et al. 2009; Zhang et al. 2011; Murta et al. 2014a, b). In mouse and rat ovarian *corpora lutea* (CL), receptors Notch1-4, Dll1, Dll4 and Jagged1 are expressed, and NICD (1 and 4) are detected (Johnson et al. 2001; Hernandez et al. 2011; Murta et al. 2014a, b). The ovarian expression patterns of Notch components during the estrous cycle are schematically illustrated in Fig. 2.

In the mammalian ovary, Notch is important for the initial formation and growth of follicles, and for regulating the proliferation and differentiation of follicular granulosa cells during the periovulatory period (for review see Vanorny and Mayo 2017).

Hormonal activation of the luteinizing hormone-receptor in the prepubertal mouse ovary, induces a Jagged1 localization shift from oocytes to somatic cells, and following *Jagged1* RNA interference knockdown, the loss of Jagged1 led to suppression of granulosa cell differentiation and steroid secretion (Prasasya and Mayo 2018). One remarkable feature of ovarian function is that germ cells signaling through Jagged1 may activate Notch in granulosa cells, which is essential for function of these cells (Hubbard et al. 2019).

Notch1, Notch3, Notch4, Jagged1 and Dll4 are expressed in ovarian blood vessels, which is associated with ovarian neovascularization during follicle and CL development (Johnson et al. 2001; Vorontchikhina et al. 2005; Hernandez et al. 2011; Zhang et al. 2011; Fraser et al. 2012; Tanriverdi et al. 2013; Jovanovic et al. 2013; García-Pascual et al. 2013). In fact, ovarian neoangiogenesis plays a crucial role in follicular growth and CL formation (Wulff et al. 2002; Zimmermann et al. 2003; Fraser et al. 2005; Fraser et al. 2006). Later on, Dll4 was found to be central in the regulation of CL angiogenesis and function (Fraser et al. 2012; García-Pascual et al. 2013). Although not interfering in follicular development (Fraser et al. 2012; Jovanovic et al. 2013), Dll4 induced Notch signaling exerted a luteotropic role by promoting luteal cell viability and steroidogenesis (Hernandez et al. 2011). Notch signaling inhibition with DAPT decreases *in vitro* production of progesterone by rat luteal cells due to a decrease in P450scc levels, also increasing apoptosis (Accialini et al. 2015).

At each estrous/menstrual cycle, the female reproductive tubular tract undergoes extensive remodeling, to render it receptive for the developing embryos. These changes involve a finely tuned synchrony of cellular proliferation, apoptosis and differentiation, along with extracellular matrix turnover, angiogenesis and leukocyte infiltration, in response to sex steroids (estradiol and progesterone). Presence of Notch components in the uterus was first shown in human samples through protein localization (Mazella et al. 2008; Cobellis et al. 2008) and gene transcription (Mikhailik et al. 2009). Later, animal models allowed to describe the Notch enrolment in uterine physiology (Afshar et al. 2012a; b; Degaki et al. 2012; Murta et al. 2015). Receptors Notch1-4, Dll4, Jagged1, Jagged2 and effectors Hes1, Hes2 and Hes5 show a dynamic expression pattern in the

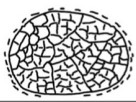
Structure/cells	Notch components	Species	Technique	Authors
Primordial follicles Granulosa cells 	Notch2[3,7]; Notch effectors[7]; Jagged1[7]; Dll4[7]	Mouse	Immunostaining	Trombly et al. (2009)[3]; Murta et al. (2014)[7]
Primary follicles Granulosa cells 	Notch2[3,5,7]; Notch3[7]; Notch effectors[5,7]; Jagged1[7]; Dll1[7]; Dll4[7]	Mouse	Immunostaining[3, 5, 7]; *in situ*[5]	Trombly et al. (2009)[3]; Zhang et al. (2011)[5]; Murta et al. (2014)[7]
Secondary follicles Granulosa cells 	Notch2[1, 5-7]; Notch3[1, 7]; Notch effectors[1, 5, 7]; Jagged1[7]; Jagged2[1]; Dll1[7]; Dll4[7]	Mouse	*in situ*[1, 5]; Immunostaining[5-7]	Johnson et al. (2001)[1]; Zhang et al. (2011)[5]; Jovanovic et al. (2013)[6]; Murta et al. (2014)[7]
Antral follicles Granulosa cells 	Notch2[1, 5-7]; Notch3[1, 7]; Notch effectors[1, 5, 7]; Jagged1[7]; Jagged2[1]; Dll1[7]; Dll4[7]	Mouse	*in situ*[1, 5]; Immunostaining[5-7]	Johnson et al. (2001)[1]; Zhang et al. (2011)[5]; Jovanovic et al. (2013)[6]; Murta et al. (2014)[7]
Oocytes 	Notch2[5, 7]; Notch effectors[3, 5, 7]; Jagged1[1, 3, 5, 7]; Dll4[7]	Mouse	*in situ*[1, 5]; Immunostaining[3, 5, 7]	Johnson et al. (2001)[1]; Trombly et al. (2009)[3]; Zhang et al. (2011)[5]; Murta et al. (2014)[7]
Corpus luteum cells 	Notch1[4, 7]; N1ICD[4]; Notch2[7]; Notch3[1, 7]; Notch4[4, 7]; N4ICD[4]; Notch effectors[7]; Jagged1[7]; Dll1[7]; Dll4[4, 7]	Mouse[1, 7]; Rat[4]	*in situ*[1]; Immunostaining[4, 7]	Johnson et al. (2001)[1]; Hernandez et al. (2011)[4]; Murta et al. (2014)[7]
Blood vessels 	Notch1[1, 2, 4, 6]; Notch3[6]; Notch4[1, 2, 4, 6]; Jagged1[2, 6]; Dll4[4, 6]	Mouse[1, 2, 6]; Rat[4]	*in situ*[1]; Immunostaining[2, 4, 6]	Johnson et al. (2001)[1]; Vorontchikhina et al (2005)[2]; Hernandez et al. (2011)[4]; Jovanovic et al. (2013)[6]

Fig. 2. Cellular localization of gene transcription and expression of Notch components in the mouse and rat ovary.

oviduct and uterine epithelia along the mouse estrous cycle (Murta et al. 2015). The uterine expression patterns of Notch components during the estrous cycle are schematically illustrated in Fig. 3. Notch signaling in the uterus was associated with uterine angiogenesis (Mazella et al. 2008; Degaki et al. 2012) and cyclic cellular events (Cobellis et al. 2008; Mikhailik et al. 2009; Murta et al. 2015).

Notch role in physiological and pathological scenarios within the mammalian reproductive tract are now well demonstrated, although the mechanisms behind are still poorly

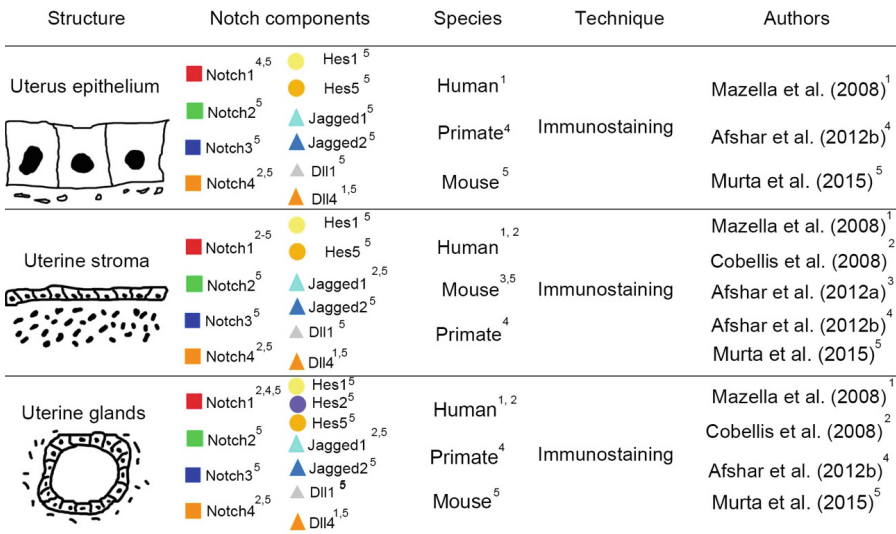


Fig. 3. Cellular localization of gene transcription and expression of Notch components in the mouse and rat uterus.

understood. Constitutive Notch signaling induced abnormal development of the oviducts, abnormal angiogenesis, and cyst formation in the mouse (Ferguson et al. 2016). Studies have shown relevant involvement in pregnancy establishment, decidualization and placentation, and several pathological conditions of pregnancy and of the genital tract. Notch1 and Dll1 were implicated in decidualization and embryo implantation (Degaki et al. 2012; Afshar et al. 2012a, b). In Notch gain-of-function transgenic mouse, conditionally overexpressing Notch1 intracellular domain (N1ICD) in the reproductive tract, results in complete infertility as a consequence of multiple developmental and physiological defects, including the absence of uterine glands and dysregulation of progesterone and estrogen signaling (Su et al. 2016). Also, Notch signaling is involved in endometrial–trophectoderm interactions during the initiation of attachment and angiogenesis in the placenta, and aberrant signaling is found in diseases of gestation, such as eclampsia and intra-uterine growth restriction (Cuman et al. 2014).

As in the male, Notch signaling is now considered a central regulatory cellular pathway of female reproductive events, encompassing the cyclic reproductive tract changes, embryo-maternal communication, implantation and placentation. Therefore, it is not surprising that aberrant signaling is associated with pathological scenarios in the above events. Current research aims to elucidate the complex mechanistic relationships of Notch, that operate in mammalian reproductive biology.

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Endothelial Dll4/Notch Signaling as a Target for Cancer and Wound Healing Therapy

A. Trindade[1(✉)], D. Djokovic[1,2P], L. Mendonça[1], M. Badenes[1,3P], L. Lopes-da-Costa[1], and A. Duarte[1]

[1] Reproduction and Development Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
adtrindade@fmv.ulisboa.pt
[2] Nova Medical School, Nova University of Lisbon, Lisbon, Portugal
[3] Instituto Gulbenkian de Ciência, Oeiras, Portugal

Abstract. Notch signalling is an evolutionarily conserved pathway that metazoan use to regulate cell-fate determination during development and maintain adult tissue homeostasis.

Numerous mutations in Notch pathway components have been shown to produce embryonic lethal phenotypes, notably linked to endothelial function. Our laboratory has studied the vascular function of the Notch pathway ligand Dll4 and used it as a target for novel therapeutic strategies. The study of Dll4 function by genetic manipulation revealed its importance for arterial endothelial cell specification and branching angiogenesis. Dll4 is highly expressed in tumoral vasculature and blocking Dll4 function with a novel biopharmaceutical, Dll4-Fc, was effective in reducing tumour growth in various *in vivo* cancer models. This effect resulted from an exaggerated vascular response to tumoral proangiogenic stimuli, that leads to an increased proportion of poorly functional tumoral blood vessels and thus reduced tumour blood supply.

It was also found that Dll4 exerts angiocrine effects over neighbouring tumour cells, regulating cancer stem cell biology, differentiation, and epithelial-to-mesenchymal transition, with the end result being an effect on metastasis onset. All these effects on cancer establishment, growth and progression into metastasis, regulated by Dll4, makes this a relevant target for cancer therapy.

Keywords: Dll4 · Notch · Cancer · Angiogenesis · Biopharmaceutical

1 The Notch Signalling Pathway

The Notch pathway is an evolutionarily conserved signalling mechanism that mediates intercellular communication in multicellular organisms (Artavanis-Tsakonas et al. 1999). Using a simple framework, the pathway transduces signals from the cell surface to the nucleus, regulates the expression of particular target genes and, in this fashion, determines cell fate decisions influencing cellular proliferation, differentiation or apoptosis. It has been shown to be fundamentally important in diverse metazoan developmental

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and physiological processes and to be associated with an array of human inherited and late onset diseases (Hansson et al. 2004).

In humans and mice the Notch family of proteins is composed of four receptors (Notch1-4) that are canonically activated by five ligands of the so-called DSL [Delta, Serrate (Jagged in mammals), Lag2] family: Delta-like (Dll) 1, 3, 4 and Jagged (Jag) 1 and 2. The Notch receptors and their ligands are single-pass transmembrane protein molecules with large extracellular domains that basically consist of epidermal growth factor (EGF)-like repeats (Artavanis-Tsakonas et al. 1999). Ligand binding promotes two proteolytic cleavages in the Notch receptor that are catalysed by ADAM-family metalloproteases and a γ-secretase enzymatic complex, respectively. The second cleavage results in the release of the Notch intracellular domain (NICD), which translocates to the nucleus where it interacts with the DNA-binding protein CSL [named after Cbf1, Su(H) and Lag1] and its co-activator Mastermind (Mam) to promote gene transcription. The known target genes involve helix-loop-helix type transcription factors known as hairy and enhancers of split (Hes – *e.g.*, Hes1, 5 and 7), and Hes-related repressor proteins (Herp – *e.g.*, Herp1, 2 and 3; also named Hesr, Hrt or Hey proteins) (Guruharsha et al. 2012).

2 Dll4 Signalling in Vascular Development

While Notch signalling regulates many aspects of metazoan development, it is critically involved in the cardiovascular system development. This has been shown early on by the identification of mutations in genes encoding the Notch pathway components causing inherited human diseases that exhibit vascular malformations, such as Alagille syndrome and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy CADASIL (Guruharsha et al. 2012).

Our lab has been involved in the study of the function of the Notch ligand Dll4 during embryonic development and in physiological and tumour angiogenesis. In order to study Dll4 function and simultaneously map Dll4 expression in mouse embryos and selected adult tissues we created a *knock-in* mouse line containing a *lacZ* reporter gene inserted downstream of the *Dll4* endogenous promoter by homologous recombination in embryonic stem cells. The resulting Dll4-specific beta-galactosidase expression allowed for high sensitivity and single cell resolution analysis (Duarte et al. 2004). Expression was detected in several tissues where Notch signalling was known to control cell-fate decisions, like the vascular system, the nervous system, the gastrointestinal system, and the thymus. Throughout embryonic cardiovascular development, Dll4 expression was seen only on endocardial cells and endothelial cells (ECs) of the arteries (Fig. 1), arterioles, and capillaries, being absent from vascular smooth muscle cells and veins. In the nervous system, expression was detected in the brain, neural tube, retina, and, for the first time, in the olfactory epithelium, vomeronasal organs and para-aortic bodies. Extensive Dll4 expression was also observed in the gut. This detailed expression analysis revealed new clues for both endothelial and non-endothelial Dll4 function in different organs (Benedito and Duarte 2005).

To try to understand the role of this gene in mammalian development and tissue homeostasis our group, in collaboration with the Rossant laboratory, was the first to report the

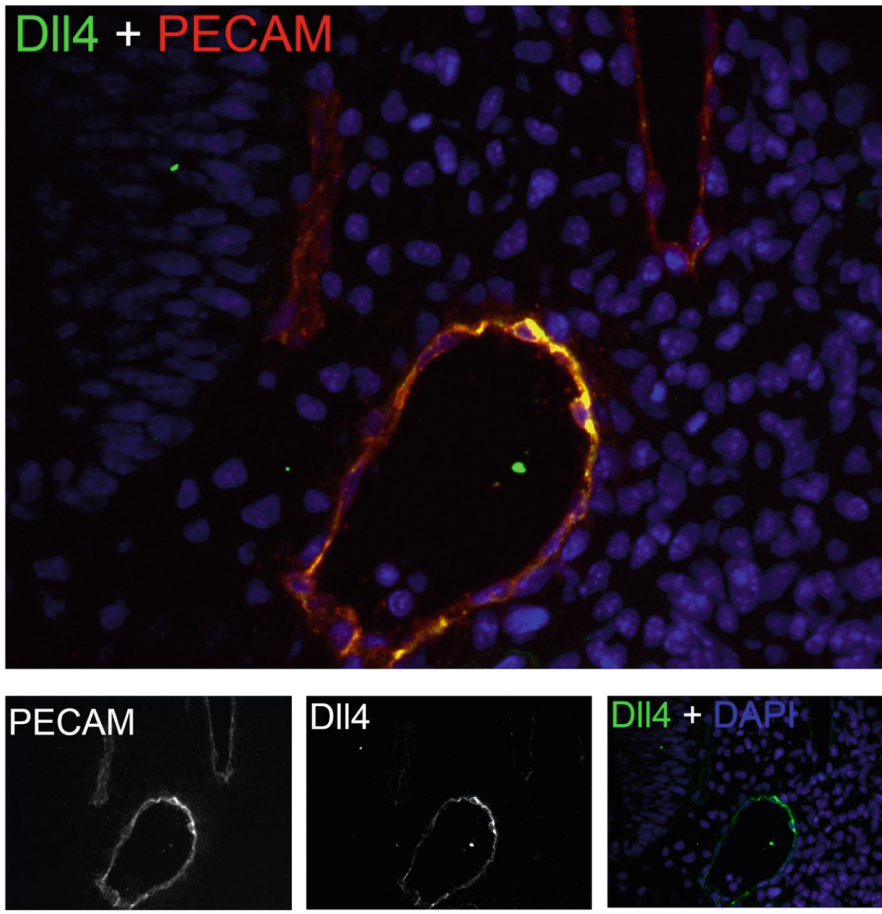

Fig. 1. Dll4 is expressed in the arterial endothelium of the developing mouse embryo. Immunofluorescence image of E9,5 embryo cryosection stained against Dll4, in green and PECAM, in red, with DAPI as nuclear counterstain, in blue. Dll4 is detected in all endothelial cells of the dorsal aorta, bottom center, but not in the endothelial cells of the anterior cardinal vein, top right, or endothelial cells from the perineural vascular plexus, top left.

loss-of-function phenotype of *Dll4*$^{-/-}$ mutant mice (Duarte et al. 2004). Remarkably, the requirement for Dll4 during mouse embryonic development was found to be strictly dosage-dependent. The heterozygous lethal phenotype was observed to be variably penetrant on an outbred genetic background (between approximately 60% and 100%), while deletion of both *Dll4* alleles led to a more severe and fully penetrant lethal phenotype. *Dll4*$^{+/-}$ heterozygous mice appeared at normal Mendelian frequencies at E8.5, began to exhibit increasingly severe defects at E9.5, with embryonic lethality occurring at approximately E10.5. *Dll4* hemizygosity was found to cause early pericardial oedema, absence of arterioles in the yolk sac, decreased arterial lumen in the embryo proper and a marked reduction of the dorsal aorta calibre. In addition, increased number of vascular sprouts

and branches, reduced arterial ECs while augmented endothelial venous phenotype, and induced premature fusion between the arterial and venous compartments was observed. Predictably, *Dll4*$^{-/-}$ embryos showed more severe and precocious vascular defects than the heterozygotes. Although the major blood vessels were formed in *Dll4*$^{-/-}$ embryos, their later development was completely impaired, none of the studied arterial markers was expressed in the endothelium, and at E10.5 no viable embryos could be found. This Dll4 mutant phenotype resembled that of the Notch1 and Notch4 double-mutants, suggesting that the biologic effects of Dll4 in embryonic development are essentially exerted through the activation of these two receptors (Krebs et al. 2000). This was the second gene, after that coding to Vegf, *Vegfa,* found to be hemizygous embryonic lethal due to severe defects in the developing cardiovascular system (Carmeliet et al. 1996; Ferrara et al. 1996). A subsequent, more detailed, analysis of these mutant embryos revealed that *Dll4*$^{-/-}$ ECs are more proliferative and more loosely connected to the basement membrane. This, in conjunction with a higher expression of Vegfr2, causes the ECs from the dorsal aortae to migrate dorsally, in response to a Vegf gradient produced by cells of the neural tube, thus causing the reduction or absence of these major embryonic vessels (Benedito et al. 2008).

The expression of Dll4 and its receptors, Notch1 and Notch4, was first reported to be mostly restricted to the arterial endothelium in early embryonic development (Villa et al. 2001). However, in collaboration with the Jones laboratory, we have shown that *Notch1* transcription in embryonic ECs starts with the onset of vascular flow and is initially pan-endothelial. Interestingly, it is the arterial specificity of Dll4 expression, and presumable activation of Notch1 there, that maintains Notch1 expression in the arterial endothelium, whereas in the venous endothelium it is gradually shut off. This means that *Dll4* is one of the earliest genes expressed specifically in arterial ECs (Jahnsen et al. 2015).

Given the embryonic lethal phenotype described above, we next developed a conditional *Dll4* knockout mouse line using the cre/LoxP system. This line was used to study tissue-specific *Dll4* loss-of-function phenotypes in adult tissues, starting by producing endothelial specific *Dll4* knockout mice by breeding with a VE-cadherin-cre-ERt2 mouse line. In these mice the administration of tamoxifen resulted in the deletion of the floxed *Dll4* allele in ECs. When administered to pregnant females, the double transgenic offspring recapitulated the same endothelial phenotype that we first described in the constitutive knockout.

Furthermore, to characterize possible gain-of-function phenotypes, we created a conditional overexpressing *Dll4* transgenic mouse line using the TetO7 inducible promoter. Two different founders were obtained, displaying identical phenotypes when crossed with the endothelial specific tie2-rtTA-M2 transgenic line. Double transgenic embryos displayed grossly enlarged dorsal aortae and died before E10.5, showing variable degrees of premature arteriovenous fusion. Veins displayed ectopic expression of arterial markers. Other defects included reduced vascular sprouting, EC proliferation, and migration. Dll4 overexpression also inhibited VEGF signalling and increased fibronectin accumulation around the vessels. *In vitro* and *in vivo* studies of DLL4-FL (Dll4–full-length) in ECs recapitulated many of the Dll4 transgenics findings, including decreased tube formation, reduced vascular branching, fewer vessels, increased pericyte recruitment, and increased fibronectin expression (Trindade et al. 2008).

Analysis of vessels in the embryonic hindbrain and post-natal retina of *Dll4*$^{+/-}$ mice, in collaboration with the Eichmann laboratory, revealed a striking phenotype, with greatly increased number of filopodia-extending endothelial tip cells (Suchting et al. 2007). Tip cell marker genes were ectopically expressed in most ECs, instead of being restricted to the tip cells as in the wild-type littermates. These data suggest that Dll4/Notch signalling is involved in suppressing endothelial tip cell identity. Formation of filopodia by tip cells in the retina is dependent upon Vegfa stimulation (Gerhardt et al. 2003). We observed that inhibition of VEGF signalling by sequestering Vegf or blocking Vegfr2 activation resulted in loss of filopodia in *Dll4*$^{+/-}$ mouse retinas. In addition, heterozygous mice showed reduced expression of *Vegfr1* and increased expression of *Vegfr2* in the retinal blood vessels, which may account for their increased sensitivity to Vegfa. Thus, Dll4 and Vegfa appear to act antagonistically to regulate vascular sprouting, with Dll4 inhibiting the formation of tip cells in response to Vegfa stimulation, resulting in only a few selected cells being able to form a new sprout. As the tip cell receives more Vegfa signal, this in turn upregulates Dll4 expression. Dll4 on the tip cell membrane signals to the adjacent cells, reducing their sensitivity to Vegfa and therefore preventing their differentiation into tip cells and directing them instead to a stalk cell fate.

A transcriptomic analysis of retinal ECs isolated from *Dll4*$^{+/-}$ and wild-type mice, allowed the identification of a number of tip cell-enriched genes that could be divided in three major groups: genes encoding for extracellular matrix degrading enzymes, for basement membrane components and for secreted molecules. Thus, tip cells may regulate angiogenesis via matrix remodeling, production of basement membrane, and release of secreted molecules, some of which regulate stalk cell behaviour (del Toro et al. 2010).

In summary, data obtained using our *Dll4* mutant mouse lines strongly suggests an involvement of the Notch signalling pathway, mediated through the Dll4 ligand in a cell-autonomous manner, in the establishment of the endothelial arterial cell phenotype in mouse embryos. In addition, this ligand was shown to have a central role in the regulation of vascular branching morphogenesis, endothelial proliferation and migration and in the induction of vascular maturation by controlling endothelial expression of transmembrane receptors for pro-angiogenic growth factors.

3 Dll4 Signalling in Cancer Neoangiogenesis

Angiogenesis, the formation of blood vessels from pre-existing vasculature, is involved in the pathogenesis of a variety of disorders. In particular it was identified as one of the original hallmarks of cancer (Hanahan and Weinberg 2000). Given the role of angiogenesis in tumour growth, the identification of Dll4/Notch signalling as a key regulator of vascular development and homeostasis led to a series of studies examining Dll4 expression in different tumour types. Indeed, tumour ECs were found to express Dll4, in both animal tumour models and in different human tumours (Mailhos et al. 2001; Gale et al. 2004; Patel et al. 2005). In human tumour xenografts implanted in mice, Dll4 was found to be highly expressed in microvessels and small arteries while poorly detectable in venules and larger tumour vessels (Gale et al. 2004). When we analysed subcutaneous tumour grafts in *Dll4*$^{+/-}$ mice using lacZ as reporter, the expression of Dll4 was found to be much higher in the tumour vasculature than in adjacent normal vessels (Fig. 2) (Scehnet et al. 2007).

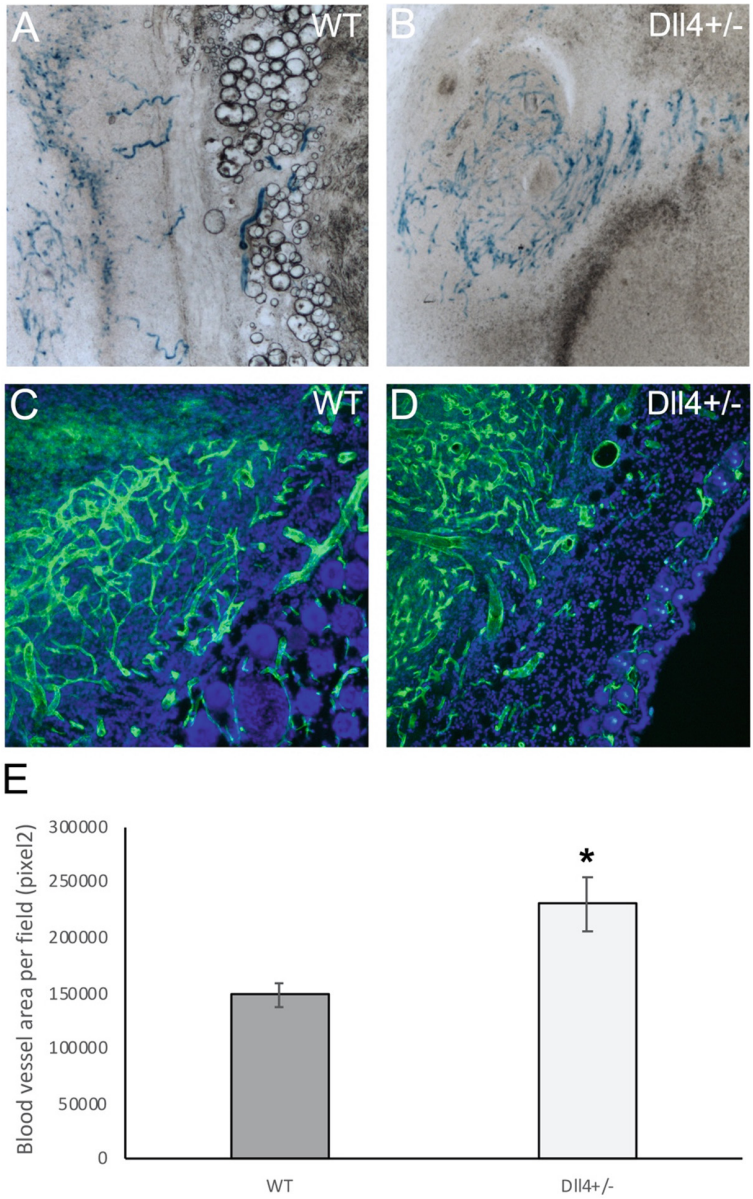

Fig. 2. Tumor vasculature expresses Dll4 and is more dense in Dll4 heterozygous mice ($Dll4^{+/-}$). (A) 80 μm Cryosection of a tumor showing expression of the lacz reporter, under control of the endogenous dll4 promotor, in the vasculature. (B) 80 μm cryosection of a tumor showing strong lacz expression in the tumor and weak in the adjacent skin area. (C) PECAM immunofluorescence of 40 μm cryosection of tumor growing in a WT mouse. (D) PECAM immunofluorescence of 40 μm cryosection of tumor growing in a $Dll4^{+/-}$ mouse. Note that the vasculature of the Dll4 heterozygous mouse's tumor appears to have a more finely branched vascular network than the vasculature on the tumor in the WT mouse. (E) Tumor vessel density is increased in $Dll4^{+/-}$ mice, relative to tumors grown on WT mice. Graph shows the average area of PECAM positive tissue per microscopy field of 40 μm cryosections of tumors analyzed as well as standard deviation for each group. Adapted from Scehnet et al. (2007).

Around the same time, pioneering work by Noguera-Troise and colleagues showed that the blockade of Vegfa in mice bearing tumour implants resulted in profound *Dll4* down-regulation (Noguera-Troise et al. 2006). Thus, the high levels of Dll4 expression in tumour endothelium observed by us and others were assumed to be a consequence of relatively higher levels of Vegfa signalling in tumours in comparison to normal tissues.

To define the function of increased expression of Dll4 in tumour vessels, a comparative analysis of the vasculature of tumour cell implants grown in age-matched wild-type and $Dll4^{+/-}$ mice was performed. The tumour vascular response in $Dll4^{+/-}$ mice was marked by highly increased vascular density. However, in contrast to the wild-type mice, the neovessels in the mutants displayed narrow caliber, exaggerated and aberrant branching patterns and lacked hierarchic organization. In addition, recruitment of vascular smooth muscle cells to the newly forming tumour capillaries was significantly reduced in $Dll4^{+/-}$ mice (Fig. 2). Thus, tumour angiogenic response was increased but defective, and vessel maturation and function were severely compromised. In parallel, the expression of a blocker of Dll4/Notch signalling (soluble Dll4 – Dll4-Fc) by transduced tumour cells was demonstrated to result in the formation of dense, highly branched and markedly irregular networks composed of fine and weakly perfused interconnections, while the expression of the full-length membrane bound form of Dll4 caused Notch activation in the tumour blood vessels and resulted in a reduction of tumour vessel sprouting and straighter branch formation (Noguera-Troise et al. 2006; Scehnet et al. 2007). Altogether, this data indicated that the Dll4-mediated signalling in tumours served as a negative regulator of sprouting angiogenesis and an important pro-maturation factor required for both regular branching and mural cell recruitment, contributing to the formation of a productive tumour vasculature.

4 Targeting Dll4 Signalling in Cancer

This understanding of the role of the Dll4 ligand led to the proposal to test anti-Dll4 therapy as a tool in the treatment of cancer. We and others developed anti-Dll4 therapeutic proteins of two different types: 1) Dll4 soluble proteins, in our case Dll4-Fc (a fusion between the extracellular domain of Dll4 and the Fc region of immunoglobulin) that binds to Notch receptors and acts as dominant-negative, preventing Notch signalling activation by endogenous Dll4 (Scehnet et al. 2007) and 2) anti-Dll4 antibodies that bind Dll4 proteins and prevent its activation of Notch receptors (Noguera-Troise et al. 2006).

Pharmacological Dll4/Notch inhibition, by intraperitoneal administration of Dll4-Fc in xenograft-bearing mice, was observed to lead to the defects in tumour vessels that are qualitatively identical to those caused by targeted *Dll4* allele deletion (Scehnet et al. 2007). More importantly, it was evident that systemic Dll4-Fc administration markedly decreased tumour growth. Notch receptors are activated by membrane-bound ligands and binding of the soluble Dll4-Fc prevents endogenous Notch activation, as confirmed by the down-regulated expression of the Notch target genes *Hey* and *Hes* in Dll4-Fc treated mice. By blocking Dll4 function, Dll4-Fc causes newly forming vessels to proliferate excessively, leading to deficient maturation and perfusion. Although the vascular density rate generally correlates with tumour growth, this poorly functional vascular network

resulted in reduced tumour perfusion that slowed malignant cell proliferation. Systemically administered soluble Dll4 variants and antibodies blocking Dll4/Notch signalling were shown to enhance immature and non-productive vascular responses, resulting in decreased perfusion, tumour hypoxia and suppressed tumour expansion, with little or unobserved systemic toxicity (Noguera-Troise et al. 2006; Ridgway et al. 2006; Scehnet et al. 2007). While Vegfa stimulates tumour endothelium to proliferate, Dll4/Notch signalling, induced by Vegfa itself (Noguera-Troise et al. 2006; Williams et al. 2006), suppresses chaotic sprouting and branching, and promotes vessel wall assembly, vessel lumenization and perfusion. In this way, Dll4/Notch signalling ensures the functionality of newly formed vascular plexus. Thereby, it can be assumed that combined inhibition of Vegfa and Dll4/Notch signalling, when appropriately balanced, is likely to be more effective in tumour growth suppression than Vegfa inhibition alone, resulting in both reduced and non-productive tumour angiogenesis.

To assess the potential therapeutic relevance of Dll4/Notch targeting on tumour angiogenesis, both genetic and pharmacological approaches were used to evaluate the signalling inhibition in autochthonous tumour models (Djokovic et al. 2010, 2015). One of the tumour models used was the transgenic RIP1-Tag2 (RT2) mice that develop pancreatic β-cell tumours (insulinomas). In this mouse model, host/tumour interactions are more reliably reflected then in tumour xenografts, while predictable stepwise progression and angiogenic switch makes it a particularly useful tool in the investigation of tumour angiogenesis and its inhibitors (Hanahan 1985). The consequences of *Dll4* allelic deletion on insulinoma onset, growth, associated vascular response and mouse longevity were analysed (Djokovic et al. 2010). *Dll4* hemizygosity did not influence oncogene-driven β-cell transformation as no difference in tumour number per mouse between RT2 $Dll4^{+/+}$ and RT2 $Dll4^{+/-}$ littermates was observed. However, remarkably reduced tumour growth and consequently increased RT2 $Dll4^{+/-}$ mouse longevity pointed out the importance of Dll4/Notch function for tumour development and the beneficial effect of Dll4/Notch inhibition, as previously observed in xenograft-based experiments. Mechanistically, in autochthonous insulinomas as well as in ectopic grafted tumours, *Dll4* allelic deletion resulted in increased vessel sprouting and led to the establishment of dense vascular networks with fragile branches that lacked adequate mural cell stabilization and, clearly, the capacity to provide appropriate blood supply to the tumours (Djokovic et al. 2010).

This excessive and aberrant vascular response associated with reduced Dll4 expression levels in RT2 tumour vessels, was next confirmed to reflect increased tumour vessel sensitivity to Vegfa (Djokovic et al. 2010), as observed in post-natal retinal angiogenesis upon genetic or pharmacological Dll4/Notch inhibition (Lobov et al. 2007; Suchting et al. 2007). It was also shown that Dll4/Notch impairment promoted Vegfa production, probably in response to deteriorated tumour oxygenation. There was also an increase in Vegfr2 levels and reduced expression of Vegfr1. Vegfr2 is the Vegfa principal receptor, whereas Vegfr1 binds Vegfa with much greater affinity then Vegfr2 but functions as a Vegfa trapper, having extremely weak signal-transducing properties (Shibuya and Claesson-Welsh 2006). Therefore, increased Vegfr2/Vegfr1 ratio in $Dll4^{+/-}$ RT2 mice enhanced Vegfa signalling and thus increased endothelial proliferation and vascular density.

As the *Dll4* allelic deletion resulted in tumour suppression in spontaneous RT2 insulinomas and the morphological and molecular basis of this effect became more apparent, this model was next used to assess the efficacy of our putative therapeutic protein, Dll4-Fc. It was tested alone and in combination with sEphB4 (Djokovic et al. 2010). This soluble monomeric form of the extracellular domain of EphB4 functions as an antagonist of Ephrin-B2/EphB4 signalling, having been previously documented to suppress sprouting angiogenesis in tumour models (Kertesz et al. 2006; Scehnet et al. 2009). Both Dll4-Fc and sEphB4 (Djokovic et al. 2010) proteins reduced insulinoma growth rates when applied as monotherapies in RT2 mice. sEphB4 was highly effective while, in the chosen inhibitory concentrations, Dll4-Fc resulted in even more pronounced tumour suppression (Fig. 3). As in the case of Dll4 hemizygosity, the number of neoplastic lesions per mouse remained substantially unchanged in both Dll4-Fc- and sEphB4-treated mice when compared with untreated controls. Thus, malignant transformation in the RT2 model was confirmed to be independent of Dll4/Notch and Ephrin-B2/EphB4 signalling, while the tumour growth was retarded by the inhibition of these signalling pathways. Combination of the two inhibitors was superior to each antagonist alone. Dll4-Fc resulted in enhanced endothelial proliferation in RT2 insulinomas, consistent with previously observed Vegfa/Vegfr2 activation resulting from Dll4/Notch inhibition while sEphB4 had the opposite effect, in accordance with findings that Ephrin-B2 positively controls Vegfr2 internalization and signalling (Sawamiphak et al. 2010). Both compounds, however, were evidenced to compromise tumour neovessel maturation defined by reduced mural cell recruitment, enhanced vessel leakiness and impaired lumenization. Considering that the combination of the two proteins resulted in a slight reduction of vessel density in relation to the controls, it appears that reduced vascular competence was the determining factor in the inhibition of tumour growth (Fig. 3).

As Dll4 induces Ephrin-B2 expression (Iso et al. 2006), this latter protein is downregulated in $Dll4^{+/-}$ mice. This, in combination with reduced Tie2 expression, contributes to the vessel wall assembly impairment associated with Dll4 deficiency. Although Dll4-Fc markedly increases and sEphB4 therapy significantly reduces tumour microvessel density, both lead to the paucity of pericyte recruitment and the formation of poorly functional blood vessels. Importantly, simultaneous Dll4/Notch and Ephrin-B2 inhibition was shown to have cumulative efficacy in blocking vessel maturation and perfusion of the tumour. Thus, inhibition of Dll4/Notch and Ephrin-B2/EphB4 provide effects that are not entirely overlapping even though Dll4/Notch signalling induces Ephrin-B2 expression. In addition, inhibition of Ephrin-B2 induces hypoxia and consequently upregulates the expression of Vegfa and Dll4 (Selkoe and Kopan 2003; Scehnet et al. 2009).

Regarding safety concerns related to Dll4 inhibition, *Dll4* allelic deletion in CD1 and RT2 mice did not cause vascular or other lesions in vital organs such as heart, lung, kidney, brain, liver and intestinal tract (Scehnet et al. 2007; Djokovic et al. 2010). Likewise, no toxic side-effects were observed to follow the intermittent administration of tumour-suppressive doses of Dll4-Fc, alone or in combination with sEphB4, in RT2 mice (Djokovic et al. 2010). In contrast, chronic application of Dll4/Notch antagonists

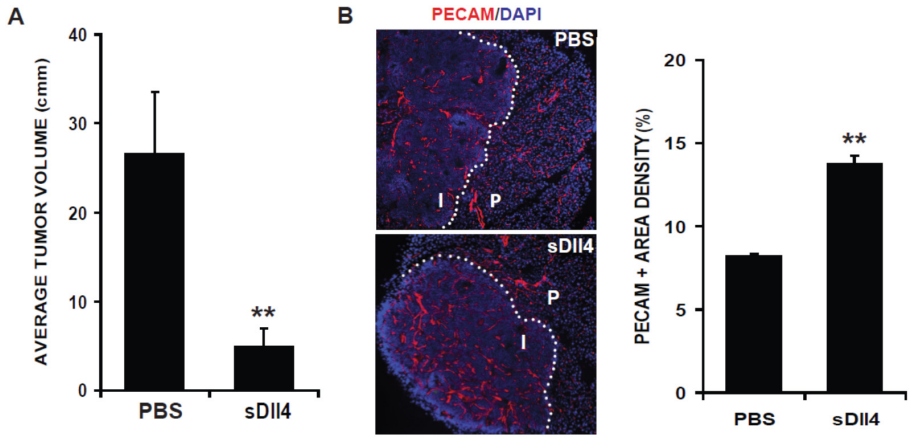

Fig. 3. sDll4 effects and mechanism of action assessed in RT2 interventional trials. *A,* Average tumor volumes developed by 13.5-week old RT2 male mice previously treated for 3.5 weeks with drug vehicle (PBS, i.p. 3x/wk, control group) or sDll4 (10 mg/kg i.p., 3x/wk). *B*, Representative images of PBS and sDll4-treated tumors immunostained to PECAM (*left*). *Dot lines* mark tumor border. *I* and *P* indicate insulinoma and normal pancreatic tissue surrounding the tumor, respectively. Vascular density was estimated as percentage of PECAM-positive area per tumor section surface and presented for two experimental groups (*right*). Adapted from Djokovic et al. (2010).

had been found to induce benign vascular neoplasms in rat livers as well as prominent atrophy of mouse thymus, where Dll4/Notch1 signalling is involved in the regulation of T-cell development (Yan et al. 2010). Using a conditional, endothelial specific *Dll4 knock-out* mouse line, we observed that these mutants also develop alterations in liver architecture. These include macroscopic micro-nodular liver surface aspect, areas with markedly dilated sinusoids and excessive subcapsular vessel proliferation with sporadic haemangioma-like structure formation (Djokovic et al. 2010). Encouragingly, endothelial *Dll4 knock-out* mice did not show evidence of vascular proliferative lesions when treated with sEphB4. In other words, simultaneous blockade of Dll4/Notch and Ephrin-B2/EphB4 abolished hepatic alterations seen when only Dll4/Notch signalling is impaired. Further specific studies are required to provide a comprehensive understanding of the side effects arising from long-term therapeutic Dll4/Notch inhibition. Special attention should be paid to the sites where physiological and reparatory angiogenesis occur, namely the female reproductive tract, bone fractures and soft tissue traumatic and ischemic lesions.

5 Exploring *Dll4* Gain-of-Function as an Approach to Cancer Therapy

Following the assessment of the Dll4 role in tumour angiogenesis by antagonizing its signalling, the effects of *Dll4* gain-of-function on tumour vascular response and growth were explored by using Tie2-rtTA-M2; TetO7-Dll4 conditional transgenic mice that overexpress endothelial *Dll4* upon tetracycline or doxycycline-induction (Trindade et al. 2008).

The endothelial *Dll4* up-regulation was examined in three tumour models: Lewis Lung Cancer (LLC) xenografts, chemically-induced skin papillomas and RIP1-Tag2 insulinomas. The analyses provided very consistent results leading not only to the confirmation of the cellular and molecular basis underlining the fundamental Dll4 involvement in tumour vascular biology, but also to the establishment of a concept that endothelial Dll4 function agonists represent an alternative anti-cancer strategy (Trindade et al. 2017).

In all three models, enhanced Dll4/Notch signalling in ECs restricted their proliferation, resulting in reduced tumour vessel density and improved vascular maturity and function, as indicated by larger branch formation, increased mural cell recruitment and improved vessel network perfusion. Despite the vascular normalization, the effect of vascular sprouting suppression was predominant since *Dll4* overexpression significantly inhibited tumour growth in both LLC xenografts and in autochthonous chemically-induced skin papillomas and RT2 insulinomas. The increase in Dll4/Notch function reduced Vegfa/Vegfr2 and Vegfc/VEgfr3 signalling, explaining the reduced EC activation.

Based on these results, *Dll4* overexpression joins the Dll4 inhibition as a modality that might improve cancer control, being exempt of toxic side-effects, as far as the evidence shows. Improved vessel competence seen in endothelial *Dll4* overexpressing mice resulted in better cytostatic delivery at the tumour site while improved vessel wall assembly probably played a part in decreasing the malignant cell penetration to the circulation and metastization (Trindade et al. 2017).

6 A Potential Role for Dll4 Signalling in Metastasis Development

As described above, endothelial *Dll4* loss-of-function or therapeutic targeting of Dll4 creates an immature vasculature with multiple points of access to the bloodstream (Scehnet et al. 2007; Djokovic et al. 2010, 2015; Badenes et al. 2017; Mendonça et al. 2019). Endothelial *Dll4* overexpression, on the other hand, enhances vessel wall coverage with smooth muscle cells, that constitute a barrier for tumour cells intravasation to the bloodstream (Trindade et al. 2017). It could therefore be assumed that the disorganized vasculature in endothelial *Dll4* loss-of-function mice would facilitate metastasis formation.

When we evaluated tumour growth in the endothelial-specific *Dll4* loss of function mutants (eDll4cKO), the first observation was that, in general, the angiogenic phenotype produced was identical to that observed after treating tumour-bearing mice with Dll4-Fc (Mendonça et al. 2019), a first confirmation of the cell-autonomous nature of the effect of this therapeutic protein. The second major observation was that, contrary to our expectation, the number and burden of distant site metastasis foci was substantially reduced (Fig. 4). One possible explanation could be that tumour cells easily escape through tumour neovessels but fail to achieve the bloodstream due to the dysfunctional state of these vessels. Alternatively, endothelial *Dll4* loss-of-function could be affecting two early metastasis mechanisms: epithelial-to-mesenchymal transformation (EMT) and cancer stem cell (CSC) maintenance (Mendonça et al. 2019). In fact, immunostaining and gene expression analysis of LLC tumour transplants in eDll4cKO mice revealed a downregulation of the EMT markers *Snail-1*, *Twist*, *Slug*, *TGF-β* and upregulation of

the epithelial marker (Mendonça et al. 2019). Additionally, a reduction in the tumour immunostaining of two cancer stem cell markers (CD49f and p63) was observed. The reverse phenotype, an increase of EMT markers, was observed in endothelial *Dll4* over-expression mice. However, in this case, metastasis formation was reduced, probably as a consequence of the increased smooth muscle cell coverage of the neovasculature of the primary tumour preventing intravasation (Trindade et al. 2017).

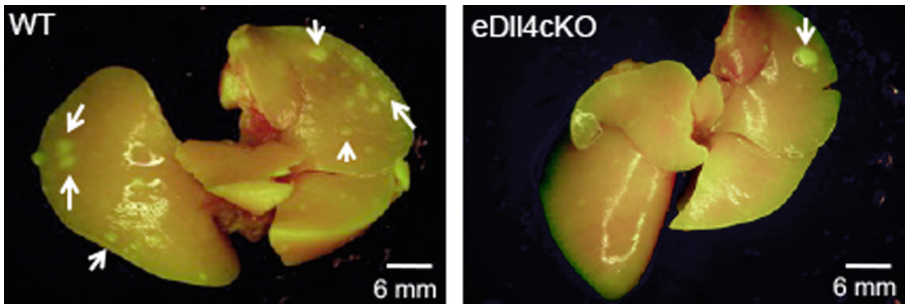

Fig. 4. Effect of endothelial-specific *Dll4* loss-of-function mice in metastasis formation from primary LLC xenografts. LLC tumour lung macro-metastases stereoscope photographs illustrative of the two mouse groups (the white arrows are pointing macro-metastases). Adapted from Mendonça et al. (2019).

The tumour neovasculature is therefore determinant in the intravasation step of the metastatic cascade. Tumour immunostainings of endothelial *Dll4* loss-of-function mice revealed an important reduction in the number of GFP-tagged tumour cells inside the blood vessels. Whether this effect is mainly directed by the reduction in EMT and in the size of the cancer stem cell pool, *per se*, awaits further clarification (Mendonça et al. 2019). Surprisingly, in the context of Dll4 loss-of-function, hypoxia, a well-known metastatic driver, is uncoupled from metastatic initiation (Mendonça et al. 2019).

7 Targeting Notch Signalling to Improve Physiological Angiogenesis

In mammals, physiological angiogenesis occurs mainly during tissue regeneration, such as in healing wounds, and in the cycling female reproductive tract. Wound healing assays constitute a fast, easy, and reliable *in vivo* model of physiological angiogenesis for studying the molecular mechanisms involved in the formation and remodeling of vascular structures (Suchting et al. 2007). Wound healing involves complex interactions of extracellular matrix (ECM) molecules, soluble mediators, resident and infiltrating inflammatory cells. As a critical regulator of angiogenesis, inflammation and cell-fate determination, Notch signalling contribution to wound healing responses has been thoroughly investigated.

Our group has shown that both endothelial-specific *Dll4* over-expression and loss-of-function led to delayed wound healing. In the first case this was due to decreased vascular density with increased vessel maturation, and in the latter, it was caused by increased vascular density with reduced vessel maturation, both phenotypes leading to

reduced tissue perfusion. Contrastingly, *Dll4* heterozygous mice presented accelerated wound regeneration with associated improved vascular density as well as near-normal levels of blood vessel perfusion. This led to testing low dosage inhibition of Dll4/Notch signalling, using the Dll4-Fc therapeutic protein, which proved to be effective in accelerating the healing response by improving overall vessel functionality (Trindade et al. 2012). These experiments revealed how Dll4 regulation of endothelial branching and vascular maturation have different signalling thresholds. This explains why low-level inhibition of Dll4 signalling, although sufficient to increase angiogenesis, does not interfere with normal vessel organization and maturation. It therefore results in a moderate increase in vessel density that leads to improved perfusion and accelerated wound healing. This principle was later confirmed to be applicable to improve the resolution of tissue ischemia. Using two different models, hindlimb ischemia and skin flap surgery, in collaboration with the Gill laboratory, we showed that low dosage inhibition of Dll4 allowed faster recovery from vascular and tissue injury (Liu et al. 2012). This finding suggests the possibility of developing therapies based on Dll4 inhibition to promote recovery from vascular injury and restoring blood supply to ischemic tissues.

8 Conclusion

Endothelial Dll4/Notch signalling serves as a potent modifier of angiogenic vessel growth. The present research shows that both loss- and gain-of- endothelial Dll4 function interferes with tumour growth in distinct experimental settings by promoting non-productive angiogenesis or suppressing the tumour angiogenic response, respectively. Dll4 is part of a regulatory feedback loop that includes the VEGF pathway and is responsible for the normalization and maturation of nascent vasculature. This represents a rational basis for the development and implementation of new, antagonistic and agonistic, Dll4/Notch-based therapies, and continued studying of the currently existing ones, that, upon validation, may substantially contribute to control cancer.

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Feline Mammary Carcinoma: Past, Present and Future

A. Gameiro[1], F. Almeida[1], M. Soares[2], J. Correia[1], and F. Ferreira[1(✉)]

[1] Pathology Laboratory, Center for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Avenida da Universidade Técnica, 1300-477 Lisbon, Portugal
fernandof@fmv.ulisboa.pt

[2] Research Center for Biosciences & Health Technologies, Faculdade de Medicina Veterinária, Universidade Lusófona de Humanidades e Tecnologias (ULHT), Campo Grande 376, 1749-024 Lisbon, Portugal

Abstract. The Pathology Lab at CIISA/FMV has been conducting efforts towards the understanding of the molecular mechanisms underlying the feline mammary carcinoma (FMC). So far, the detection and quantification of three novel molecular biomarkers (HER2, SDF-1, CXCR4) were optimized in tumor tissue samples and sera, and their clinical usefulness was also validated. In parallel, six FMC immunophenotypes were reported and characterized, showing similar clinicopathological features to the ones described in human breast cancer patients. In a prospective study, our group reported that queens showing triple negative basal-like or HER2-positive mammary carcinoma subtypes are associated to shorter overall survival, contrasting with queens presenting luminal A/luminal B and triple negative normal-like molecular subtypes. We also demonstrated that the frequency of HER2 overexpression in FMC is similar to what is reported in women (about 30%), although no gene amplification was detected. More recently, studies on the role of CXCR4-SDF-1 axis and of miRNAs in FMC were performed in order to explore their role in oncogenesis and to validate new diagnostic/prognostic serum biomarkers. Finally, the antitumor activity of new TK inhibitors is under evaluation, using FMC HER2-positive and HER2-negative cell lines towards the support of new specific molecular therapies.

Keywords: Feline mammary carcinoma · Biomarkers · Diagnosis · Prognosis · HER2 · SDF-1/CXCR4 axis

1 Introduction

Cats are popular companion animals in Europe and in the USA (Downes et al. 2009; Murray et al. 2010; Murray et al. 2015). In 2011, the estimated UK cat population was 10 114 764, which was quite similar to the estimated dog population – 11 599 824

A. Gameiro and F. Almeida—Contributed equally to the work.

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(Murray et al. 2015). In parallel, in the USA, data from 2012 indicates that there were approximately 74 059 000 cats and 69 926 000 dogs (American Veterinary Medicine Association 2012). Cancer in companion animals is one of the major causes of death and morbidity, and at least 4 million cats develop this disease every year. The incidence of Feline Mammary Carcinoma (FMC) is increasing, probably due to a humanized lifestyle, an unbalanced diet and unregulated environmental factors. Moreover, better prevention and efficient treatment of infectious diseases also contribute to this reality. Despite this scenario, few studies have been published reporting the diagnostic, therapy and prognostic assessment of FMC. Although histopathology remains as a gold standard for proper management of cancer in pets, there is an urge in the Veterinary oncology field to expand the knowledge about the molecular mechanisms of cancer (at DNA, RNA and protein levels). This will enable the development of new diagnostic tools and to uncover mechanism-based therapeutics. The cat's recently partially annotated genome (Murphy et al. 2007; Davis et al. 2009) will enable further advances on the characterization of the different types of tumors in this species. Recent molecular cytogenetic studies showed that the genome of the cat was subjected to a very small number of genomic rearrangements, when compared to the human genome.

Additionally, there is growing evidence that mammary tumors in pets can be good biological models for breast tumors in women (Vail and MacEwen 2000). There is a strong pressure from the scientific community for the use of new tumor models that would allow valid extrapolations to human medicine (Zappulli et al. 2005; Paoloni and Khanna 2008; Pinho et al. 2012).

According to Dorn et al. (1968), mammary tumors are the third most common tumor type in female cats (after skin tumors and lymphoma), representing 12% of tumors in feline, regardless of the sex. More recently, Vascellari and colleagues (2009) reported that mammary tumors were the second most common tumor in cats (representing 16.3% of the tumors).

Feline mammary tumors are almost exclusive of the female sex, as in human breast cancer (Sorenmo et al. 2013). According to the literature, and as described for women and bitch, feline mammary carcinoma incidence increases with age, and the disease is predominantly seen in middle-aged to older cats. The mean age of diagnosis is between 10 to 12 years of age, with risk increasing up to 14 years of age (Weijer and Hart 1983; Sorenmo et al. 2013; Zappulli et al. 2015).

Concerning the breed, two studies registered that Siamese cats appear to be overestimated when compared to other breeds (Hayes et al. 1981; Ito et al. 1996). Finally, the exposure to ovarian hormones is also strongly implicated in mammary tumorigenesis in cat. Overley et al. (2005) concluded that sexually intact cats have a higher risk than spayed cats to develop mammary carcinomas ($p = 0.001$, odd ratio – OR = 2.7). Moreover, cats spayed prior to 6 months of age had a 91% reduction in the risk of developing FMC, while in cats spayed prior to one year, the risk reduction was lower (about 86%). Besides the ovarian hormones, there is also a higher risk of developing mammary cancer in queens subjected to regular administration of progestogens (Misdorp et al. 1991). More recently, Overley and colleague (2005) concluded that given birth did not affect mammary tumor development.

Most of mammary tumors in cats are malignant (85% to 95%), and present an aggressive biological behavior, with early lymphatic invasion and lymph node metastases. The malignant tumors types in cats are predominantly adenocarcinomas, particularly the simple carcinomas such as tubulopapillary and solid carcinomas. Others types of mammary tumors include non-infiltrative (*in situ*) carcinoma, cribriform carcinoma, invasive micropapillary carcinoma, squamous cell carcinoma, mucinous carcinoma, lipid rich carcinoma and inflammatory mammary carcinoma (Misdorp et al. 1999; Pérez-Alenza et al. 2004; Kamstock et al. 2005; Seixas et al. 2007; Millanta et al. 2012). In contrast, carcinosarcomas, sarcomas and other non-epithelial neoplasias are very rare mammary tumors in cats (Misdorp et al. 1999; Sorenmo et al. 2013).

2 Clinical Presentation

As indicated above, cats with mammary carcinomas are often old and may be sexually intact or spayed. Tumors are usually easy to detect on physical examination as they appear as firm and discrete masses in the mammary gland, which can be detached or attached to the underlying tissue. Multiple tumors are common and 60% of cats have more than one tumor at the time of diagnosis, according to Hayes and Mooney (1985). All glands are susceptible to tumor development although anterior glands are less commonly involved (Weijer and Hart 1983). For this reason, a careful examination of the remaining mammary glands is always recommended, and for each mass the following features should be recorded: size of the mass, consistency, mobility and the presence of skin ulceration (Weijer and Hart 1983). Tumor size is a very important feature as it presents prognostic value and depends on how early the tumor is detected and how aggressively the tumor behaves. Usually, larger tumors may become ulcerated, inflamed and infected (Weijer and Hart 1983; MacEwen et al. 1984; Ito et al. 1996).

FMC are typically very aggressive and present high degree of metastization to regional lymph nodes and to distant organs such as lungs and pleura, the organs most commonly affected (Weijer and Hart 1983). Moreover, authors report that 50% to 93% of cats with mammary carcinoma show metastasis at necropsy, rendering the inspection of the local lymph nodes in the clinical examination crucial for diagnosis and prognosis (Hayden and Nielsen 1971; Hahn et al. 1994). Considering the high incidence and morbidity of FMC, a clinical and complete work-up is recommended and includes a complete physical examination, a complete blood count, serum biochemical profile, serum T4 concentration, urinalysis, three-view thoracic radiographs (ventrodorsal, right and left lateral views), abdominal ultrasound or a computed tomography (CT) scan. All mammary masses and any palpable regional lymph node should be subjected to a fine-needle aspiration (FNA) or to a biopsy. Ulcerated lesions should be scraped and fluids from the affected glands should be examined, in order to achieve a diagnosis before surgery. All the mammary lesions and corresponding regional lymph nodes should be analyzed by histopathology.

Similarly, to humans and dogs, the mammary disease in cats is also staged (McNeill et al. 2009). However, this staging system should not be used for mammary gland sarcomas. The inflammatory mammary carcinoma is a rare but a clinical important type of mammary tumor, which is also described in women and bitch. In these cases, the entire

mammary chain or the mammary gland affected may appear edematous, swollen, warm and painful. The clinical identification of this type of carcinoma is very important, as these animals show poor prognosis due to the secondary postsurgical complications, like no healing incisions, edema and suture rejections (Pérez-Alenza et al. 2004).

3 Treatment Options

Surgery is the most widely used treatment for FMC, either alone or in association with chemotherapy (MacEwen et al. 1984). Cats with mammary lesions are usually subjected to a simple lumpectomy, mastectomy, regional mastectomy, or a chain mastectomy that can be unilateral or bilateral. The goal of surgical intervention is to remove the current tumor or tumors with clean margins, in order to prevent the emergence of new tumors in the remaining mammary glands. In cats, radical mastectomy (unilateral for cats possessing a single tumor or a 2-staged bilateral chain mastectomy for animals with bilateral tumors) results in a significant larger disease free survival (DFS) when compared with cats receiving a conservative chain mastectomy (MacEwen et al. 1984). Thus, unilateral or staged bilateral mastectomy is the recommended treatment for FMC. In some cases a bilateral mastectomy could be performed in a single surgery, if the postsurgical tension is minimal (when the animal presents excessive mammary or adipose tissue, for example). For tumors that are fixed, muscular fascia or portions of the body wall should also be resected (Sorenmo et al. 2013).

The assessment of the regional lymph node is justified by the high metastatic potential of FMC and by the poor prognosis that is associated with the presence of lymph node metastasis. This could be evaluated by an ultrasound-guided FNA or through the histopathological evaluation of the resected regional lymph node, during surgery (Sorenmo et al. 2013).

Early detection and radical surgery (including prophylactic chain mastectomy) can result in long-term survival in cats with early stage mammary tumors. However, cats with late diagnosis or later stages of the disease are not treated effectively with surgery alone. In those cases the use of adjuvant doxorubicin-based chemotherapy should be considered (Novosad et al. 2006; McNeill et al. 2009). In one multi institutional retrospective study, a comparison between cats with mammary carcinomas receiving surgery *versus* cats receiving surgery with adjuvant chemotherapy demonstrated that the subset of cats having unilateral chain mastectomy followed by chemotherapy presented an longer overall survival (OS) than the other group of animals (McNeill et al. 2009).

Alternative chemotherapy protocols include a combination of doxorubicin and cyclophosphamide or doxorubicin-based chemotherapy and a nonsteroidal anti-inflammatory drug (meloxicam). However, survival studies based on these protocols have not been performed, so their efficiency cannot be determined (Mauldin et al. 1988; Sorenmo et al. 2013; Borrego et al. 2009). Concerning others treatments, as hormonal therapy, they are unlikely to be effective, as several studies suggest that FMC presents a low expression of hormonal receptors (de las Mulas et al. 2002; Millanta et al. 2005; Millanta et al. 2006; Burrai et al. 2010). Recently, promising results were obtained using an oncolytic virus (Adelfinger et al. 2014). This therapy is based on the capacity of oncolytic viruses infecting and promoting cancer cells lysis without extensively damaging the surrounding normal tissue. Finally, and despite the high incidence of distant

metastasis in these animals, no studies were reported about the efficiency of adjuvant systemic treatments.

4 New Insights on the New Biomarkers: HER2 E CXCR4/SDF-1 Axis

As mentioned above, FMCs present several similarities with the human breast cancer disease (age incidence, histopathology, pattern of metastasis, and response to therapy), and are also classified in the same subtypes (luminal A, luminal B, luminal B-like, epidermal growth factor receptor type II-positive and triple negative) (Goldhirsch et al. 2013). Moreover, these companion animals share a common environment with humans and therefore exposure to environmental contributors to the development of cancer should be similar. Due to these resemblances, the domestic cat has been proposed as a model for comparative oncology (Vail and MacEwen 2000).

Pet owners are increasingly requesting the use of the most advanced diagnostic and therapeutic tools to improve time and quality of life of their companion animals. However, the currently available methodologies are insufficient to answer those demands. Although the knowledge of the oncogenic mechanisms of breast cancer is increasing, still, there is a lack of innovation in accurate biomarkers for diagnostic and prognostic.

In human breast cancer, the evaluation of the human epidermal growth factor receptor-2 proto-oncogene (HER2), along with the estrogen receptor (ER) and progesterone receptor (PR), has become critical for the assessment of tumor status, progression, prognostic and therapy planning (Wolff et al. 2007; Wolff et al. 2014). HER2 is a 185 kDa transmembrane glycoprotein that comprises three domains: an extracellular domain (ECD), a short transmembrane region and an intracellular domain with tyrosine kinase (TK) activity (Coussens et al. 1985; Carney et al. 2013). HER2 belongs to the epidermal growth factor receptor (EGFR) family and is involved in a variety of molecular pathways associated with tumor growth and progression (Vollan and Caldas 2011). Clinical oncologists evaluate HER2 protein levels typically by immunohistochemistry (IHC), and classify tumors attending to HER2 expression values. Molecular epidemiology studies showed that about 20–30% of human breast cancer patients display HER2 overexpression (Wolff et al. 2007; Wolff et al. 2014). This lead to the development of a directed therapy based on a humanized anti-HER2 monoclonal antibody (Trastuzumab; Roche, Basel, Switzerland) (Stebbing et al. 2000). The feline counterpart of HER2 is well conserved in the cat with over 90% similarity with the human HER2 (Santos et al. 2012; Santos et al. 2013). Reports have shown also an association between the feline HER2 overexpression and mammary carcinogenesis (Millanta et al. 2005; Ordás et al. 2007; Maniscalco et al. 2012). However, few studies are available and the data is somewhat inconsistent due to different protocols and evaluation criteria. Further studies on the feline HER2 protein overexpression are needed.

In order to achieve reliable and reproducible results, IHC and fluorescence *in situ* hybridization (FISH) were routinely applied in human oncology, with well-defined criteria that include specific experimental conditions, scoring definitions and interpretation standards (Wolff et al. 2007; Goldhirsch et al. 2013; Wolff et al. 2014). Focusing on these guidelines, our lab developed a protocol optimized for the immunodetection of feline

HER2 in tumor samples (Soares et al. 2013). The optimized IHC and FISH protocol applied to feline tumor samples emphasized the relevance of immunostaining standardization in veterinary diagnosis, and revealed that the feline HER2 is overexpressed in about 33% of FMC cases, a number very similar to the one found in human breast cancer, highlighting the importance of the molecular characterization of this molecule in cats (Soares et al. 2013).

Our lab then applied this methodology to characterize in detail the clinical and the pathological features of a population of cats with FMC (Soares et al. 2016a). The authors used a large population of female cats presenting mammary carcinomas (n = 102) and aimed to characterize the existent cancer subtypes, along with statistical analysis for overall survival (OS) and disease free survival (DFS). We were able to demonstrate the molecular heterogeneity of feline mammary carcinomas, and identified and characterized six different molecular cancer subtypes, presenting several similarities with the human breast cancer disease. However, unlike humans, where synchronous bilateral breast carcinomas are uncommon, the presence of multiple masses found at the moment of the diagnosis is very common in cats. This poses a serious difficulty not only for the interpretation of the results and comprehension of tumor disease behavior, but also for disease staging, and clinical and therapeutic management. Overall, this study opened new perspectives for a better prognostic evaluation and for the development of directed treatments for the improvement of the OS and the DFS of the feline patients with mammary cancer (Soares et al. 2016b).

The gold standard methods for the classification of breast cancer, either in human or in veterinary oncology, still involve an invasive step of tissue collection from the affected regions. This constitutes a significant limitation for the continuous follow-up of patients after the initial invasive surgery. Efforts have been made to develop new non-invasive techniques to quantify HER2 and classify breast tumors, as the case of the analysis of protein levels in patient serum samples, which are much easier to obtain than tissue samples. The HER2 extracellular domain (ECD) is released from the surface of tumor cells into the bloodstream via protease activity, allowing its detection in sera by quantitative biochemical assays (Esteva et al. 2005; Ludovini et al. 2008). These studies indicated that the measurement of HER2-ECD in serum samples could be a valuable tool to follow the tumor disease progression, and that elevated HER2-ECD levels could be associated with higher relapse rates and worse prognosis (Esteva et al. 2005; Ludovini et al. 2008). Our lab evaluated for the first time the usefulness of measuring the HER2-ECD levels in cats with FMC (Soares et al. 2016c). Soares and colleagues aimed to detect and quantify the serum HER2 levels in a population of 16 female cats with mammary carcinoma, by using the already established ELISA method (approved to measure HER2 levels in sera from women) and another less expensive biochemical assay, the Dot blot assay. The results showed that, as well in humans, the feline HER2 protein can release its extracellular domain into the extracellular space, and that HER2 levels in the sera of cats with HER2 overexpressing mammary carcinomas are significantly higher than in cats with HER2-negative mammary carcinomas. Nevertheless, analysis of the clinical and pathological data of the cat patients revealed that HER2 protein levels both in serum and in tissue were associated with less aggressive features, contradicting what is described for humans. Altogether, this study validates ELISA and Dot blot assay to replace the

IHC technique for diagnostic purposes and for monitoring the response to therapy in cats, not only due to their efficacy but also because of the lower costs.

Uncontrolled cell proliferation is a hallmark of cancer malignancy and has emerged as an important diagnostic and prognostic tool in human breast cancer. Therefore, additional prognostic and predictive factors that may provide useful insights into the tumor biology and the proliferation index of the tumor are important to achieve a more efficient therapy and a better follow-up of patients (Perou et al. 2000; Park et al. 2012). Several targets have been considered but only a few produced useful results. The Ki-67 is a protein found only during cell division. The expression of this protein is low in early stages of the cell cycle and increases until mitosis takes place. This distinctive pattern of expression makes the Ki-67 antigen a useful nuclear marker of cell proliferation and reliable in the prognosis of human breast cancer (Cheang et al. 2009; Yerushalmi et al. 2010; Dowsett et al. 2011). As in the cat little information was available on the use of Ki-67, our lab assessed the prognostic value of the Ki-67 proliferation index in female cats with mammary carcinoma (Soares et al. 2016a). This study included 96 primary mammary tumors, in which the Ki-67 index was determined and compared to other clinical and pathological features of cats with FMC. Soares and colleagues demonstrated that the Ki-67 index can be used as a prognostic biomarker in cats with mammary carcinoma. Moreover, Ki-67 values above 14% were associated with lower OS and with other aggressive clinical and pathological features. On the basis of this analysis, the authors proposed that a Ki-67 cutoff of 14% should be adopted to identify tumors with high risk of disease progression (Soares et al. 2016b).

There is a continuous effort to search for more accurate biomarkers for FMC. Our lab also evaluated a family of chemotactic molecules known by chemokines. Chemokines and their specific receptors are known to be involved in the interplay between tumor microenvironment and cancer cells, modulating cellular proliferation, and other cellular responses (Ferreira, 2017). Recent studies showed that the binding of stromal cell-derived-factor-1 (SDF-1) to the C-X-C chemokine receptor 4 (CXCR4) increases the proliferation rates of breast tumors (Luker et al. 2012; Mego et al. 2016). CXCR4 overexpression has also been observed in breast cancer patients, especially in HER2 positive cancer subtypes. Moreover, since the SDF-1/CXCR4 axis elicits the activation of multiple kinase pathways, its inhibition may represent a new therapeutic strategy for the treatment of mammary tumors (Müller et al. 2001; Salvucci et al. 2006). Taking into account the relevant role of the CXCR4/CXCL12 axis in breast cancer progression and its potential as a therapy target, our lab addressed the contribution of the CXCR4/CXCL12 axis in FMC (Marques et al. 2017). A total of 115 female cat mammary tumors, and blood samples from 42 queens with mammary disease and 5 healthy female controls, were used to assess the expression levels of CXCR4 and SDF-1 by IHC and immunofluorescence, as well as the association between CXCR4 and SDF-1 tissue status and serum levels with clinical and pathological data from the diseased cats. The authors found a signature for CXCR4 and its ligand SDF-1 in FMC, indicating that serum SDF-1 levels have diagnostic value in cats with mammary carcinoma and determined the best cut-off value to discriminate cats with mammary carcinoma from healthy ones ($\geq$2 ng/ml, similar to what was reported for human breast cancer patients). Altogether, this work identified a new serum biomarker for feline mammary carcinoma, especially for HER2

positive tumors, and not only highlighting the importance of these proteins as a possible target for diagnostic and therapy of FMC, but validating also the cat as a suitable model for comparative oncology.

An increasing number of human HER2 positive breast cancer cases are presenting immunotherapy resistance to HER2 directed therapies, due to sequence variations (SV) in the HER2 gene (Siddig et al. 2008; Watrowski et al. 2015). Several SVs were also found in the cat HER2 gene (Santos et al. 2012; Santos et al. 2013). Currently, our lab is interested in validating the SVs and the haplotypes previously reported using a larger population of cats with mammary carcinoma. The HER2 ECD and TK DNA regions from 25 healthy cats and from 100 cats with FMC are currently being amplified and sequenced, and the SVs detected will be evaluated for possible associations with clinical and pathological data. Preliminary results indicate that there is a high percentage of identity among the four cell lines analyzed, and despite the number of SVs detected, in comparison to previous studies, the majority is located within intron regions. To this point, no mutations previously associated with therapy resistance were found. Our final goal is to use the information of the SVs to predict disease progression/prognosis and to understand the incidence of FMC that could present immunotherapy resistance.

In the last few years, our lab provided solid knowledge of the biology of the feline mammary tumor to support veterinary medicine and substantiate the improvement of diagnostic/prognostic/therapy tools. Still, there is room for other methodologies to be optimized and introduced in the field. We aim to continue and unveil new molecular targets for the development of novel diagnostic and therapeutic options for FMC.

5 Understanding the Role of miRNAs on FMC

miRNAs were firstly identified in *Caenorhabditis elegans* by Lee et al. (1993) and among the group of small RNAs they are the most abundant in somatic cells, and found in most of the organisms. To date, more than 17 000 miRNA sequences, in more than 140 different species have been cataloged in the miRBase database, which has increased exponentially, and has nearly tripled in the last years (Griffiths-Jones 2004).

MicroRNAs (miRNAs) constitute a class of small non-coding RNAs (ncRNAs), being composed by sequences of 18 to 25 nucleotides (Lee et al. 1993) and encoded in genomic regions of non-coding DNA. Their biological role involves the control of gene expression, and mutations in their sequences have been associated with breast carcinoma in women (Frères et al. 2015). Indeed, the function of each miRNA can be quite elaborate. They can play an important role in post-transcriptional regulation through the suppression or degradation of various types of mRNAs, and/or may play an important role in the regulation of other miRNAs (Bartell 2004; Wagner 2013; Bertoli 2015; Kabir 2015; Wang and Luo 2015). In humans, miRNAs play an important role in the regulation of more than 60% of encoding genes, being implicated in many diseases such as Alzheimer's disease, cardiovascular diseases and various types of cancer diseases (Wagner et al. 2013). Errors in miRNAs regulation may have several origins such as chromosome anomalies, epigenetic mechanisms, faults in miRNAs synthesis, dysregulation of transcription factors' activity, and even nucleotide point mutations at miRNA binding sites (single nucleotide polymorphisms - SNPs) (Garzon et al. 2010;

Iorio and Croce 2012; Ha and Kim 2014; Hayes et al. 2014; Hata and Lieberman 2015; Rupaimoole and Slack 2017).

Although several studies point out the promising role of these molecules as diagnostic and prognostic biomarkers, further investigation is needed towards a deeper understanding of their importance in different pathologies and in homeostatic cellular activity. In oncological diseases, miRNAs can be used in early diagnosis, as biomarkers of prognosis, namely related to survival times, and may also indicate the presence of distant metastases and predict chemotherapy response, as well as possible resistances to targeted treatments, anti-endocrine therapy and radiation therapy (Fleischhacker et al. 2013; Hagrass et al. 2015; Casey et al. 2016; Komatsu et al. 2016). In cancer patients, errors may affect tumor suppressors miRNAs or miRNAs that are oncogenic, so in these cases the miRNAs may also be used in therapy, through their substitution or inhibition, respectively (Gambari et al. 2016).

The synthesis of miRNAs begins at a nuclear level location where their transcription occurs and ends at the cytoplasmic level where they accumulate. The miRNAs are present in all tissues of the body and are also released to all organic fluids, such as blood, either by secretion phenomena or due to tissue injury or even cell apoptosis or necrosis. The fluids where miRNAs can be found are blood, serum, plasma, urine, saliva, semen and cavity effusions, and also in exosomes or linked to proteins or lipoproteins, from which it is convenient to carry out patients' sample collections for subsequent analysis (Ling et al. 2013; Graveel et al. 2015; Tiberio et al. 2015).

Although, most of the miRNAs are conserved between the mammalian species, few data is reported on tumors of dogs and cats, even though they can serve as an alternative model to the mouse (Weber et al. 2015). In the Pathology lab, the serum levels of different miRNAs were evaluated in cats with mammary carcinoma and compared with healthy animals. Significant differences were found in serum levels of miR-21, let-7a, miR-10b, miR-200b and miR-200c between these two groups of animals (Santos 2018), as well as a significant correlation between serum levels and specific oncological characteristics (e.g. the presence/absence of tumor, tumor size, tumor histological subtype, presence of tumor necrosis, lymph node metastasis, tumor molecular subtype, serum concentrations of derived stromal-1 (SDF-1) factor, DFS and OS (Santos 2018). In the future, and since breast pathology, both in human and animal species will remain a worldwide and serious problem, our group intend to pursue studies in order to further understand the role of miRNAs' in feline mammary carcinoma, giving new insights to develop novel diagnostic and therapeutic tools.

6 Exploring Efficacy of TKIs, HDACIs and MTIs in FMC

Considering the increasing incidence of FMC and the lack of efficient therapeutics, our lab aims to evaluate new therapeutic strategies targeting HER2. Recently, two non-redundant sequence variants (SVs) associated with malignant/metastatic-status coupled with loss of heterozygoty in malignant/metastatic tumor samples were identified in cat (Santos et al. 2012). This raised the hypothesis of their utility as biomarkers in cancer diagnosis/prognosis and reinforced the idea that the use of tyrosine kinase inhibitors (TKI) is an alternative strategy to future therapeutic antibodies. Therefore, we planned

to identify efficient TK inhibitors for their putative use in FMC-HER2 + and also study the use of microtubules inhibitors (MTi) and histone deacetylase inhibitors (HDACI).

Until now, promising results were achieved considering the TK inhibitors, which block the HER2 pathway. On this study, two TK inhibitors approved for the treatment of breast cancer HER2 + were exploited and their antitumor effects were characterized. For the study, three feline cell lines (CAT-M, FMCp and FMCm) were used, as well as one human cell line, as control (SkBR-3). To evaluate the possible resistance to the TKI therapy, the HER2-IC domain was sequenced in the feline cell lines and in clinical samples, to infer about putative resistance, a phenomenon already reported in humans (Feldinger and Kong 2015; Shi et al. 2016). Results from immunoblot analysis revealed that SkBR-3 and FMCm cell lines are HER2-positive, whereas CAT-M and FMCp cell lines show a basal HER2 expression.

The cytotoxic effect of TKI in the feline cell lines was similar to the effect in human cell line SkBR-3. Considering the TKI Lapatinib, a 100% cytotoxicity was obtained for all the cell lines and the IC50 value was calculated (SkBR-3 = 14.35 μM; CAT-M = 7.59 μM; FMCp = 11.21 μM; FMCm = 22.65 μM). For the other TKI (Neratinib), only around 40% cytotoxic effect was observed for SkBR-3 cell line and 60% for the feline cell line FMCp (IC50 = 30.89 nM). The CAT-M and FMCm cell lines have a different behavior, showing no cytotoxicity, suggesting that an effect of resistance is activated for cell survival, in the presence of TK inhibitors (Hegedüs et al. 2015). Indeed, using a small concentration (0.195 nM), 42.7% of cytotoxicity was measured for CAT-M and 50.7% for FMCm cell line, whereas only 13% and 23.3% was found at higher concentration (1500 nM).

Concerning Rapamycin (an inhibitor of the mTOR pathway) which alone does not have effective cytotoxicity, it was found to be very useful in combination with others, allowing the use of small drug concentrations. Our results confirm that Rapamycin alone has no cytotoxic effect in all the cell lines. In combinatory experiments, the best results were achieved with FMCp cell line, when 6,25 nM Rapamycin were added to 0.78 μM Lapatinib. With this combined treatment, it was possible to achieve 63.3% cytotoxicity, comparing with 3.8% cytotoxicity when Lapatinib was used alone. Our data suggest that the addiction of two drugs have a potentiation effect on the cytotoxicity of the TKI alone, as others described (Li and Li 2013). Then, in order to evaluate which molecular mechanisms are disrupted by the treatments, the phosphorylated levels of the proteins related to ERK, AKT, and mTOR pathways were analyzed by immunoblot, in the different cell lines. In general, TK inhibitors reduce the phosphorylated levels of the proteins related with the HER2 pathway. In parallel, the mTOR inhibitor leads to a decrease of phosphorylated ERK, AKT and HER2 forms.

Knowing that point mutations in HER2-IC domain lead to therapy resistance, to Lapatinib and Neratinib (Tate et al. 2018), this region was sequenced in tumor tissue samples and FMC cell lines. The feline gene corresponding to the HER2-ICD was amplified by PCR and sequenced by Sanger technique. All sequences were compared with the gene sequence available in the NCBI database (*Felis catus, Cinnamon breed Abyssinian- NC_018736.3:40780250-40804241).* Considering the feline cell lines, the majority of SVs are present in intron regions, as reported by Santos et al. (2012), being unrelated to therapy resistance. In CAT-M and FMCm cell lines, only three mutations

were found. Considering the FMCp cell line several mutations were found, most of them in intronic regions (66/86), namely substitutions (59/66), insertions (4/66) and deletions (3/66). Taking into account only the exonic sequences, 20/86 mutations were detected; 17/20 of them were synonymous mutations and 3/30 were nonsynonymous. One of them shared the same location (20715 bp) of a mutation reported to confer resistance to Lapatinib therapy in human breast patients (L869R/Q) (Tate et al. 2018). In this case, the reported mutation encodes for a different codon, a valine, not inducing Lapatinib´s resistance. Fortunately, none of the clinical tumor samples showed mutations that may interfere with the therapy.

In summary, Lapatinib is the TKI that showed the most promising results, demonstrating its use in therapy of cats with FMC. The drug combination enhances the cytotoxic effect of single treatments, even at low concentrations, which could be useful in a therapeutic application, decreasing the secondary effects and acquired resistance (Gayle et al. 2012). Furthermore, the mutations found in the HER-ICD, both in tumor samples and feline cell lines, do not appear to interfere with the binding capacity of TKIs.

Beyond the TKI, the effect of HDACIs and MTis were analyzed in our lab. This line of research has the general goal of discovering new options for treatment of FMC. So far, we have studied the cytotoxic effect of six HDACIs (CI-994, Panabinostat, SBHA, SAHA, Scriptaid and Trichostatin A) and four MTis (Colchicine, Nodocodazole, Paclitaxel and Vinblastine) in the three feline cell lines above mentioned, and using the SkBR-3 cell line, as control.

About the HDACIs, 100% cytotoxicity was detected in all cell lines exposed to Scriptaid (IC50 SkBR3 = 5.7 μM; CAT-M = 4.0 μM; FMCp = 2.7 μM). Considering CI-994 the following IC50 values were obtained: SkBR3 = 17.9 μM; CAT-M = 25.7 μM). When Panobinostat was used the following IC50 values were calculated: SkBR3 = 0.02 μM; CAT-M = 0.05 μM, for SAHA: SkBR3 = 4.0 μM; CAT-M = 4.6 μM; FMCp = 2.9 μM, for SBHA: SkBR3 = 66.2 μM; CAT-M = 68.6 μM and, finally for Trichostatin A: SkBR3 = 0.6 μM. Interestingly, the FMCm cell line exhibits a multidrug resistance pattern, also found in TKIs treatments, especially, at higher drug concentrations. Taking into account the above, Panobinostat may be a promising anticancer drug for FMC treatment, as reported for human breast cancer (Chun et al. 2015). Concerning MTIs (Colchicine, Nocodazole, Paclitaxel and Vinblastine), all drugs show cytotoxic activity in all feline cell lines (except in FMCm cell line), with Colchicine showing the more promising cytotoxic effects (IC50: SkBR3 = 70.0 nM; CAT-M = 2.3 nM; FMCp = 6.8 nM).

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Novel Diagnostic and Therapeutic Immunologic Strategies to Overcome Infectious, Oncologic and Neurodegenerative Disorders

F. Aires-da-Silva(✉), J. Dias, S. I. Aguiar, F. Marques, A. André, S. Gil, and L. Tavares(✉)

Microbiology and Immunology Lab, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
fasilva@fmv.ulisboa, ltavares@fmv.ulisboa.pt

Abstract. In the scope of viral, oncologic, inflammatory and neurodegenerative diseases, animal models have been used to assess novel immunology based therapeutic strategies, such as the use of new generation monoclonal antibodies to treat immunodeficiency, lymphoma and neurodegenerative disorders. Here we describe the methods used in the development of engineered single domain antibodies (sdAbs) of rabbit origin against different antigenic targets using techniques that include rabbit immunization, construction of antibody libraries and selection by phage display. The potential of these antibodies as therapeutic tools is vast, varying with the antigen they target. They can be potent anti-viral products, if they are directed against viral spike glycoproteins, or anti-tumor cytotoxic reagents, if they target a tumor specific antigen, or precise therapeutic carriers, able to deliver pharmaceutical agents to locations where their antigen can be found. Applications of these methods are addressed in this review, exemplified with current work pursued in our lab. Namely development of: 1) specific recombinant sdAbs against the gp120 HIV-1 glycoprotein; 2) recombinant sdAbs and small molecules for diagnosis and treatment of naturally occurring canine lymphoma; and 3) recombinant sdAbs for brain targeting and drug delivery across the Blood-Brain Barrier.

Keywords: Immunotherapy · Antibody engineering · Recombinant antibodies · Antibody fragments · Phage display · Drug delivery systems

1 Antibodies (Igs) as Immunotherapeutic Tools

The immune system has long been recognized as the most important line of defence against disease in general and, specifically, disease caused by pathogenic microorganisms, neoplastic degeneration and metabolic disorders. The immune system relies on a complex network composed of multiple defence systems interacting with each other to restore the organism's homeostasis by detecting, protecting and eradicating foreign antigens and neoantigens that may enter or arise in the body (Lopez Gelston and Mitchell

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2017). These include physical barriers, normally considered as the first line of defense, that exclude invaders, innate immunity, that affords rapid initial protection and adaptive immunity, that provides prolonged effective immunity (Tizard 2018). Innate immunity is readily activated when a pathogen overcomes the physical barriers and invades the organism, aiming at the rapid elimination of the pathogen. The adaptive immunity involves the proliferation of T and B lymphocytes, triggered by the binding of antigens (non-self molecules) to cellular receptors (Goldsby et al. 2003). Innate and adaptive immune systems differ in their use of cell surface receptors and soluble molecules to recognize foreign invaders. Lymphocytes possess surface receptors (Igs on B lymphocytes and the T-cell receptors on T lymphocytes) that recognize antigens with extraordinary specificity (Yatim and Lakkis 2015). Antibodies (Abs) play a particularly determinant role since the destruction or neutralization of the invaders is mediated by specific Abs which result from the rapid proliferation and differentiation of B lymphocytes (Goldsby et al. 2003; Tizard 2018).

Abs are a class of soluble glycoproteins called immunoglobulins (Igs) that are produced by the immune system in response to the presence of antigens (Ag) (Tizard 2018). The comprehensive knowledge accumulated over the years regarding antibody structure and remarkable high specificity has allowed researchers to develop and engineer antibodies that have evolved from scientific tools to powerful therapeutic agents for a wide range of diseases (Buss et al. 2012). The objective is to eliminate or neutralize the infectious agent or disease target, by blocking the action of specific molecules through direct binding; targeting specific cells and eliminate them through antibody dependent cell cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC); or crosslinking receptors that intervene in cell division or cell death (Brekke and Sandlie 2003). In addition to its use as a therapeutic agent on its own, antibodies can also be constructed as drug transport shuttles. To take advantage of this feature, antibodies are being engineered to transport cytotoxic drugs, radiotherapeutic molecules, or even immunocytokines greatly increasing the applicability of these biodrugs.

Most therapeutic Mabs consist of a full-length IgG molecule because of its predominance in human serum, importance for immune response, and excellent specificity characteristics (Aires da Silva et al. 2008a). This typical antibody is usually of a "Y" shape composed of two heavy chains and two light chains (Fig. 1A). Each heavy chain is organized into three constant domains (CH1, CH2 and CH3) and one variable domain (VH) while the light chain is composed only by one constant (CL) and one variable domains (VL). The fragment antigen binding (Fab) region of the antibody comprises the variable domains (VL and VH) along with the CL and CH1 regions. This fragment is connected to the CH2 and CH3 domains, both components of the fragment crystallizable (Fc) region by a flexible sequence (hinge). The variable domains are responsible for the antibody specificity and affinity toward the antigen. The Fc region is mainly responsible for antibody effector functions through complement and receptor binding (Fig. 1A).

Nevertheless, although most marketed antibodies are comprised of a full-length IgG molecule, this conventional antibody format presents some drawbacks that limit their clinical use and there is a range of therapeutic applications in which other antibody formats may be more appropriate. First, due to high molecular weight (~150 kDa), IgG antibodies are known to penetrate poorly into densely packed tissues, to have an impaired

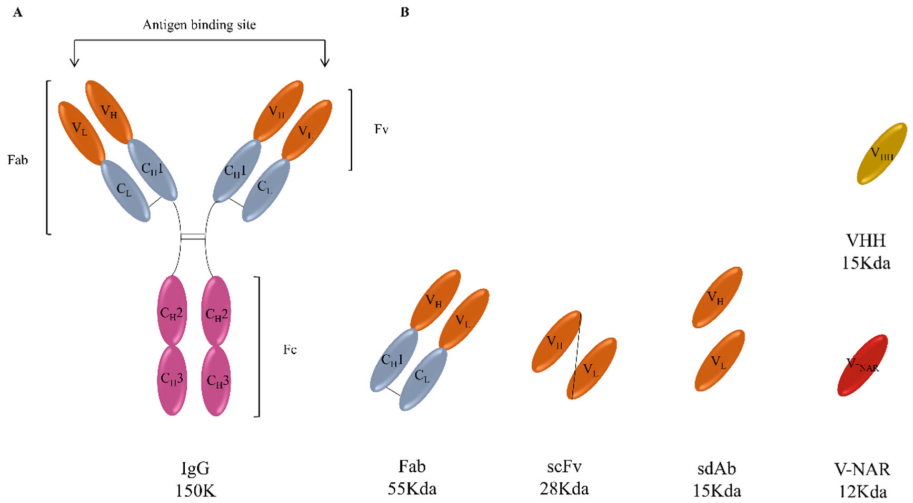

Fig. 1. Schematic representation of the structure of a conventional IgG antibody and antibody fragments of biotechnological and clinical interest. (A) IgG antibodies comprise a pair of identical heavy and light chains linked by disulphide bonds. Light chains contain one constant domain (CL) and one variable domain (VL), while heavy chains contain three constant domains (CH1, CH2 and CH3) and one variable domain (VH). The variable domains of both the heavy and light chains are responsible for the antigen-binding site of the molecule. The Fc constant region recruits effector functions of the immune system. Constant light (CL) and heavy (CH) chain domains are represented in orange and gray. Variable light (VL) and heavy (VH) chain domains are represented in green. B) The engineering of antibody fragments that can be generated from an intact conventional IgG: antigen-binding fragment (Fab), single-chain Fv fragment (scFv), heavy and light single domains antibodies (VL and VH sdAbs) and natural camelid variable domain (VHH) and shark variable domains (V-NAR).

reaching of difficult targets and to present a slow clearance rate. Second, inappropriate activation of Fc receptor-expressing cells sometimes leads to massive cytokine release with subsequent toxic effects. As such, with the advances of genetic engineering, smaller antibody molecules are being developed such as the antigen binding fragment (Fab) or the variable fragment (Fv) promoting the development of new recombinant molecules and antibody fragments with multiple applications (Fig. 1B). (Aires da Silva et al. 2008a; Holliger and Hudson 2005).

A promising alternative to conventional IgGs are single-domain antibodies (sdAbs). sdAbs are the smallest functional antigen-binding fragments of an antibody that can be isolated from conventional IgGs (from human, murine, or rabbit) or obtained from naturally occurring antibodies devoid of light chain that were discovered in two types of organisms, the camelids (camels and llamas) and cartilaginous fish (wobbegong and nurse shark) (Fig. 1B) (Aires da Silva et al. 2004; Greenberg et al. 1995; Hamers-Casterman et al. 1993; Holt et al. 2003). Due to their small size, sdAbs present improved tissue penetration and are able to reach targets not easily accessible by conventional

IgG molecules, such as enzyme active sites or canyons in receptors molecules. Moreover, such as Fab and scFv, sdAbs lack the Fc domain of a full IgG antibody, presenting a low nonspecific uptake in tissues that highly express Fc receptors. Additional important advantages include their high stability, low immunogenicity, and lower manufacturing cost. Furthermore, sdAbs can be easily attached to other proteins, peptides, small molecules, or nanoparticles by simple molecular biology or chemical procedures (Holliger and Hudson 2005). Taking into consideration all these advantages and properties, Fab, scFv, and sdAb antibody scaffolds are definitely a promising alternative to conventional antibodies for immunotherapy applications.

2 Selection and Screening of Recombinant Antibodies

The field of immunology was revolutionized in 1975 by Kohler and Milstein's development of an *in vitro* method for monoclonal antibody (Mab) production using a mouse hybridoma technique that consisted on fusing B-cells from the spleen of a previously immunized mouse with cells from a murine myeloma cell line (Köhler and Milstein 1975). Fusion would give rise to a hybrid cell that inherited the ability to produce Abs from the progenitor B-cell and the capacity to grow *in vitro* from the myeloma progenitor (Köhler and Milstein 1975). This discovery, and Nobel prize winning technology, opened a new era in biomedicine and, since then, a myriad of applications for Mabs have been conceived for cancer, autoimmune, cardiovascular, respiratory, ophthalmologic, neurologic and infectious disease, and even organ transplantation (Suzuki et al. 2015). Nevertheless, murine mAbs present several properties that limit their clinical utility, namely folding problems, low solubility and high aggregation tendency. For this reason, during the past decade several display and screening methods have been developed to improve human/humanized high-affinity antibodies. One of such approaches is the Phage Display, an amazing and innovative technology that revolutionized the selection of antibodies. The 2018 Nobel prize in chemistry was awarded to George P. Smith and Sir Gregory P. Winter, for pioneering the phage display methods for selection of peptides and antibodies.

The Phage Display was developed as an alternative to traditional hybridoma technology to select diverse antibodies. Described initially by George Smith in 1985 (Smith 1985) and later by Sir Gregory P. Winter (McCafferty et al. 1990), Phage Display is a molecular technology that allows the potential of the immune system to be exploited to produce ligands for practically any structure. Its combination with modern protein engineering technology makes it viable to generate antibodies with high affinity and specificity for any diagnostic or therapeutic target (Barbas et al. 1991; Winter et al. 1994). The genes encoding the antibody's variable domains are fused to genes encoding bacteriophage coat proteins. The fused genes can be integrated in bacteriophage particles that also display the heterologous proteins on their surfaces and thus, a physical linkage is established between phenotype and genotype (Smith 1985). The most commonly used phage protein is the pIII minor coat protein located at the tip of the long, thin filamentous phage M13 (Brekke and Sandlie 2003). Phage Display vectors can be classified according to the coat protein used for display; whether the protein to be displayed can be fused to all copies of the coat protein or to only a fraction of them;

and whether the recombinant fusion is encoded on the phage genome or on a separate genome. They also vary according to the genome used for expressing the coat protein fusion: the genome can range from a wild type (M13) to a modified type that can be propagated as a plasmid under antibiotic selection; to a phagemid (a plasmid that carries the recombinant coat protein gene as well as a phage origin of replication) (Barbas et al. 2004). M13 pIII is often the first choice for Phage Display fusions because of its tolerance for large insertions (Georgieva and Konthur 2011). However, in phagemid vectors a truncated version of pIII is used for fusion which brings advantages, such as a more efficient display by reducing proteolysis of the fusion protein and reducing the size of the phagemid vector. Moreover, phagemid vectors, compared to phage vectors have other advantages, such as ease of cloning, capacity to accommodate a larger foreign DNA fragment, transformation efficiency, availability of a variety of restriction enzyme recognition sites convenient for DNA recombination and gene manipulation, and genetic stability under multiple propagations (Qi et al. 2012).

The pComb3X vector is a phagemid vector that is regularly used for Phage Display. Phagemid vectors contains a plasmid origin of replication and a phage-derived origin of replication and, like typical plasmids, an antibiotic-resistance marker is provided to allow selection, but on the other hand, unlike plasmids, the phagemid genomes can be packaged in the phage coat (Barbas et al. 2004). After creating an antibody library, the PCR products are cut by restriction enzymes and cloned into the pComb3X vector in frame with the truncated pIII. *Escherichia coli* (*E. coli*) cells are transformed by electroporation with the resulting phagemids and, as pComb3X does not have all the other genes necessary to encode a full bacteriophage, then they are infected with the helper phage (Barbas et al. 2004; Levisson et al. 2014). The helper phage provides all of the phage derived proteins and enzymes required for phage replication, and structural proteins that encapsulate both the helper phage and phagemid genomes, "helping" the process of replication and packaging of the phagemid genome. The helper phage contains a kanamycin resistance gene, which, along with the ampicillin resistance gene carried by the phagemid, assists in the selection of bacterial cells containing both the helper phage and the phagemid genomes. Therefore, the result is a library of phages, each expressing an antibody on its surface and harboring the vector with the respective nucleotide sequence within (Barbas et al. 2001).

Phages that display antibody fragments can be isolated and amplified by panning. Panning is the antibody selection process in Phage Display where antibody-displaying phage library is incubated with the antigen of interest and nonbinding phage are eliminated by washing. Bound phages are eluted by conditions that disrupt the interaction between the displayed protein and the target molecule and then amplified by re-infecting *E. coli* cells with new addition of helper phage. Selection rounds are subsequently repeated (usually, three to six rounds) using washing steps with increasing stringency, ultimately resulting in a phage population enriched in a limited number of variants with the desired binding affinity and specificity (Fig. 2) (Barbas et al. 2004; Levisson et al. 2014; Rader and Barbas 1997).

In general, three types of phage antibody libraries may be used: immune, naïve and synthetic (Aires da Silva et al. 2008a; Hoogenboom 2005). Immune libraries are derived from the IgG mRNA of B cells from an immune source, such as immunized

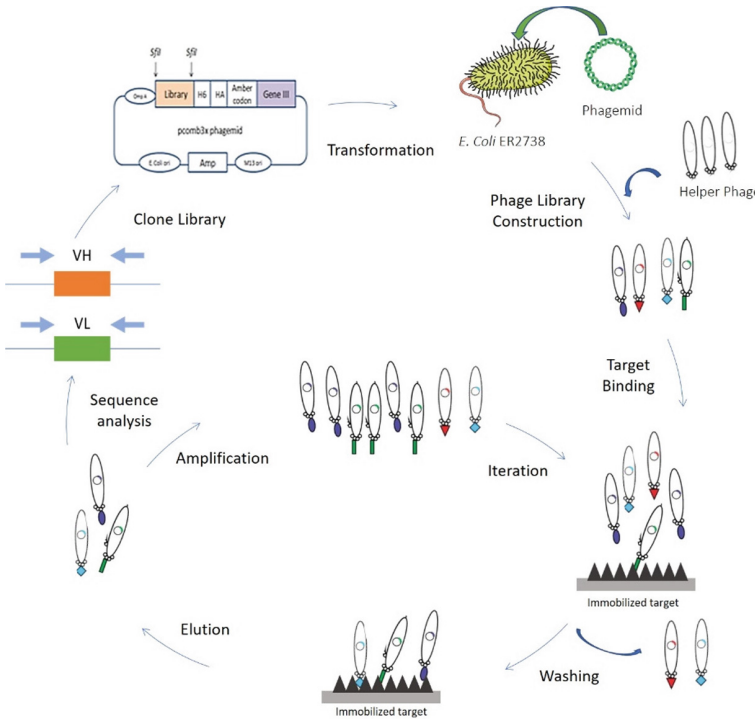

Fig. 2. Schematic representation of a Phage Display selection round (panning). A library of antibody DNA fragments, encoding random variants, is created and cloned into a phagemid vector (pComb3X). *E. coli* cells are transformed with the obtained phagemid and superinfected with helper-phage to create a library of phages, each displaying an antibody variant. The resulting antibody library is exposed to an immobilized target molecule and the nonbinding phages are washed away while bound phages are eluted and then amplified by infecting *E. coli cells.* The cycle of selection and amplification process can be repeated as necessary using in each round (panning) more stringent washing conditions in order to obtain phages with the displayed antibody of interest with the highest target-binding affinity.

animals or from B cells recovered from immunized patients. These libraries can result in higher affinity antibodies than those obtained from hybridomas. Naïve libraries are obtained from large antibody repertoires recovered from non-immunized donors. Synthetic libraries are entirely constructed *in vitro*. Each type of antibody library presents advantages and disadvantages, nevertheless in terms of antibody specificity and antigen affinity, immunized libraries are considerably more appealing than the other two (Aires da Silva et al. 2008a). For this reason, one of our group's strategies is to develop rabbit immunized antibody libraries in order to obtain highly specific antibodies towards antigens in their natural conformation status. In the next section, a brief overview of rabbit antibodies will be presented with a focus on single domain antibodies and their promising potential use as therapeutic scaffolds.

3 The New Generation of Recombinant Antibodies of Rabbit Origin

The rabbit antibody repertoire has been used for decades in diagnostic applications in the form of polyclonal antibodies (Rader et al. 2000; Weber et al. 2017). Now it also holds great promise as a source for generation of therapeutic mAbs. Rabbits, which belong to the order Lagomorpha (lagomorphs), are evolutionarily distant from mice and rats, which belong to the order Rodentia (rodents) (Popkov et al. 2003; Weber et al. 2017). As a result, epitopes conserved between rodent and human antigens that are invisible to rodent mAbs (and also human mAbs generated from transgenic mice with human immunoglobulin genes) can often be recognized by rabbit antibodies. This is of particular interest for the development of therapeutic antibodies that are to be evaluated in mouse models and are required to recognize both the human antigen and its mouse homologues. Rabbits also elicit a better immune response and few antibody gene segments are rearranged and accessed during rabbit immune responses, which reduces the number of PCR primers required for cloning of a repertoire (Aires da Silva et al. 2004; Barbas et al. 2004). Moreover, most strategies to generate mAbs are based on the recovery of B cells from spleen, bone marrow and/or blood, which are present in higher quantities in rabbits than in mice due to their larger body size (Barbas et al. et al. 2004; Feng et al. 2011; Popkov et al. 2003). In addition, and as previously demonstrated by us and others, rabbit antibodies can be converted to humanized antibodies that retain both high specificity and affinity for the antigen (Aires da Silva et al. 2004; Rader et al. 2000; Steinberger et al. 2000).

Over the past decade, we have been showing that rabbit derived single-domain antibodies (sdAbs) can be efficiently developed against several disease targets and that these minimal scaffolds show great potential for therapeutic applications (Aires da Silva, et al. 2005; Aires da Silva et al. 2004; Cunha-Santos et al. 2016; Oliveira et al. 2012). As mentioned above, these recombinant antibody molecules are the smallest functional antigen-binding fragments of an antibody that can be isolated from conventional IgGs (Holt et al. 2003). Due to their small size, sdAbs show improved tissue penetration and are able to reach targets not easily accessible by conventional antibodies, such as enzyme active sites or canyons in receptors molecules. Moreover, since sdAbs lack the Fc domain of a full IgG antibody, the nonspecific uptake in tissues that highly express Fc receptors is low. Additional important advantages of rabbit derived sdAbs include their high stability, low immunogenicity and lower manufacturing cost. Furthermore, sdAbs can be easily attached to other proteins, peptides, small molecules or nanoparticles by simple molecular biology or chemical procedures. Within this context, rabbit derived sdAbs are a promising platform and an alternative to conventional antibodies. Therefore, one of the main research lines in our Microbiology and Immunology lab has been the development of engineered sdAbs of rabbit origin against different antigenic targets using a combination of techniques that include rabbit immunization, antibody library construction and selection by Phage display technology. The sequence of techniques employed in this antibody production method is schematized in Fig. 3.

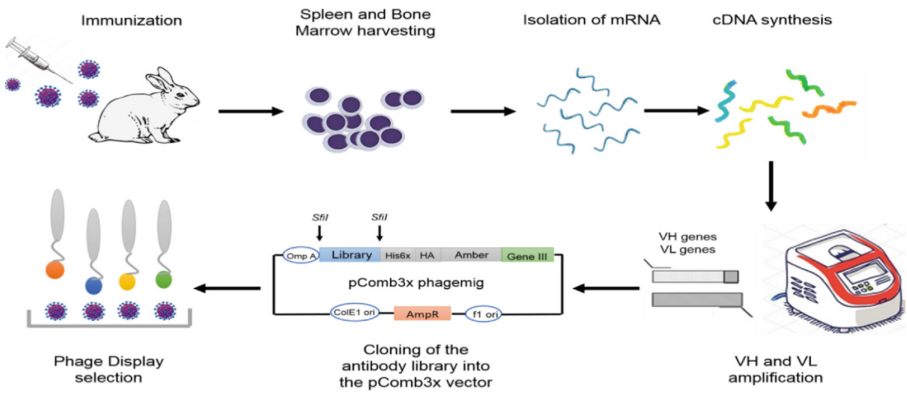

Fig. 3. Schematic representation of the methodology used in the development of rabbit derived recombinant Abs in our lab. After rabbit immunizations with the target antigen, the rabbit bone marrow and the spleen are extracted, the mRNA is isolated, and cDNA synthetized. The cDNA is then used to amplify separately the VH and VL antibody genes regions (sdAbs) with a panel of specific primers. The antibody fragments are then purified and cut with the *SfiI* enzyme and cloned into the pComb3x phagemid. Afterwards, sdAb-phages are produced and used for phage display selections.

4 Potential Applications of Rabbit Derived sdAbs

The potential of rabbit derived sdAbs as therapeutic tools is immense and largely dependent on the antigen they target. They can be potent anti-viral products, if they are directed against viral spike glycoproteins, or effective anti-tumor cytotoxic reagents, if they target a specific tumor antigen, and even important therapeutic carriers, able to deliver pharmaceutical agents to specific locations where their antigen can be found. In the next section, we will review examples of the above mentioned potential uses of recombinant antibodies of rabbit origin, currently being pursued in our laboratory.

5 Development of Specific Recombinant sdAbs Against gp120 HIV Glycoprotein

Human immunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS), a condition in which progressive failure of the immune system allows life-threatening opportunistic infections to succeed. One of the most important factors in the worldwide spread of HIV is its enormous genetic variability and rapid evolution. The revealing of all stages of HIV replication cycle led to the identification of potential therapeutic targets in order to decrease the replicative process.

Soon after HIV was isolated and confirmed as the cause of AIDS in the early nineteen-eighties, it was widely expected that an effective treatment would rapidly become available. However, almost forty years later, the scientific community is still struggling to develop an effective treatment. The extraordinary variability of the virus, its capacity to evade adaptive immune responses and the inability to induce binding neutralizing Abs against HIV-1, constitute barriers to the development of an effective therapy, able to

overcome the current resistances that the virus has gained to existing therapies. The limitations of currently available drugs and the difficulty encountered to develop an effective vaccine against HIV-1 infection, justify the search for new therapeutic strategies for AIDS treatment, including immune reagents, such as sdAbs which target steps in the viral replication cycle that are not affected by currently available drugs. The process of virus entry into the host cell, an essential step of the virus replication cycle mediated by the CD4–gp120 high affinity interaction, is a strategic step to target HIV replication. Therefore, the development of sdAbs capable of inhibiting this stage by blocking the glycoprotein gp120, is extremely promising.

The research line pursued in our lab aims to select sdAbs specific for gp120 from an immunized antibody library. The purpose is to produce sdAbs, which, because of their small size, may be able to bind inaccessible regions of the gp120 protein, thereby blocking the binding process and preventing the entry of the virus into the host cell. With this objective, the potential of rabbit derived sdAbs as therapeutic molecules is explored in our lab. For that, VH and VL immunized antibody libraries were constructed and specific Abs against gp120 were selected using the Phage Display technique. Antibody of high diversity were obtained, and the profiles attained on the Phage Display were consistent with the stringent conditions applied in each selection, resulting in antibodies recovered with high affinity for gp120.

This work is part of a project whose main goal is to develop a recombinant bispecific antibody (bsAb). bsAbs are essentially artificially designed antibodies that combine two antigen-recognizing elements into a single construct. A bsAb molecule can simultaneously address different targets involved in distinct pathophysiological processes or different epitopes in the same target and thereby increase therapeutic efficacy. This dual-specificity property of bsAbs has been successfully explored to develop therapeutic antibodies mainly for cancer therapy and inflammatory diseases. In our group we aim to explore the potential of bsAb for infectious diseases, particular for HIV. For that, we have engineered a bsAb, where one of the arms consists of an anti-gp120 sdAb optimized to specifically block the epitope responsible for interaction with the CD4 receptor. The other arm, will consists of an anti-gp41 antibody (F63) that we have recently developed and showed to have a broad and potent inhibition activity against HIV-1 and HIV- 2 (Cunha-Santos et al. 2016). With this strategy we aim to have a double neutralizing action in the virus entry process, able to prevent the fusion between HIV-1 and the host cell (Fig. 4).

The rabbit antibody repertoire shows great promise as a source for generation of therapeutic antibodies for blocking HIV infection (Aires da Silva et al. 2005; Aires da Silva et al. 2012; Steinberger et al. 2000). Nevertheless, recently other species, such as bovines, started also to be explored. Due to the characteristics of their immune system and their extraordinary long HCDR3, they seem to have good potential for the research of new strategies for immunization and development of antibodies. Therefore, it will be interesting to further explore the immunization of cows, as this may provide an opportunity to rapidly generate antibodies with prophylactic and therapeutic properties, which can address disease agents that have evolved to escape human antibody responses, such as HIV. Indeed, in a very promising study, researchers from the Scripps Research Institute injected HIV immunogens into the flanks of four calves and waited for their

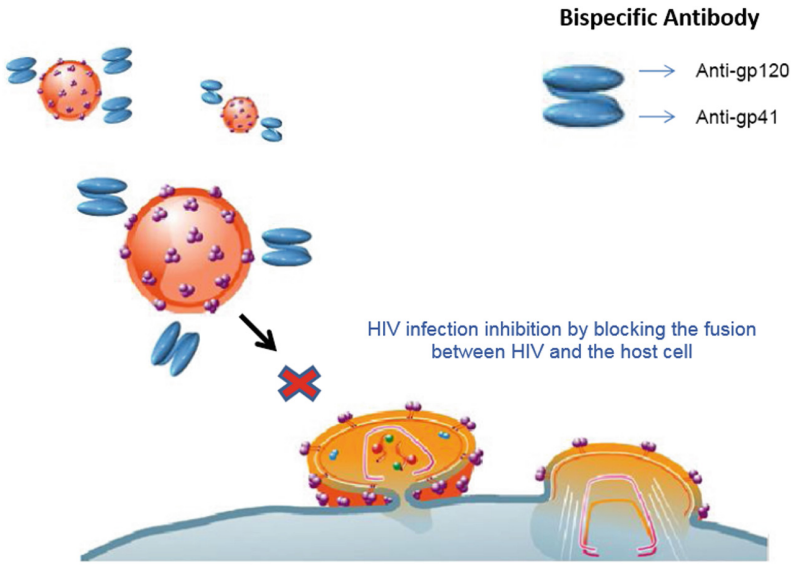

Fig. 4. Schematic representation of our strategy to developed bispecific antibodies for HIV-1. The developed bispecific antibodies will have the capacity to bind to the gp120 and gp41 proteins that are exposed to the surface of the viral particle. This binding will block the binding of gp120-CD4-gp41 and the process of HIV fusion and entry into the host cell.

immune systems to respond. All four cows developed neutralizing antibodies to HIV in their blood as rapidly as 35 to 50 days following two injections (Sok et al. 2017). While bovine antibodies are not likely suitable for clinical use in humans in their current form, exploring this rapid production may help in the development of new therapies for HIV. Another animal species that has caught the attention of the scientific community is the cat. The cat, being an animal that can also be naturally infected by a lentivirus, Feline immunodeficiency virus (FIV), causing an acquired immunodeficiency syndrome similar to HIV infection in humans, is actually the smallest natural model for the study of lentivirus infections. Due to the enormous structural and pathophysiological similarities between both viruses and their respective infections, the cat has great potential as a study model for the development of therapeutic strategies for HIV. The FIV mechanism of entry into the host cell is very similar to the interaction between SU gp120-receptor shown in HIV, since both virus use a primary binding and entry receptor (although different) for infection (Elder et al. 2010; Taniwaki et al. 2013). Thus, it would be interesting to evaluate the immune system response of the cat. Indeed, it could be interesting to verify whether, as in rabbits and cows, neutralizing antibodies would be elicited, using the same immunogen. It would be also interesting to develop new immunogens, derived from FIV Env glycoproteins, and evaluate the response obtained. The fact that cats can be naturally infected with the virus represents an advantage, because the immunizations protocol can be skipped, and serum can be directly obtained from FIV positive animals. Serum can be evaluated for the detection of neutralizing antibodies to determine which immunogen elicits its production, thus contributing to the development of an effective therapy and

vaccine. More than just providing an important study model for HIV, applying these techniques in the cat, may increase the knowledge of FIV, making it possible to obtain new molecules to improve the diagnosis, treatment, and eventually to revolutionize the prophylaxis of the disease.

6 Development of Recombinant Antibodies and Small Molecule Compounds for Diagnosis and Treatment of Naturally Occurring Canine Lymphoma

Naturally occurring tumors in dog mimic features of those observed in humans, difficult to reproduce with other models. This represents a unique opportunity to study long-term efficacy and toxicity of cancer immunotherapies. To overcome limitations associated with conventional preclinical models, we validate the naturally occurring canine B-cell lymphoma as an animal model for the development of immunotherapeutic strategies and new diagnostic tools for non-Hodgkin lymphoma (NHL). A multidisciplinary approach is being used for (1) construction of canine and human lymphoma biobanks, (2) comparative immunotherapeutic target characterization, (3) selection and characterization of innovative therapeutic molecules such as recombinant antibodies and small molecules compounds against clinically validated targets (*e.g.*, CD20) and novel receptor targets associated with lymphomas, (4) efficacy and safety studies of the selected therapeutic candidate molecules on xenograft mouse models. A multidisciplinary team is conducting this research line to strengthen the collaboration between the Medical and Veterinary Science involving excellent national and international institutions of research.

The current scenery of cancer research is experiencing a profound transformation with the introduction of immuno-oncology as the fourth pillar for cancer therapy. Not only have immunotherapies resulted in unprecedented clinical responses, rapid drug development and several first-in-class approvals from the FDA in the past few years, but the advent of such innovative therapies is also revolutionizing treatment procedures in current oncology and hemato-oncology clinical practice (Kelly 2018). As a result, clinical and translational research needs to adapt to a rapidly changing scenario to effectively translate novel concepts into sustainable and accessible therapeutic options for cancer patients (Golan et al. 2017). Historically, the success rate in developing new oncology drugs has been surprisingly low as compared to other areas of medicine, particularly in late phases of development. The complexities and challenges of the new era of immuno-oncology strongly emphasize the need to identify new strategies, models and paths to develop, fast, successful, and cost-effective therapies (Golan et al. 2017; Ventola 2017).

Advances in cancer immunotherapy relies on faithful predictive preclinical investigation and rodent models have been the foundation of preliminary basic research and safety assays (Malaney et al. 2014). However, these induced-tumor models underrepresent the heterogeneity and complex interaction between the human immune cells and tumors, and have often failed to correlate with clinical success rates (Biemar and Foti 2013; Kohnken et al. 2017; Kola and Landis 2004). The use of alternative animal models is pivotal to bridge the translational gap between murine models and human clinical studies. The inclusion of a canine model in the drug development path of cancer immunotherapies is being widely recognized as a valid solution to overcome several hurdles associated

with conventional preclinical models (Decker et al. 2017). Dogs with naturally occurring tumors are highly translational models that represent an opportunity to investigate the clinical potential of novel immunotherapies in a comprehensive manner and rapidly translate the results obtained from canine patients to human patient management, with benefits for both species (Barutello et al. 2018; Klingemann 2018).

Driven by the great success achieved with the application of immunotherapies in the treatment of human non-Hodgkin lymphoma (hNHL) and by the remarkable similarities of canine lymphoma to its human counterpart, canine lymphoma has been one of the main focus of comparative research regarding the development of immunotherapeutic approaches for dogs (Ito et al. 2015; Jain et al. 2016; Marconato et al. 2015; O'Connor and Wilson-Robles 2014; Weiskopf et al. 2016). These efforts are also motivated by the great clinical demand to improve the poor prognosis of dogs diagnosed with canine lymphoma (cNHL), considering that the majority of dogs, regardless of the treatment options used, relapse with terminal, drug-resistant disease (Vail et al. 2007). To date, two monoclonal antibodies (mAbs) - CD20-positive B cell (Blontress®) and CD52 positive T cell lymphoma (Tactress®) - have been approved by the US Department of Agriculture and are commercially available in the USA and Canada (Regan and Dow 2015). Nevertheless, the reported therapeutic efficacy of these mAbs is suboptimal and substantially inferior to results reported in human patients, demonstrating an urgent and unmet need to develop effective immunotherapeutic strategies for cNHL (Klingemann 2018).

The research underway in our lab aims at the development of a novel sdAb-based drug delivery system for canine NHL, and the characterization and validation of an animal model of human NHL. A multidisciplinary strategy addressing several distinct complemental objectives was carefully designed. Considering that high quality basic, clinical and translational cancer research requires prompt access to well-preserved biological samples, a canine lymphoma biobank has been established, with samples collected from several positively diagnosed dog patients. These samples were used to assess the cytokine mRNA profiles of circulating and intratumoral environments in multicentric canine lymphoma. Overall, it was determined that, similarly to its human counterpart, local and systemic dysregulation in cytokine response might be involved in cNHL pathogenesis and tumor survival promotion, progression and immune escape. Potential cytokine candidates to be used as targets for therapeutic intervention either by cytokine inhibition and/or immunomodulatory strategies were identified (Dias et al. 2018a, b). Preliminary studies have reported that chemotherapy protocols, while prompted complete tumor responses, appeared to fail to restore immune response in cNHL, possibly reducing its ability to re-establish control over residual disease (Axiak-Bechtel et al. 2014). Also, active immunotherapy strategies such as cancer vaccines have also proven to be successful in mounting an immune response and improving survival in dogs with NHL, when in combination with conventional chemotherapy (Marconato et al. 2014, 2015; Peruzzi et al. 2010).

In the last decade, the scientific community has been reporting cases of therapeutic success using mAbs in human lymphoma treatment, encouraging the establishment of similar therapeutic options in veterinary settings (Adler and Dimitrov 2012). However, the recently approved mAb-based therapies have presented disappointing scientific and

clinical results, demonstrating that the discovery of an effective antibody for the treatment of cNHL may require a more complex and innovative approach.

The anti-CD20 mAb Rituximab has revolutionized the treatment of Human B-cell malignancies and is now used in a growing range of clinical settings (Ito et al. 2015; Motta et al. 2010). This unprecedented success has shown the ability of mAb to improve outcomes and catalyzed the interest in the pharmaceutical industry to develop the next generation anti-CD20 mAbs (Alduaij and Illidge 2011; Chames et al. 2009). A few 1st generation mAbs targeting canine CD20 were recently developed and one has obtained clinical approval. However, unsatisfactory clinical results have highlighted the need for 2nd and 3rd generation mAbs and an urgent demand to gain better understanding of antibody effector functions present in the canine immune system (Ito et al. 2015; Jain et al. 2016; Klingemann 2018; Rue et al. 2015; Sinha 2014). With this in mind, we have conducted the first comprehensive target characterization of canine CD20 using our cNHL biobank. Data obtained under gene and protein expression studies on lymphoma and normal canine cells demonstrated an overexpression of this receptor on canine lymphoma cells, validating the canine CD20 as a potential target for veterinary immunotherapeutic strategies. Additionally, a new sequence of canine CD20 was identified in our biobank samples, diverging from previous published sequences. Furthermore, we reported the use of a novel strategy for the generation of recombinant anti-CD20 monoclonal antibodies sdAbs that bind both canine and human epitopes (Dias 2018a, b). This resulted in the selection of a panel of novel sdAbs, that may become useful tools for exploring the development of novel therapeutic alternatives for comparative oncology.

Among mAb-based therapies, antibody-drug conjugates (ADCs) are considered one of the most promising strategies, combining the tumor selectivity, pharmacokinetics and biodistribution properties of antibodies with the cytotoxic potency of small molecules (Diamantis and Banerji 2016) (Fig. 5). The selection of the target antigen is the first and most important determining factor for a successful ADC, directly affecting its efficacy, therapeutic window and toxicity profile. During the development of sdAbs against novel cNHL targets, we focused in selecting sdAbs with binding and internalization properties, in order to develop a highly selective and potent new anti-cancer ADC therapeutic molecule. For that purpose, a highly diverse library of rabbit sdAbs against primary canine NHL cells has been successfully constructed, to ensure the presence of antibodies against any potentially relevant target that is overexpressed in the context of the disease environment. By coupling subtractive antibody selection rounds on whole-cells and a high-throughput screening, we have selected a panel of novel sdAbs targeting cNHL. The obtained data showed that these selected sdAbs have great promise. In fact, recently a final *in vivo* selection on a human and canine NHL murine model confirmed that these sdAbs populations were indeed highly selective and specific against NHL (Dias 2018a, b). With this novel approach, the repertoire of targetable NHL tumor receptors may be expanded, while simultaneously confirming the availability of the epitope *in vivo* and generating new antibodies for targeting. In the future, we expect to use these promising sdAbs as tools for the development of a novel ADC for NHL, by coupling a potent cytotoxic drug payload to the selected sdAbs.

Among the main components of an ADC – payload, linker and antibody – the payload is an important and crucial constituent of the therapeutic ADC molecule. In broad

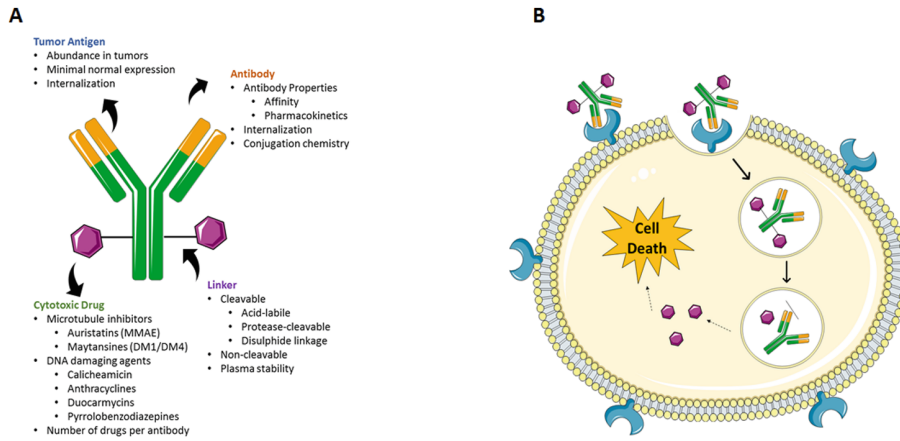

Fig. 5. Conventional ADC molecule and its mechanism of action. A) ADCs consist of a cytotoxic drug conjugated to a monoclonal antibody by means of a select linker. B) Delivery of cytotoxic drugs to cancer cells by ADCs. The monoclonal antibody component of an ADC selectively binds a cell-surface tumor antigen, resulting in internalization of the ADC-antigen complex through the process of receptor-mediated endocytosis. The ADC-antigen complex then traffics to lysosomal compartments and is degraded, releasing active cytotoxic drug inside the cell. Free drug then causes cell death through either tubulin polymerization inhibition or DNA binding/damage depending on the drugs mechanism of action.

terms, payloads for ADCs can range from small cytotoxic molecules to protein toxins, enzymes, other proteins and radionucleotides (Strohl and Strohl 2012). In the first-generation ADCs, researchers used clinically approved chemotherapeutics with known clinical profiles like doxorubicin, methotrexate, and 5-flurouracil as payloads. However, this ADC class was underwhelming due to the insufficient potency of the payload and/or high toxicity as a consequence of the instability of the ADC and systemic loss of the drug (Dan et al. 2018; Vankemmelbeke and Durrant 2016). Since then, the development of second-generation ADCs focused on improving linker stability. Moreover, it has resorted to more potent (100–1000-fold) tubulin-targeting agents such as the auristatins and maytansinoids, or DNA-targeting agents such as the calicheamicins. That has led to the relatively recent US FDA approval of two microtubule-inhibiting ADCs: brentuximab vedotin (SGN-35, Adcetris®) for Hodgkin and anaplastic large cell lymphoma and ado-trastuzumab emtansine (T-DM1, Kadcyla®) for metastatic HER2-positive breast cancer. Third generation ADCs focused on site-specific conjugation to ensure homogenous ADCs with well-defined DAR. At the same time, efforts are being made to explore payloads with novel modes of action, with special focus on agents that present activity against non-proliferating cancer cells, which would widen the target area to include tumor-initiating cells and possibly overcome resistances (Vankemmelbeke and Durrant 2016).

Within this context, a screening of multiple drugs, acting via different mechanisms, was conducted to assess their cytotoxic effect on cNHL, aiming to identify potential payloads to be coupled with our sdAbs. A panel of seven HDACis was initially tested

on the well characterized CLBL-1 canine B-cell lymphoma cell line and panobinostat was identified as the most promising compound. Panobinostat was therefore thoroughly characterized and showed strong *in vitro* and *in vivo* antitumor properties (Dias et al. 2018a, b)

The execution of this work prompted the establishment of a murine xenograft model of canine B-cell lymphoma using CLBL-1 cell line, required to test the antitumor effect of panobinostat *in vivo*. This model established with a stable CLBL-1 expressing firefly luciferase and GFP reporters proved to be a rather efficient non-invasive and quantitative method to monitor the outgrowth of cNHL. Thus, it represents an innovative preclinical tool for comparative medicine (Dias et al. 2018a, b).

The approach taken so far allowed, not only the characterization of a new potential therapeutic antibody for lymphoma treatment, but also the establishment of a workflow that shows tremendous potential for the development of similar molecules. The methodologies applied to identify potential targets, select and characterize the best therapeutic antibody, characterize a highly potent payload and to develop and characterize a bioluminescent model for *in vivo* testing, resulted in a thorough process that can be easily translated to other drug development research projects. This work opens up perspectives in comparative oncology as it contributes for the validation of the naturally occurring canine B-cell lymphoma model for translational immune-oncology research (Fig. 6).

7 Development of Recombinant sdAbs for Brain Targeting and Drug Delivery Across the Blood-Brain Barrier

Neurological disorders affect up to 1 billion people worldwide and are responsible for 12% of the global deaths, ranking the 5th leading cause of global deaths in 2016 and having more than doubled between 2000 and 2016 (WHO). A new wave of innovative antibody-based therapies promises significant breakthroughs in a range of diseases. Yet, in the field of CNS, drug discovery and development researchers are experiencing difficulties in developing drugs that can complete clinical trials and reach regulatory approval. In fact, as reviewed by Strohl, not a single CNS antibody-based molecule has been approved by FDA (Strohl 2017). Despite intensive research on the structure of the CNS and the advances of medical and nanotechnology, many neurological diseases remain undertreated by effective therapies and this is not due to the lack of good candidate drugs. A major bottleneck in successful development of CNS drugs is the discovery and design of drugs that can cross the Blood Brain Barrier (BBB) (Chen and Liu 2012).

The BBB main function is to maintain the homeostasis of the brain by regulating the exchange of substances between blood and brain while protecting it against undesirable molecules and pathogens (Neves et al. 2016; Pardridge 2016, 2017; Shen 2017). Unfortunately, the BBB also serves as a barrier to potentially beneficial drugs for treatment of CNS diseases (Abbott et al. 2010; Boado et al. 2007; Neves et al. 2016; Pardridge 2016; Pardridge and Boado 2012). Consequently, only small molecules (<400 Da) with appropriate lipophilicity, molecular weight (MW) and charge can freely diffuse across the BBB. Higher MW substances essential to the brain can only transpose by either transport proteins, specific receptor-mediated transcytosis (RMT), or adsorptive transcytosis (AMT). As a result, the majority of small molecules (MW > 500 Da), proteins,

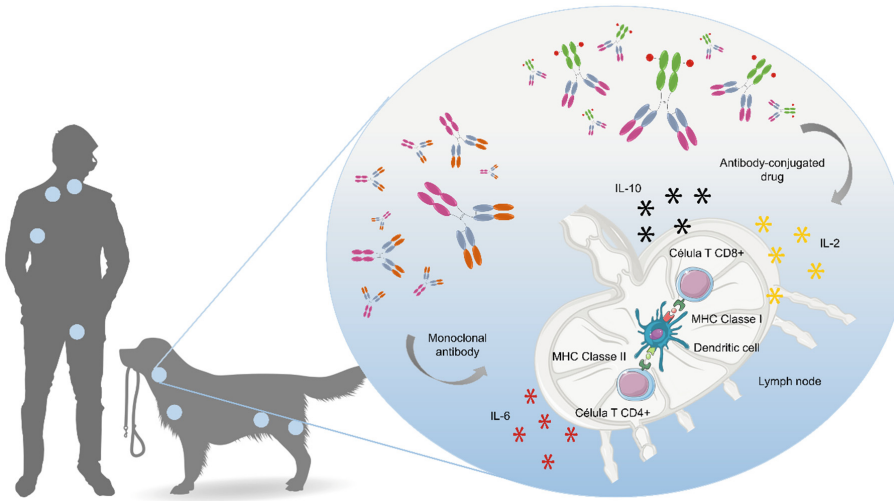

Fig. 6. Schematic representation of the approaches explored in the development of recombinant antibodies and small molecules compounds for diagnosis and treatment of naturally occurring canine lymphoma in our lab. Overall, our research group is particularly interested in the development of innovative mAb-based therapies. By focusing on therapeutic areas with a high unmet medical need, such as cancer, we aim to provide solutions that improve patient's quality of life and prolong lives. At the core of our oncology program is the validation of canine lymphoma as an animal model for immunotherapeutic approaches in comparative medicine. Notably, the Canine Lymphoma Project provides an integrated drug discovery platform that maximize interdisciplinary cooperation and leverage commonalities across humans and dogs, for the development of novel immunotherapies against non-Hodgkin lymphoma, benefiting both species. Currently, this work is actively investigating new immunotherapies that mobilize the patient's own immune system to combat cancer and mAb-based drug delivery systems that selectively direct potent payloads to the site of the disease and promote cancer cells death.

and peptides do not freely cross the BBB which results in 100% of large protein drugs and >98% of small molecule drugs unable to reach its CNS target, representing a major obstacle for the accumulation of the majority of biologically active molecules in the brain (Neves et al. 2016; Pardridge 2012).

A promising approach to circumvent the BBB impenetrability is to develop CNS drug delivery strategies that take advantage of BBB transport systems. Due to the specificity of the interaction, RMT is being strategically used for brain targeting and drug delivery. In this pathway, specific and highly expressed receptors, such as insulin receptor (IR), transferrin receptor (TfR), when bound to its ligand, internalize the ligand–receptor complex and release the ligand at the abluminal (brain) side of the brain capillary endothelium, allowing the delivery of macromolecules important for brain function. To take advantage of this transport system, mAbs targeting endogenous BBB receptors are being reengineered as "Trojan Horses" to cross the BBB without damaging the cells (Lajoie and Shusta 2015; Pardridge 2012; Pardridge and Boado 2012). mAbs are particularly appealing brain shuttles because not only can they be designed to specifically target BBB receptors that undergo RMT, but also be conjugated with other molecules

or nanoparticles with therapeutic features as previously mentioned. Several studies have already validated the use of anti-IR and anti-TfR IgG antibodies to transpose the BBB (Pardridge 2016; Boado et al. 2007; Pardridge et al. 1995; Pardridge 2012). Moreover, fusion of these antibody to different lysosomal enzymes (Boado et al. 2012, 2014; Boado and Pardridge 2017; Pardridge 2012), peptide radiopharmaceuticals (Wu et al. 1997), bi-specific antibodies (Niewoehner et al. 2014; Yu et al. 2011) and pegylated liposomes (Xia et al. 2008; Zhang et al. 2003), validated the use of receptor-specific antibodies for brain transport and demonstrated their potential applications in terms of therapeutic and diagnostic of neurological diseases. Nevertheless, despite the validation of antibodies toward insulin and transferrin receptors as potential brain shuttles, these are ubiquitous receptors. As a result, the approaches currently available generally result in a low fraction (1%) of the injected dose actually reaching the drug targets in the brain (Neves et al. 2016). Within this context, there is a pressing need to identify and develop new molecules as well as to discover novel BBB endogenous receptors which could provide a more selectively delivery into the brain.

A promising alternative to conventional mAbs molecules are the sdAbs. As previously mentioned, fragmentation of antibodies results in modified physiochemical features of these therapeutic molecules particularly interesting for BBB targeting, such as reaching targets not easily accessible by conventional antibodies. More importantly, these fragments can be easily attached to other proteins, peptides, small molecules, or nanoparticles by simple molecular biology or chemical procedures, which makes them ideal to function as brain-targeting drug delivery vectors (Abulrob et al. 2005; Aires da Silva et al. 2008b; Nelson et al. 2010). In line with this, our group has been focused in the development of sdAbs targeting brain RMT receptors for BBB translocation and drug delivery. To develop highly specific brain targeting sdAb we are exploring the potential of our rabbit derived sdAb platform and in vivo Phage display selection. It has been argued that *in vivo* phage display selection procedures offer an advantage over in vitro screening procedures in that the antibodies that are displayed at phage surface can be selected in the complicated milieu of the animal based on desired pharmacokinetic and targeting specificity properties. Moreover, antibodies are identified and tested functionally and must overcome natural mechanisms of degradation. Also, it is known that *in vivo* BBB endothelial cells get stimulated by their surrounding cells and intraluminal blood flow. This contributes to the complexity of the BBB and regulates the expression of specific receptors at the cell surface in a polarized fashion. For these reasons, screening for BBB transmigrating antibodies should preferably be performed *in vivo*. Within this context and since rabbit derived antibodies have strong cross-species reactivity, we are exploring the potential of *in vivo* phage display screening on mice models (Fig. 7). With this approach we were already able to identify a panel of novel and highly specific BBB crossing sdAbs. Currently we are exploring the use of these brain drug delivery systems in several CNS disease models including Alzheimer, Parkinson, glioblastoma and meningitis. We believe that the ability of delivering brain-specific therapies will allow drug targeting to the disease and an increase of drug concentration at the disease site increasing efficacy of the treatment and also a reduction of systemic distribution of drugs, which leads to decreased toxicity and side effects.

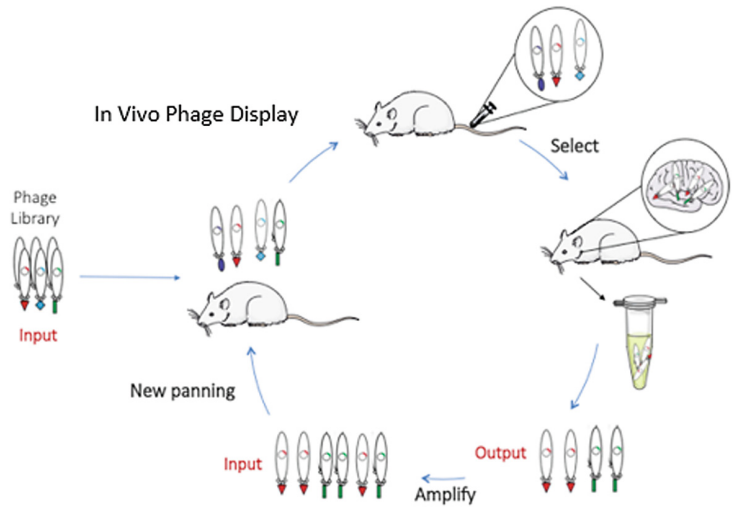

Fig. 7. Schematic representation of the in vivo Phage Display selection explored in our lab to develop highly BBB specific antibodies. In the *in vivo* phage display technique phage libraries are injected intravenously into the mice tail and then phages are allowed to circulate for a selected period of time. Then, mice are perfused with sterile PBS to remove unspecific antibodies. Brain and other organs (e.g.: liver, kidney, lung, etc.) are collected, weighed, homogenized and phage are eluted, titrated and amplified for a new round of selection. A total of 3–4 rounds of *in vivo* selections are performed until a high enrichment is observed. The amount of infectious sdAb-phage particles in the blood and other organs are also determined during each panning to determine the percentage of sdAb-phage that is actually crossing the BBB.

8 Conclusions

Enormous strides have been made in the past two decades towards the discovery, optimization, and therapeutic application of antibodies. Paul Ehrlich dream of antibodies as elegantly sensitive therapeutics has been finally fulfilled. A century later, after his vision of antibodies as "magic bullets" a total of 87 antibody-based molecules have been approved as therapeutic agents for a wide range of indications, such as cancer, autoimmune diseases, and infectious diseases, among others. With more than 150 candidates in late-stage clinical studies, it is clear that antibodies are becoming the most promising and rapidly growing class of human therapeutics. Indeed, in 2017 the top five therapeutic antibodies (Rituxan, Remicade, Herceptin, Humira, and Avastin) reached the top 10 best-selling innovative drugs (Strohl 2017) and the global antibody sales revenue was estimated to be valued at USD 95 billion and is projected to reach USD 341 billion by 2026 end (Future Market Insight 2016).

Most of the approved antibodies are conventional full-length IgG molecules. Nevertheless, as presented in this review, the versatility of the antibody molecule and the capacity to engineering it into novel and smaller antibody molecules, such as rabbit derived single domain antibody scaffolds, allowed to design novel constructs for a range of applications proven to be far more appealing for human therapeutics. In addition to its use as a therapeutic agent on their own, the antibody molecule can also be devised as a

drug transport shuttle. To take advantage of this property, several antibody fragments and scaffolds are being engineered to transport cytotoxic drugs, radiotherapeutics molecules, or immunocytokines greatly increasing the applicability of these biodrugs for several disorders (Fig. 8). With this scenario, we will witness an increase in a next generation of antibody-based molecules such as bispecific, bifunctional antibodies, ADCs, and cell-surface-expressed antibodies (CARS), which will certainly present superior properties to those of the antibodies now in clinical use.

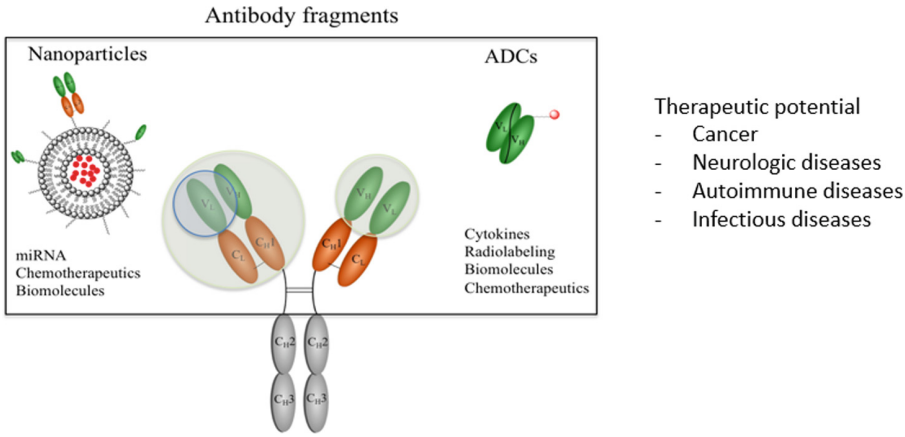

Fig. 8. Schematic representation of different antibody therapeutic applications.

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Trends in Translational Medical Research: Companion Animal Models

L. A. Mestrinho(✉), E. Delgado, M. Lourenço, and M. Niza

Clinical Research Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
lmestrinho@fmv.ulisboa.pt

Abstract. Naturally occurring diseases in companion animals can be used for the development of drugs and other treatments, devices and methods for diagnosis and prognostic studies. The identification and optimization of appropriate spontaneous disease models for a given human disease requires deep knowledge of comparative anatomy, physiology, pathology and medicine. As some companion animal models have already been identified for oncologic, inflammatory, genetic and degenerative diseases, this clinical research team has developed work in the fields of immunology and oral oncology. This manuscript aims to review three companion animal clinical research models of allergic conjunctivitis, atopic dermatitis and oral squamous cell carcinoma, illustrating the active contribution of small animal clinical investigation in biomedical sciences.

Keywords: Translational research · Companion animals · Dog model · Atopic dermatitis · Atopic conjunctivitis · Squamous cell carcinoma

1 Introduction

Physicians and veterinarians have been walking side-by-side, using similar means to resolve common problems, practicing both branches of one medicine, so from the early 20th (Bradley 1927). If initially animals were used solely with the ultimate goal of benefiting human health, now the scientific society is questioning the relevance of basic animal research to the clinical setting, different from the scientific contribution *per se*. Simultaneously, the society is demanding a reciprocal benefit for both man and animals used in research. Moreover, throughout the years, many animal experiments have been in some instances, poorly designed or conducted with major pitfalls that compromise their true benefit to humanity and lead to unnecessary waste of animal's lives.

Comparative medicine facilitates the translation of knowledge since the disease pathogenesis is frequently similar among species, but animal studies should be well designed, performed before clinical studies and the same principals of clinical trials should be applied, namely randomization and blinding. Additionally, systematic reviews of animal studies, in order to compare results with already existent clinical trials, should be routinely performed, since they are the best way of producing evidence about the value of animal research to human clinical research (Pound et al. 2004; Faggion 2015).

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In this context, multi and cross disciplinary collaborations with veterinary clinical researchers have led, more recently, to translational studies of reciprocal interest using spontaneous animal models from small animal practice. Naturally occurring diseases in companion animals can be used for the development of drugs and other treatments, devices and methods for diagnosis and prognostic studies. This synergic work has been built over the One Health concept, a worldwide strategy for expanding interdisciplinary collaborations and communications in all aspects of healthcare for humans, animals and the environment, in order to accelerate biomedical research from the 21st century and beyond.

This work will review three companion animal clinical research models focusing in the fields of ophthalmology, dermatology and oral oncology research.

2 Companion Animals in Clinical Research

The rationale behind the use of companion animals in clinical research is set upon two arguments. The first one is related with the predictability of preclinical studies in laboratory animals. Predictability from pre to clinical studies is often poor, raising questions about the use of genetically modified models. There is no doubt that they show obvious advantages providing quick and repeatable results, since the studies are performed in homogenous populations, within controlled environments. However, they do not represent the variability encountered in real life, especially with regards to environment - stressors and risks – and genetic variability. Human beings share a similar environment, common risk factors and stressors, and many genetic traits with companion animals.

Consequently, some researchers have identified companion cats, dogs, horses, rabbits, etc., as "proof of concept" opportunities, filling the major pitfalls between preclinical and clinical trials mentioned above. Veterinary clinical trials can be used as predictors of those parallel in the human field in terms of efficacy, filling this gap and perhaps accelerating approval of new therapies (Kol et al. 2015). It is well known the classical example of application of topical cyclosporine for the treatment of keratoconjunctivitis sicca, which was firstly reported by a veterinarian to treat the same disease in the dog (Kaswan et al. 1989).

It is imperative to have deep knowledge of comparative anatomy, physiology, pathology and medicine in order to identify and optimize naturally occurring disease models for a given human disease. For this reason, multidisciplinary research teams must work together, including human doctors, veterinarians, engineers, geneticists, among others. Novel information is always being published, and throughout genome research it is possible to identify genetic predispositions and gene expression patterns in diseases. Some companion animal models have already been identified for oncologic, inflammatory, genetic and degenerative diseases. With regards to cancer, cats and dogs can become potential candidates for treatment trials and studies of universal causes of cancer risk and progression (Nunney et al. 2015), since they share histological, clinical and genetical similarities with Man (Gordon et al. 2009; Nunney et al. 2015).

Seeking new options for the management of cancer disease for their beloved companions, owners are eager to enroll pre-clinical research trials. Simultaneously, the investment from pharmaceutical companies in the development of new therapies with potential

applicability in animal health has been remarkable. Consequently, veterinary clinical trials, using companion animals, usually enrolled at teaching hospitals, in order to facilitate integration in translation studies, are adapting guidelines based on human trials conduct (Page et al. 2016). Regardless the ultimate benefit for Man, many questions still remain open, most of them related to the ethical and legal implications of the models themselves (Gordon et al. 2009). One is the informed consent, obtained from a client/owner, which brings ethical and controversial implications related to client vulnerability and the safeguard of the best interest of the animal itself (Hampshire 2003). Others relate with specific aspects like the housing of pet dogs and cats, which is not the same as for laboratory animals, and best practice recommendations. Best practice recommendations for cancer trials have been published recently (Page et al. 2016).

Finally, at this point in time, infrastructures that coordinate animal and human health professionals, basic scientists, pharmaceutical companies and society are lacking or are taking their first steps. Clinical and pre-clinical trials using companion animals from veterinary practices started in a large collaborative effort with cancer research institutions, namely the National Cancer Institute (NCI, USA) throughout the Oncology Trials Consortium or the Institute of Cancer Research (ICR, UK). Some leading examples relate to the use of rapamycin's in appendicular canine osteosarcoma, targeting its future use in pediatric osteosarcoma patients (Gordon et al. 2009; Khanna et al. 2009; Paoloni et al. 2010).

3 Canine Atopic Models

Atopy is an extremely common, pruritic, life-long and frustrating disease and a challenge to treat in both people and animals. Canine atopic dermatitis (CAD), with an estimated prevalence of 20 to 30%, is a paradigmatic chronic inflammatory skin disease characterized by a complex pathophysiology and a wide spectrum of the clinical phenotype (Wilhem et al. 2010; Casimiro 2017; Marsella and De Benedetto 2017). In fact, dogs can present cutaneous manifestations (atopic dermatitis is in fact by far the most common presentation), conjunctivitis and also rhinitis. This wide spectrum includes at one end the very minor forms of atopic dermatitis (AD), such as the canine patients with minimal itching and few secondary lesions and the most severe forms of AD at the other end, with high levels of pruritus, secondary lesions such as lichenification and excoriations and a significant impairment of the quality of life both of dogs and their owners (Linek and Favrot 2010). Typical distribution of body lesions can be observed in Fig. 1.

Response to treatment varies among patients with even the most efficient drugs to fail to succeed in controlling the disease in some; additionally, changing from one drug to another can lead to better management of the disease in a particular patient (Olivry et al. 2015; Saridomichelakis and Olivry 2015). To date, there is no way to predict individual response to treatment (Marsella et al. 2018).

As we understand more about atopy in both species, we appreciate the striking similarities that exist both with regards to clinical manifestations, immunologic anomalies, the aggravating role of secondary bacterial infections and the predisposition for allergic sensitization (Fig. 2). Also, the dog shares man's environment and way of living, has a spontaneous form of the disease and is phylogenetically closer when compared with the rat, the frequent animal model used for AD trials.

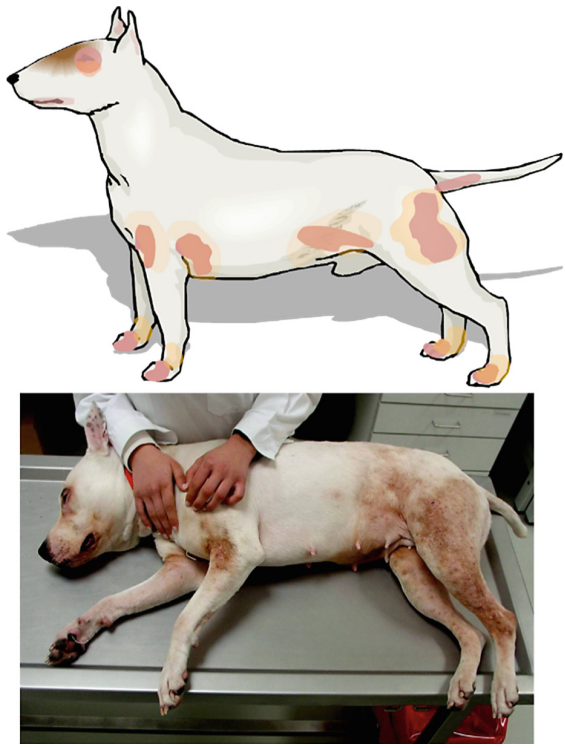

Fig. 1. Schematic representation of a patient with typical lesion distribution of canine atopy and same distribution on a real patient.

In Man, among all chronic inflammatory diseases, AD also represents a paradigmatic condition because of its genetic and pathophysiological complexity (Bieber 2008). Despite high degree of clinical heterogeneity in both species, it is unclear whether this diversity exists at a biological level. AD is still considered a single disease, usually treated according to the "one-size-fits-all" approach (Bieber et al. 2017). However, patients show different disease progression and response to treatments (Moyaert et al. 2017). It is hypothesized that AD, both in Man and in the dog, cannot be explained by one mechanism alone. In the future we have to approach AD in a more differentiated way, dissecting and stratifying clinical phenotypes into endotypes with the goal to refine the management of this condition. This methodology will not only allow an optimized treatment and prevention with the available drugs, but also hopefully help assign newly developed medicinal products to those patients who will have the best benefit/risk ratio. The identification of hypothetical endotypes, based on cluster analyses of immune mediators has been demonstrated in Man for other allergic diseases including asthma, chronic rhinosinusitis (Hinks et al. 2016).

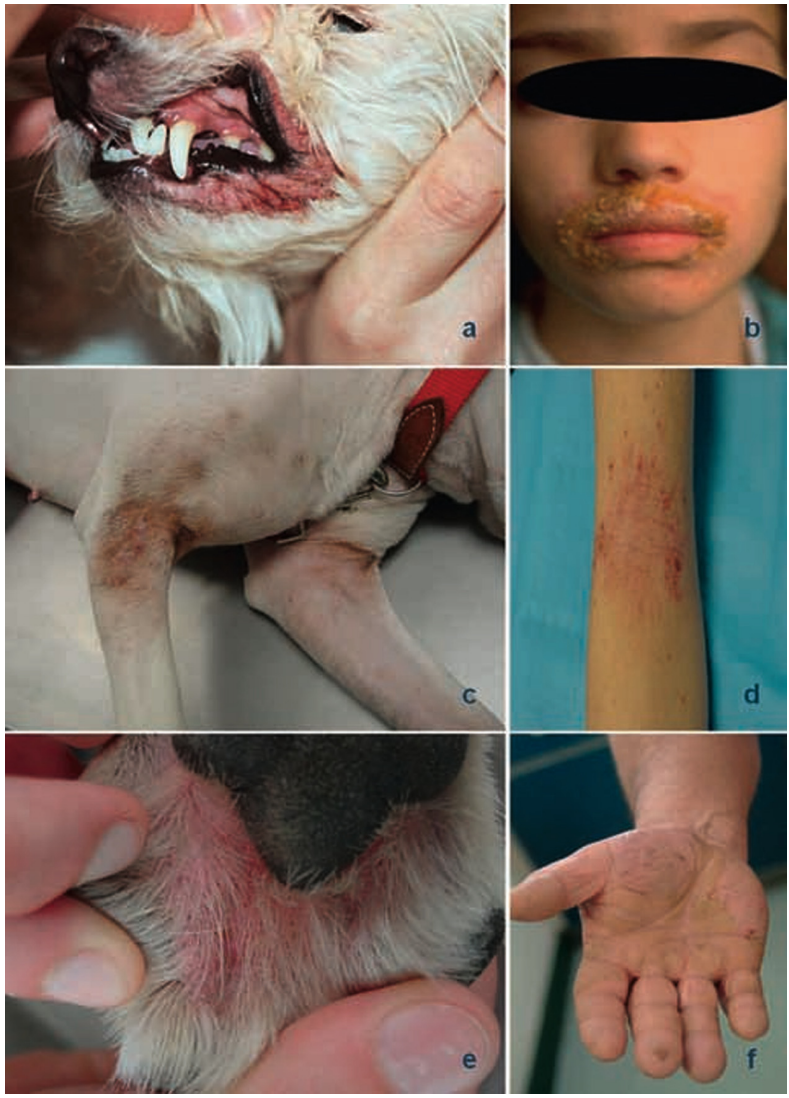

Fig. 2. a) Erythema, alopecia and severe perilabial lichenification in a cAD case; b) Erythema, scabs and perilabial lichenification in a child with AD; c) Erythema, lichenification and wheals in the flexor surface of the elbow joint in a cAD case; d) Erythema and lichenification in the flexor surface of the elbow joint in a child with AD; e) Severe foot erythema in a dog with AD; f) Lichenification and xerosis of the palms in an adult with AD. Original from Ana Mafalda Lourenço and Mário Morais-Almeida, with permission.

Joint efforts from our dermatology and ophthalmology research teams were made to deal with this shared disease. In our approach we always look for a comparative approach with the disease in Man, as we think it can be beneficial to understand its' natural course and the disparities regarding responses to existing therapies.

4 Canine Atopic Dermatitis

Growing awareness exists with respect to the resemblances between canine and human AD and the appreciation of how a comparative approach is valuable. Nowadays, in veterinary medicine, the term AD is frequently used as synonymous of environmental allergic skin disease. As such, it is expected that allergen specific IgE are present, since they are considered as one of the criteria for the clinical diagnosis of this disease. Nevertheless, clinically identical forms that lack specific-IgE have been also recognized and are currently named "atopic-like" dermatitis.

Both intradermal tests (Fig. 3) and allergen specific serology are accepted forms of determining positive sensitization that is subsequently interpreted according to the patient's clinical history. Allergens tested must be chosen in accordance to the geographic area of the patients and we have optimized a set for the area of Lisbon. Also, we have studied the possibility of using skin prick-test (SPT) as an alternative for skin testing in the dog as these are very convenient, performed without sedation and usually done in Man (Matias et al. 2014). Although doable as a technique, even without sedation, reactions were minor and difficult to interpret. Therefore, since overall intradermal skin tests offer a much more reliable interpretation, we do not advise the use of SPT for canine patients.

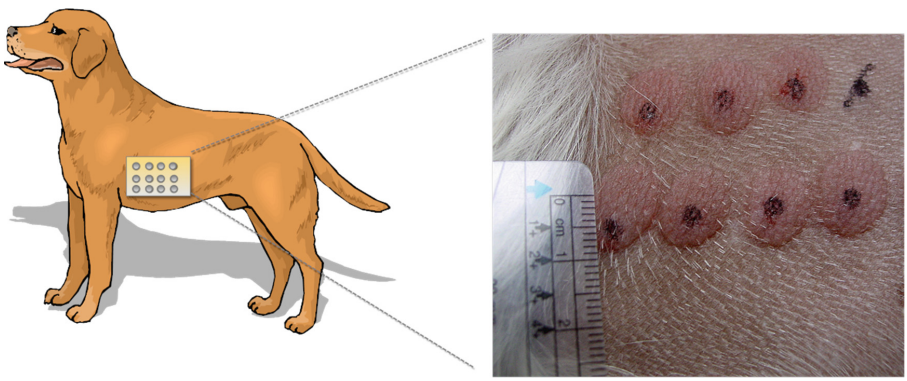

Fig. 3. Representation of intradermal test in the dog with 7 positive reactions shown in the picture to the right

Currently atopic dermatitis is not to be viewed as a single entity, but more as the illustrative term for a clinical syndrome, that may have different endotypes translating in particular clinical presentations (sometimes breed specific). Previously, the existence of substantial differences between the clinical phenotype of certain dog breeds and the phenotype in a generic population of atopic dogs has been reported (Wilhem et al. 2010). Recently, we aim to establish breed phenotype for AD in the Portuguese Water Dog. This breed presents a specific breed phenotype for AD being predisposed to the development of otitis, conjunctivitis, dry skin, yeast infections and lesions in the dorsolumbar area, front feet, pinnae and eyelids (Fig. 4) (Casimiro 2017).

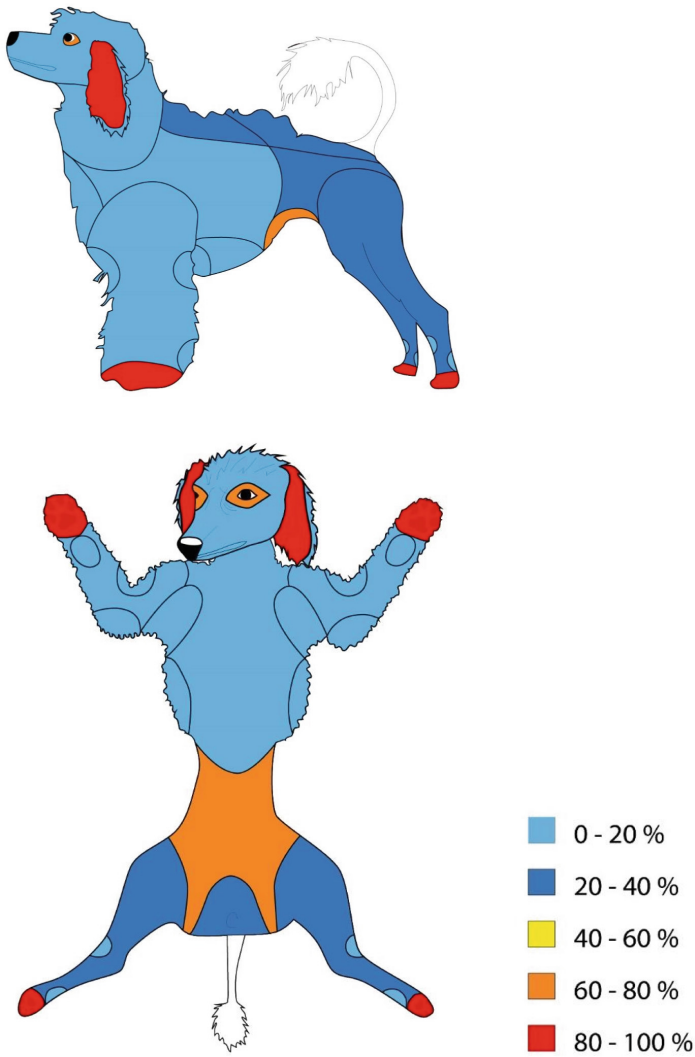

Fig. 4. Prevalence of affected areas in Portuguese Water Dog with Atopic dermatitis. Original drawing from Mariana Machado, with permission.

Diversity between canine atopic patients also applies to different and individually unpredictable responses to therapy, the latter being a potentially frustrating topic. Due to the multifactorial nature of this disease, management frequently requires a polypharmacy approach to improve the quality of life of the patient which can be deeply impaired by severe pruritus. Proactive therapy with topic steroids is one approach tried with success in canine AD by our research team (Lourenço et al. 2016) in order to prevent acute flares and was inspired by the good results achieved in human AD. Eventually this approach has made its way from "our hospital patients" to the treatment guidelines for canine AD.

Another shared aspect with human AD is the aggravating role of secondary bacterial infections of both skin and ears. Secondary infections add to the pruritus and need to be controlled but every effort must be made in order to prevent antibiotic abuse and development of bacterial resistance. Our team also looks into alternative ways to manage these infections without using to antibiotics whenever possible as recently was the case with the use of medical honey for infectious otitis (Maruhashi et al. 2016). Efforts have also been made to try and identify potential targets for a *Staphylococcus* vaccine (Couto et al. 2016).

Currently we are looking for the discovery and validation of reliable biomarkers for CAD. The ability to endotype AD canine patients may contribute to precision medicine by allowing treatment to be tailored for individual patients (Marsella et al. 2018). This would not only be beneficial for patients but would also reduce health-care related costs. Also, biomarkers that might help identify the different phenotypes early in life, including of those patients who outgrow the disease, could help to predict the course of the disease.

5 Canine Allergic Conjunctivitis

An allergic disease is characterized by an exaggerated response to innocuous antigens, developed by genetically predisposed individuals. Allergic conjunctivitis is a common disease in Man and has a powerful impact in Public Health in economic and social terms. The natural occurring allergic ocular disease in dogs resembles quite closely the human seasonal allergic conjunctivitis making the canine experimental model a very important tool to investigate etiopathogenetic mechanisms and develop drugs useful to both Man and Companion Animals.

Allergic manifestations tend to occur where the body is most exposed to agents from the external environment, being the eye an ideal and frequent site for allergic reactions.

The term ‘allergic conjunctivitis’ refers to a collection of disorders that affect the lid, conjunctiva and/or cornea. Allergic conjunctivitis ranges in severity from mild to extremely severe forms, which can interfere significantly with quality of life and vision, imposing a substantial burden of disease and medical cost on allergy patients (McMenamin 2008). Nevertheless, little focus is put to the ocular symptoms on their own. According to some human ophthalmologists the prevalence of ocular allergy clearly is underappreciated and has been under diagnosed and undertreated (Bonini 2006).

Even though the diagnosis is essentially clinical, local tests such as cytology, conjunctival provocation tests (Lourenço-Martins et al. 2011) and tear mediator analysis can be performed. Although there are some experimental models of type I hypersensitivity reactions, there is a scarcity of models mimicking the more chronic forms of ocular allergy-related pathologies.

Our clinical research team has been focusing on the management of canine and feline ocular allergies. Specifically we have been investigating key mechanisms for the development of new therapeutic strategies, since mast cell stabilizers, histamine receptor antagonists, corticosteroids and immunomodulators, currently used, do not completely abolish signs and symptoms, especially in the more severe forms of ocular allergy.

We believe that the existence of standardized criteria available to general veterinary physicians and dermatologists would allow them to correctly and easily diagnose allergic conjunctivitis, especially in those cases that do not need an ophthalmological referral.

Earlier on, we performed a prospective study that aimed to (1) evaluate the prevalence of ocular signs in a population of dogs with canine atopic dermatitis and relate their severity with skin signs and (2) to perform conjunctival provocation tests with the dust mites *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* on a group of patients with atopy, ocular allergy signs and sensitization to these mites. This enabled us to establish a relationship between ocular manifestations and the presence of a clinical relevant sensitization against these mites. We found conjunctival provocation tests to be rapid and easy to perform, without the need of sedation. Furthermore, with CPT we were able to establish an etiological relationship between ocular manifestations and specific mite sensitizations (Lourenço-Martins et al. 2011; Delgado et al. 2014).

We have developed further research in this field by evaluating the usefulness of conjunctival provocation tests in atopic dogs with allergic conjunctivitis for testing the efficacy of allergen specific rush immunotherapy and by trying to understand if this treatment is of benefit in atopic patients with allergic conjunctivitis. We concluded that conjunctival provocation tests can be used to monitor clinical responses to allergen specific rush immunotherapy and atopic patients with ocular signs benefit from allergen specific immunotherapy, since all patients showed partial or complete resolution of allergic ocular signs. These results raise the possibility of a putative development of a topical allergen-specific ocular vaccine (Delgado et al. 2015) (Fig. 1 and 5).

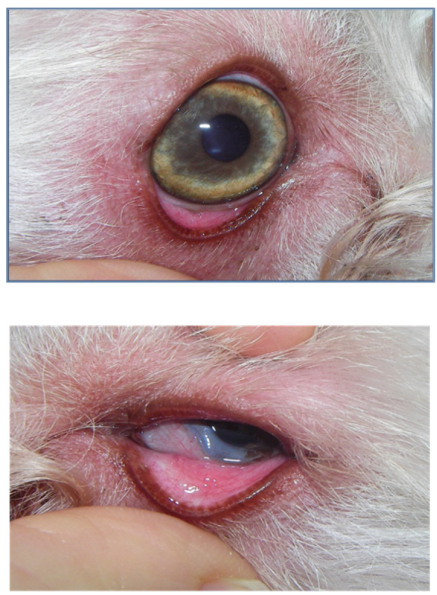

Fig. 5. Blepharitis, conjunctival hyperemia, congestion and chemosis in an atopic dog with allergic conjunctivitis.

Later on we addressed tear film osmolarity in atopic dogs with allergic conjunctivitis. Tear film osmolarity is the concentration of cations and anions dissolved in the aqueous portion of tears and hyperosmolarity has been described in human literature as a primary

marker of tear film integrity (Fig. 6) and it was evaluated using TearLab system ® (Produlab, Lisbon, Portugal).

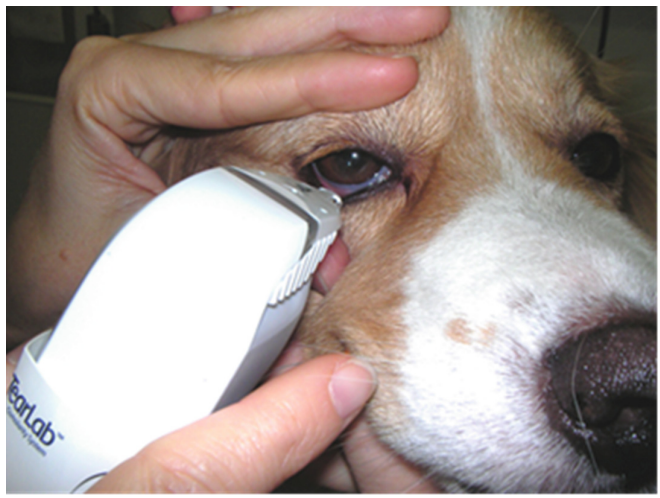

Fig. 6. Sample collection for tear film osmolarity measurement in a patient.

We found this parameter to be altered in atopic dogs with allergic conjunctivitis, which may prove useful for diagnosis and to assess the response to therapy (Delgado et al. 2014; Lourenço et al. 2017).

Tear osmolarity test has the highest accuracy and predictive value for diagnosing dry eye disease (KCS) in Humans, with tear hyperosmolarity relating with disease severity. Our research team also aimed to evaluate clinical usefulness of tear film osmolarity for diagnosing and assessing KCS severity in dogs. In our study Tear osmolarity test performed particularly poorly when tear production was severely impaired, as osmolarity was unexpectedly below the measurement threshold. Being so, tear film osmolarity does not appear to be a clinically relevant test for KCS diagnosis and staging in dogs. Further studies are necessary in this field (Conceição et al. 2016) (Fig. 5).

Little has been reported about the characteristics of the immune response in canine allergic conjunctivitis. There are no studies concerning the presence or absence of inflammatory cytokines in the conjunctiva of allergic canine patients. Presently we are developing research in an attempt to correlate the clinical involvement of patients with allergic conjunctivitis and their cytokine profile in relation with the immune response. By identifying the presence of inflammatory cytokines in the patient's conjunctivas, we will contribute to a better knowledge of this disease and to the use of dogs with allergic conjunctivitis as a model to test diagnostic tools and new therapeutic approaches that can be translated to Man (Corte-Real et al. 2016).

More recently the results of our research suggest an increase of pro-inflammatory cytokines, which are strongly involved on the innate immune response, and a Th1 subset activation. These results pinpoint to new therapeutic approaches involving immunotherapy in patients with allergic conjunctivitis (Varandas et al. 2017).

6 Oral Squamous Cell Carcinoma Spontaneous Models

Oral squamous cell carcinoma (OSCC) is an invasive epithelial neoplasm and one of the most common cancers in the oral cavity of the dog and cat. In man this is a potentially devastating disease, in which management, like what is performed in veterinary patients, includes radical surgical intervention and extremely aggressive chemo-radiotherapy treatments. Prognosis appears to have improved in recent years for both human and companion animals, although improvement is needed since morbidity and mortality still remain high.

The potential of the dog and cat in OSCC comparative oncology studies has been recognized since the 1990's. For this tumor, the dog and cat can be valuable models with multiple potential advantages. There are strong molecular similarities and comparable heterogeneity between canine and feline OSCC and human head and neck squamous cell carcinoma (HNSCC) (Liu et al. 2015; Cannon 2015), as well as similarities in histologic subtypes (Nemec et al. 2012) and expression patterns of markers identified by immunohistochemistry and molecular techniques (Wypij 2013; Sobczynska-Rak et al. 2014; Supsavhad et al. 2016; Mestrinho et al. 2017a, b; Nagamine et al. 2017). Additionally, OSCC in the cat shares anatomic location and passive tobacco-exposure related risk factors and molecular features with human HNSCC (Wypij 2013; Cannon 2015; Supsavhad et al. 2016).

At this point epidemiological, clinical and molecular information are still being gathered in order to understand the common features between these species.

Epithelial-to-mesenchymal transition has been a focus of this research team. It is an essential process in tumor progression, implicated in the gain of invasiveness to surrounding tissues, capacity to escape from the primary site and to metastasize (Smith et al. 2013). Two molecules that have gained attention are E-cadherin and p63. The first is a cell adhesion molecule and the second is a protein from the p53 family. Both molecules seem to regulate the epithelial-to-mesenchymal transition equilibrium in OSCC. Where p63 expression reintroduces epithelial characteristics in mesenchymal type cells, reversing the mesenchymal tendency of epithelial tumors (Olsen et al. 2013; Higashikawa et al. 2007). In dogs, this research team found an association between p63, E-cadherin expression and tumor grade, suggesting that a change in the expression of these 2 molecules could be a common event in OSCC in dogs, similar to what occurs in humans (Fig. 7) (Mestrinho et al. 2015). Further contributions have been done in the veterinary oncology field, whereas grade and proliferation were assessed in a group of dogs submitted to curative intent surgery (Fig. 8) (Mestrinho et al. 2017a, b). In this study high grade and high proliferation were related with early local recurrence (Mestrinho et al. 2017a, b).

After building the first biobank of canine OSCC, this research team has been collecting feline OSCC samples, as well. Grading and labeling for proliferation markers, E-cadherin and p63 have been also studied in 28 tumor samples from cats submitted to surgery with curative intent or palliative treatment. Part of the results were published in the form of a master's thesis (Silva 2016; Silva et al. 2017), but final results are being prepared.

Immunohistochemistry markers in OSCC are reliable and practical diagnostic tools that have been routinely used for our veterinary clinical patients. Moreover, they can

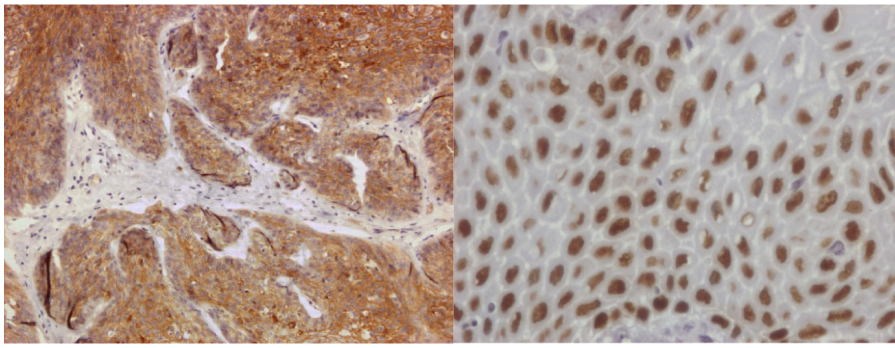

Fig. 7. Canine oral squamous cell carcinoma (OSCC). Immunohistochemical staining for proliferation marker E-cadherin (A) and P63. Histological grade 2 OSCC. (Mayers' hematoxylin counterstain, X100).

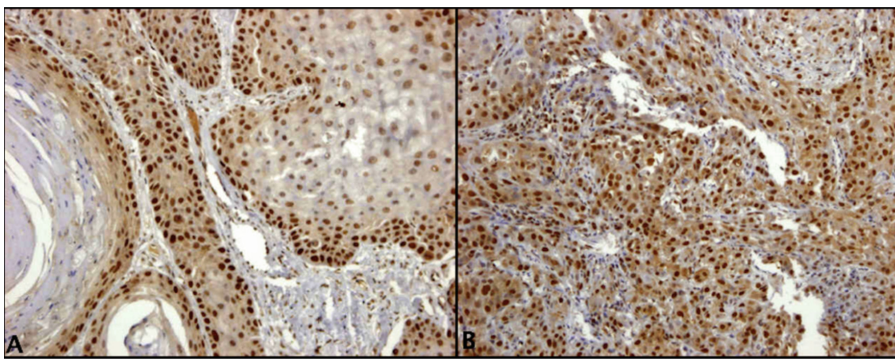

Fig. 8. Canine oral squamous cell carcinoma (OSCC). Immunohistochemical staining for proliferation marker PCNA. (A) Histological grade 2 OSCC. (B) Histological grade 3 OSCC (Mayers' hematoxylin counterstain, X100).

provide prognostic information and also guide treatment planning as some of these markers can be targets for drugs that already exist or are being developed.

Drugs targeting HNSCC have include epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR) signaling pathways or mammalian target of rapamycin (mTOR). These can be, respectively, monoclonal antibodies (mAbs) and kinase inhibitors, or mTOR inhibitors. None of them have been used, so far, in clinical trials with veterinary patients. Development of similar agents to be used in pets can not only contribute to OSCC treatment in these patients but can also provide useful comparative information.

In addition, proof-concept studies in client owned cats have been recently published using different targets, with potential interest in human patients but not been used so far, namely nanocapsules targeting protein kinase CK2α (Cannon et al. 2017) or fatty acid synthase inhibitors (Walz et al. 2018). These spontaneous models enrolled after pre-clinical trials in laboratory animals and before clinical studies were attempted.

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Neuroprotection in Glaucoma – Is Erythropoietin the Solution?

A. P. Resende, B. R. Silva, B. São-Braz, and E. Delgado(✉)

Clinical Research Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
esmeralda@fmv.ulisboa.pt

Abstract. Glaucoma is the number one cause of irreversible vision loss worldwide. Death of retinal ganglion cells (RGCs), which results in the progressive loss of visual function, occurs in glaucoma and other ocular diseases caused by hypoxia and ischemia. Although glaucoma is a multifactorial neurodegenerative disease, the only current method of treatment involves reduction of intraocular pressure and, at present, there is no effective treatment to prevent RGC apoptosis. Notably, it has been reported that erythropoietin (EPO), a cytokine hormone produced in response to hypoxia, has significant neuroprotective and neuroregenerative properties in several types of ocular disorders. All the previous studies involving EPO ocular administrations have used systemic, intravitreal or retrobulbar administration to reach retinal desired EPO concentrations. However, EPO chronic systemic administration can lead to adverse side effects related with haematopoiesis stimulation, while intravitreal or retrobulbar administrations are invasive procedures that can induce several complications such as endophthalmitis, retinal detachment, vitreitis, retinitis, choroiditis or cataracts.

Our research line aimed to find a non-invasive and safe ocular administration route without adverse effects. We also aimed to investigate if EPO delivered through this route could reach the retina both in physiological and glaucoma conditions, and if it had neuroprotective effects in glaucoma conditions.

Keywords: Erythropoietin · Ocular drug delivery · Glaucoma · Neuroprotection · Subconjunctival administration

1 Introduction

Glaucoma is the leading cause of irreversible blindness with approximately 66.8 million people affected worldwide. In 2020 this number is expected to increase to 79.6 million because of both demographic expansion and population aging (Quigley and Broman [1]). In veterinary medicine, glaucoma has a more rapid progression than in humans and represents a common and frustrating eye disease with a similar outcome, blindness (Maggio 2015). Both in humans and animals, medical therapy remains the predominant method for managing glaucoma but the failure of this first option leads to surgical and laser treatment approaches, to provide intraocular pressure (IOP) control, by increasing

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aqueous humor (AH) outflow and/or decreasing its production. Recent improvements in techniques, materials and post-operative management have resulted in better long-term outcome of IOP control. Despite the advances in the IOP control, patients progress to blindness since there is no effective treatment to prevent retinal neurodegeneration. Being so, glaucoma is considered a multifactorial neurodegenerative disease characterized in earlier stages by the degeneration and loss of RGC and their axons, leading to optic neuropathy and visual field loss.

Notably, besides EPO haematopoietic effect, several investigations have successfully outlined its molecular properties in the context of its neuro- and tissue-protective mechanisms (Aghda et al. 2016) through different pathways, including increasing resistance to inflammation, oxide-induced damage, ischemia, degeneration and permeability (Luo et al. 2015). It has been found that EPO offers protection to the optic nerve and retina when they are injured and apoptosis processes starts in retinal ganglion cells. At the present, recombinant EPO has already been tested clinically for autoimmune optic neuritis (NCT00355095); traumatic optic neuropathy (NCT01783847); methanol-associated optic neuropathy (NCT02376881) and retinopathy of prematurity (NCT00910234), with encouraging results of neuroprotection. For these reasons, EPO is actually considered a promising drug on ischemic retinal diseases, such as glaucoma.

Ocular drug administration is a difficult task due to the peculiar structure of this organ and the presence of static and dynamic protective barriers (Pescina et al. 2015). To achieve therapeutic concentrations of EPO on the retina, all pre-clinical studies used systemic, intravitreal or retrobulbar routes (Zhong et al. 2008). These routes of administration have potential risks and side effects and are difficult to use in clinical practice. In the clinical trials previously mentioned, patients were treated by i.v. administration, but with a limited number of administrations, for a maximum of three consecutive days. In patients with ocular disease such as glaucoma, an alternative route of administration is mandatory since EPO chronic systemic administration can lead to elevated blood pressure, thrombosis, chronic heart dilatation, ventricular oedema, compromised exercise performance and acute cardiac failure following challenge (Bogoyevitch 2004). On what concerns intravitreous and retrobulbar administrations routes, both may lead to ocular complications, such as chorioretinitis, retinal detachments, cataracts, vitreitis or even endophthalmitis (Ranta and Urtti 2006).

The authors postulated that EPO's neuroprotection could be achieved by a non-invasive and safe periocular administration route without complications associated with repeated systemic injections or intravitreous and retrobulbar routes.

2 In Vitro Ocular Membrane Permeation to EPO Protein

Firstly, authors evaluated the permeation to EPO protein of the different static ocular barriers: conjunctiva, cornea and sclera, using an ex vivo model. Authors concluded that the three tested ocular membranes were permeable to EPO, being the conjunctiva the most permeable membrane, followed by sclera and cornea. The lower permeability to the EPO obtained in this experiment corresponded to the thickest tested membrane, the cornea, which is in agreement with other studies where the permeability to other proteins is dependent on the structure of the tissue (Pescina et al. 2015). Being the

cornea the thicker membrane, associated to the dynamic protective barriers such as nasolacrimal drainage, tear clearance and lid blinking (Pescina et al. 2015), a reduction in bioavailability of topically applied EPO was expected. For this reason, and considering the authors' first results, the topical route for EPO administration was not the first choice for the subsequent studies but, instead, authors chose to test the subconjunctival route (Resende et al. 2017a; Resende 2018).

The subconjunctival administration is a relatively easy, safe and quick procedure, performed in ambulatory conditions, under topical anaesthesia both in human and veterinary patients. Also, the subconjunctival route seems to be more feasible for repeated administrations, with very few risks or associated complications, when compared to the other routes.

The main ocular barrier to the subconjunctival administration route are flow barriers (elimination to blood and lymphatic flows) and penetration barriers (Del Amo et al. 2017), being the scleral tissue the more important static barrier to be considered. In the *in vitro* study, authors demonstrated that EPO can permeate porcine sclera in accordance to other studies that proved that trans-scleral delivery of immunoglobulins and other large compounds to the choroid and retina is feasible (Pescina et al. 2015). Due to its easy accessibility, large surface area and relatively high permeability to a range of drug molecules, the transscleral route is suitable for delivering a wide range of therapeutic agents, from small molecules to large proteins (Srirangam and Majumdar 2012). Large molecules, such as IgG, diffuse across sclera in a manner consistent with porous diffusion through a fiber matrix (Ambati et al. 2000). Despite the promising results, barriers such as blood flow in the conjunctiva and choriocapillaris, the uveoscleral outflow and the intraocular pressure, the retinal pigmented epithelium and the blood-retinal barriers were not considered in the *ex vivo* model used. So, to bypass this limitation, it was of the utmost importance to evaluate EPO subconjunctival permeation in *in vivo* models, both in physiologic and glaucomatous conditions. Bearing this in mind, the authors designed the protocols of the second and third experiments.

3 Subconjunctival Administration in Physiologic Conditions

No studies had been previously performed to assess EPO subconjunctival permeation in *in vivo* models. So, in the second study, authors used a rat animal model to evaluate EPO´s ocular penetration after subconjunctival administration and the potential hematologic side effects associated with this route in physiologic conditions. The research team concluded that EPO crossed the sclera, which was consistent with the *ex vivo* findings, and reached several neuronal cells in all retinal layers without significant local or systemic side effects (Figs. 1 and 2) (Resende et al. 2013; Resende 2018).

The main limitation in this study was the lack of previous data regarding EPO's transscleral kinetics which made the choice of EPO dosages a difficult task. The studies that achieved EPO retinal therapeutic concentration by systemic routes used high EPO doses and, on the contrary, those which used intravitreal routes used very small EPO doses because the main ocular barriers were avoided. So, the dosage choice was based on a single previous study conducted by Zhong et al. (2008) that administered 1000 IU of rhEPO by the retrobulbar route to evaluate the RGC neuroprotection in an acute elevated

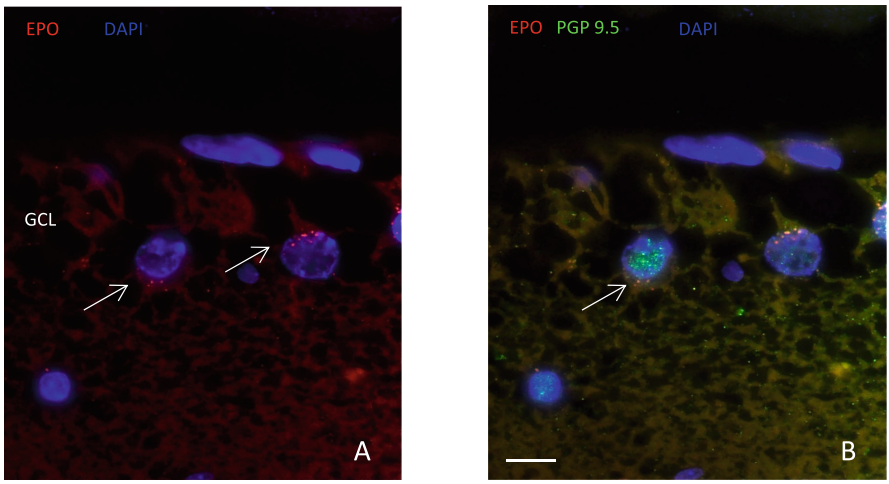

Fig. 1. A: Immunocytochemistry for cell nucleus marker of retina (DAPI, blue) and EPO antibody (red). RGC, retinal ganglion cell layer. B: The same cells are contrasted with PGP 9.5 antibody (green), showing EPO inside a retinal ganglion cell. Bar 12 μm.

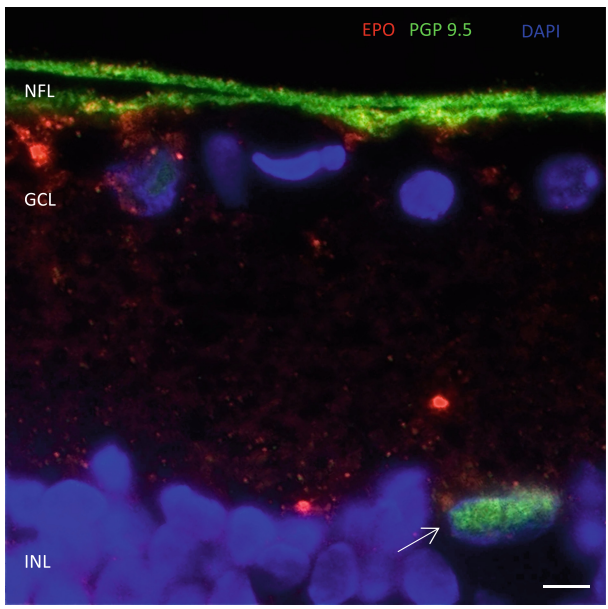

Fig. 2. Immunocytochemistry for cell nucleus marker of retina (DAPI, blue), EPO antibody (red) and PGP 9.5 antibody (green) showing EPO in others neuroretinal cells, probably a bipolar or amacrine cell once is localized in inner nuclear layer (arrow). Note the stong signal in RGC layer and in nerve fiber layer. NFL, nerve fiber layer; RGC, retinal ganglion cell layer; INL, inner nuclear layer. Bar 12 μm.

IOP model. Like in the subconjunctival route, a drug administered by the retrobulbar route comes across blood flow barriers, lymphatic flow barriers and the scleral tissue as the main penetration static barriers (Del Amo et al. 2017).

Using 1000 IU of rhEPO delivered by the subconjunctival route, authors concluded that EPO crossed the sclera, which was consistent with the *ex vivo* findings, and reached several neuronal cells, in all retinal layers. By immunohistochemistry assay, authors observed that EPO expression was more evident in the RGC layer and sixty hours after the administration there was still a strong EPO signal present in the retina of the studied animals. Regarding EPO potential systemic side-effects, subconjunctival administration did not cause any significant changes in their haematocrit values.

4 Subconjunctival Administration in Glaucomatous Conditions

The journey of the injected EPO in glaucomatous eyes involves crossing the previously mentioned structures but with high values of IOP and, in some cases, with the ocular barriers breakdown. So, after this second study where the authors demonstrated that EPO reached all the neuroretinal cell layers in physiologic conditions, by subconjunctival administration, an important question was postulated: how will IOP affect the diffusion of EPO protein across the periocular tissues? Therefore, the third study aimed to evaluate whether EPO subconjunctival administration could reach the rat's retinas in glaucomatous conditions.

In order to study the underlying mechanisms of glaucoma pathogenesis, several animal models of glaucoma have been developed. Since IOP is regulated by the balance between production and outflow of aqueous humor, experimentally disturbing of this equilibrium can induce elevation of IOP. Impairs in the aqueous humor drainage by obstruction of the aqueous humor outflow pathways can be achieved by several techniques, such as laser photocoagulation of the trabecular meshwork or the episcleral and perilimbal veins (Feng et al. 2013); cauterization of episcleral veins (Shareef et al. 1995); injection of hypertonic saline solution into the episcleral veins (Morrison et al. 1997) and injection of polysterene microbeads into the anterior chamber (Cone et al. 2010). Besides these experimentally induced models based on an increase in IOP, inherited glaucoma models also exist (Johnson and Tomarev 2010). Nevertheless, there are some disadvantages in genetic models when compared to experimentally-induced models, mainly based on the high variability and the rather slow disease progression that makes studies using these animals very expensive and time consuming. Therefore, the first part of this study was dedicated to the introduction and optimization of the experimental glaucoma rat model through cauterization of three episcleral veins (Shareef et al. 1995).

The authors followed the same methodology applied on the previous study, on what concerns EPO dosage. However, in the previous study, sixty hours after the administration there was still a strong EPO signal, so authors extended the length of the experimental design to 14 days. Using the same immunohistochemistry technique, authors have demonstrated that EPO reached all retinal layers, including the RGC layer. Surprisingly, EPO immunostaining signal was stronger in glaucomatous eyes when compared to physiological eyes. This was probably due to the ocular blood barrier breakdown that followed the rise in IOP, including blood-retinal barrier breakdown, allowing for a more

intense crossover of EPO protein. At the end of the study (day 14), EPO was still present in the retina, although its immunostaining signal was residual, which demonstrates that this protein stayed in retinal cell layers for a long time (Fig. 3) (Resende et al. 2016; Resende 2018).

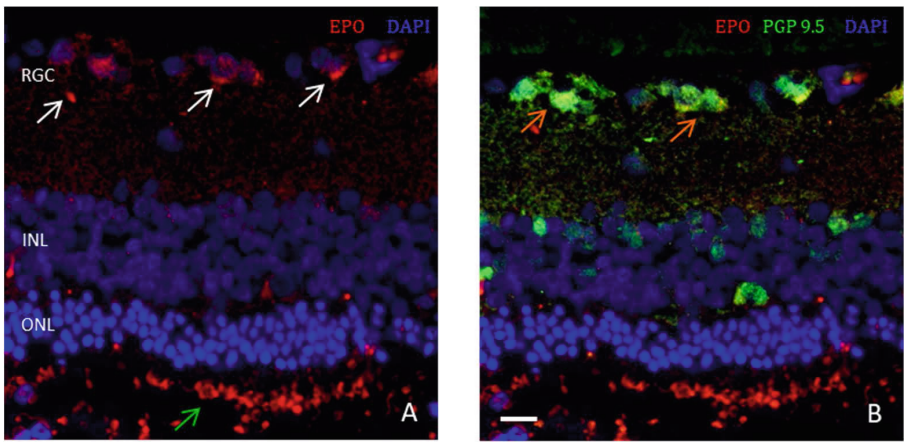

Fig. 3. Immunohistochemistry for cell nucleus marker of retina (DAPI, blue), EPO antibody (red) and PGP 9.5 antibody (green). RGC, retinal ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. A. One day after EPO administration, the EPO protein was strongly evident in RGC (white arrows). Due to the high intraocular pressure and the hemato-retinal barrier breakdown, erythrocytes are present and overspread on ONL (green arrow). B. The use of the anti-PGP9.5 antibody allowed the identification of neuronal cell bodies (orange arrows). Bar 25 μm.

5 Assessment of Functional and Structural Benefits of Subconjunctival EPO

To prove EPO neuroprotective effects in glaucoma, the authors evaluated both functional and structural benefits of the subconjunctival EPO administration in the retina of glaucomatous rats (Resende et al. 2017b; Resende 2018). So, in this fourth study, it was demonstrated that treated glaucomatous animals showed a better recovery on the ERG examination and showed thicker retinas when compared to the non-treated group. Since structure and function are highly correlated in the vertebrate retina (Hoon et al. 2014), the authors findings suggest a neuroprotective effect of subconjunctival EPO injection on the retinas of the rats with induced glaucoma (Figs. 4 and 5).

6 Limitations of the Study

Recognizing that the main focus was to prove that the subconjunctival route was an alternative route for ocular EPO delivery, several limitations must be considered on what concerns the neuroprotective effect of EPO achieved by this route.

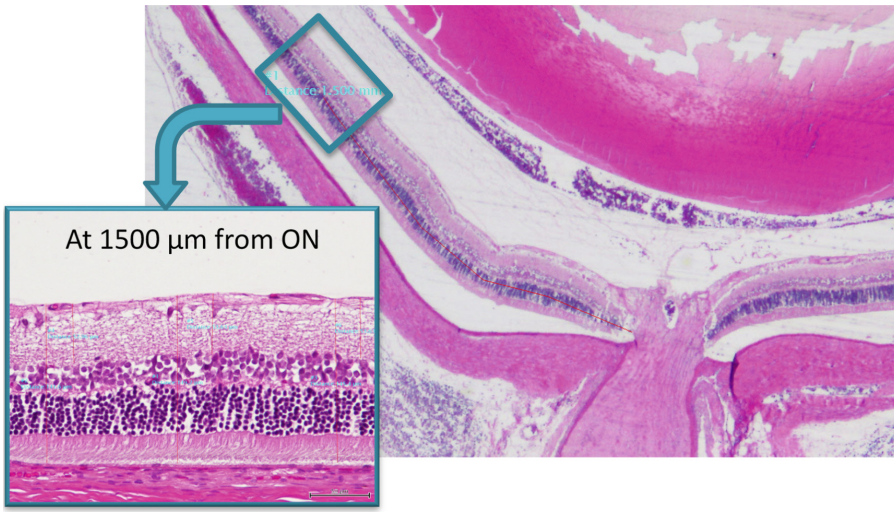

Fig. 4. H&E staining of rats' retinas at the optic nerve level. The distance of 1500 µm were measured from the optic nerve (ON) and three measurements were done in the same visual field.

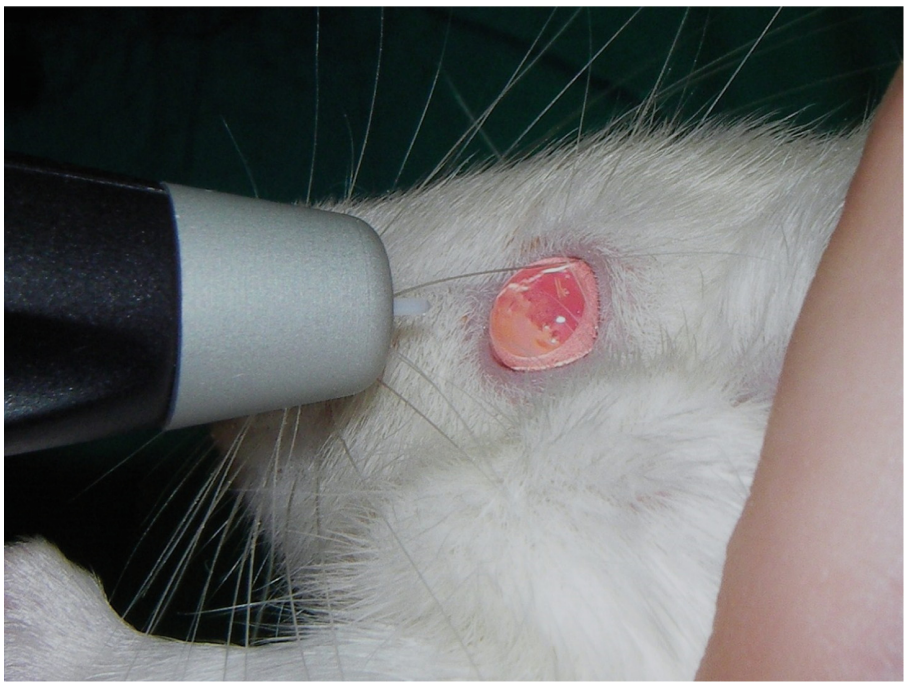

Fig. 5. Intraocular measurement in a rat using a Tonolab device (Acrivet®, Berlin, Germany)

The main limitation was the low number of animals per group for a neuroprotection study, which consequently influences the statistical results. To reduce this problem in this last study authors separated animals by gender in the two different experiments, the group of female rats was used to test visual function and the group of male rats was used to evaluate changes in retinal thickness. Nonetheless, in the visual function experiment, we did not obtain statistically significant differences in the scotopic ERG examination, due to the large individual variability in the results.

Another limitation that should be considered was the use of single EPO injections in these studies. Repeated EPO subconjunctival injections should be performed to evaluate both local and systemic potential side effects. However, even considering some systemic absorption resulting from repeated subconjunctival EPO administrations, systemic adverse side effects are not expected due to the low dosage necessary by this route of administration.

The glaucoma model used is another limitation recognized by the authors. Although this glaucoma experimental model has already been used before and showed to be reliable and reproducible (Grozdanic et al. 2007), glaucoma is a multifactorial and very complex disease. So, combining data from studies using several different glaucoma models is important in order to assess and try to understand the complete picture of EPO ocular neuroprotection in glaucoma disease. Although animal studies were useful in producing advances in human clinical approaches, they also have limitations because they do not predict the exact clinical response in humans.

In spite of the limitations stated, these studies open new perspectives concerning EPO administration in future works that target ocular neuroprotection, both in pre-clinical and clinical scenarios.

7 Future Directions

In conclusion, we have already shown that EPO, when administered subconjunctivally in a rat experimental model, can permeate the main ocular barriers and reach RGC layers, in physiological and glaucomatous conditions, without significant local or systemic side effects, having structural and functional beneficial effects on the retina after glaucoma induction.

Presently this line of investigation is trying to develop local non-invasive ocular delivery methods that increase drugs' efficacy, safety and bioavailability, developing a sustained-release system that could provide controlled, long-term drug release. This would permit improved drug flux through thinner areas of the tissue and minimize systemic drug absorption by the conjunctival vasculature.

Currently, EPO is used in clinical trials for ocular disorders. EPO supports cell proliferation and differentiation into neurons to maintain optimal tissue function. Based on the positive pre-clinical outcomes with various animal models, EPO may offer a promising future therapy for the treatment of many common ocular disorders, not only glaucoma, but also diabetic retinopathy, retinal detachment, retinopathy of prematurity, age-related macular degeneration, and optic neuritis.

Refining the preparation and administration of EPO for this purpose should be the major focus of scientists in the near future. To find local non-invasive ocular delivery

methods that increase drugs' efficacy, safety and bioavailability should be the challenge. The sustained-release systems could provide controlled, long-term drug release. This would permit improved drug flux through thinner areas of the tissue and minimize systemic drug absorption by the conjunctival vasculature.

The results obtained in this work are likely to be of great interest to the scientists and researchers both in pre-clinic and clinical trials. In accordance with other experimental evidences, trans-scleral delivery of drugs can be accomplished and constitutes a great promise in new therapeutic approaches for treating visually devastating diseases of the posterior segment of the eye. However, before considering EPO subconjunctival administration for retinal diseases in clinical patients, further studies are necessary to evaluate absorption and distribution of EPO in different concentrations, in order to characterize the kinetics of this substance when administered by this route.

Another important topic to take into account for future research works is the efficiency of EPO neuronal tissue repair. There is a change of molecule interaction in response to EPO administration in the damaged retina microenvironment. Understanding the mechanism of EPO's action could lead to novel therapeutic strategies for the treatment of neurodegenerative diseases and injuries. Thus, researchers could center their efforts on the study of specific mechanisms of tissue repair in the eye.

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No Room to Breathe: Airway Conditions Affecting the Equine Athlete

P. Tilley(✉), J. Simões, V. Pessoa, R. Fonseca, and J. P. Sales-Luis

Clinical Research Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
paulatilley@fmv.ulisboa.pt

Abstract. During exercising endoscopy, head flexion has been shown to be an important predisposing factor for upper respiratory tract collapse and is associated with conflict behaviour. Based on the substantial number of studies on the impact of hyperflexed postures on horse welfare, it was recently suggested for further research to be done on the physiological/psychological effects of a lesser degree of flexion. Our group evaluated horses ridden in two very close head positions and were able to identify significant differences for various parameters.

Inflammatory airway disease (IAD) could be the effect of repeated episodes of nasopharyngeal asphyxia, its sequel being exercise induced pulmonary haemorrhage (EIPH). EIPH and IAD account for a wide number of horses failing to perform to their potential. The American College of Veterinary Internal Medicine consensus statement proposed equine asthma syndrome (EAS) to describe horses with mild or moderate (IAD) to severe (RAO) airway disease.

Insect bite hypersensitivity has been associated with airway hyperreactivity, suggesting a probable link with EAS, and multiple hypersensitivities are significantly associated with the absence of nematode eggs in faeces.

Because severe EAS is a chronic disease with significant impact on the equine population, the development of staging methods for this disease by our group became essential to optimise equine medical care.

Keywords: Endoscopy · URT collapse · Head hyperflexion · Equine Asthma Syndrome (EAS) · Staging · Hypersensitivity

1 Upper Airway Tract

During the 20th century, horses have become progressively less important for agriculture, forestry, transport and the army. The current Olympic equestrian disciplines (dressage, showjumping and eventing) were first included in the programme in 1912 (Hedenborg 2015). Public concern about the use of horses in sport has increased in recent years, but our focus as veterinarians should not be on the moral question of whether horses should be used for sport at all, but rather on the ethical issues facing the veterinarians when the use of horses for sport is permitted by society and by law. There is an overriding, economically-driven requirement in equine sports medicine to improve performance or to

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return the athlete to competition as soon as possible, rather than a long-term preservation of the athlete's health (Campbell 2013). Nevertheless, longevity in dressage and show-jumping is critical to success and horses typically peak in performance between 12 and 18 years of age. Any training technique that severely affects a horse's welfare and health will affect longevity and be directly counter to the interests of the riders (van Weeren 2013).

Horses are elite athletes when compared with other mammalian species, but unlike them, where performance is limited by cardiovascular or musculoskeletal performance, in athletic horses it is the respiratory system that appears to be rate limiting. Therefore, all horses exercising at high intensities become hypoxaemic and hypercapnoeic, and any dysfunction in the respiratory system is likely to lead to impairment of aerobic metabolism and compromise performance further. Also, horses breathe at comparatively high frequencies when galloping due to the tight 1:1 coupling of strides to breathing (Franklin et al. 2012).

Physiological Measurements for *Performance* Evaluation. Physiological measurements described for performance exercise testing are HR and lactate. Typical heart rates are 60–80/min at a walk, 80–120/min at a trot, 120–180/min at a canter and > 180/min at a gallop, with maximal values in the region of 210–240 beats/min. Lactate is a product of anaerobic muscle metabolism and its measurement can provide information relating to both the horse's aerobic and anaerobic capacity. At rest blood lactate levels are 0,5–1 mmol/L. Peak blood lactate concentrations are substantially higher in horses when compared with human athletes and may reach 25–30 mmol/L in Thoroughbred horses after maximal exercise. Peak lactate levels may not occur for 5 min after cessation of exercise due to efflux from muscle and the measurements are most commonly determined by taking a single sample following intense exercise to fatigue. Plasma lactate concentrations are approximately 1.5 times greater than that of whole blood but whole blood is preferable because lactate in red blood cells is also accounted for (Allen et al. 2016).

Arterial blood samples should be drawn whilst the horse is exercising since rapid changes occur once exercise has ceased (within 5–10 s), which precludes the use of post exercise arterial blood gases in the evaluation of gas exchange (Sleutjens et al. 2012; Allen et al. 2016; Tilley et al. 2018). Simultaneous measurement of body temperature is also necessary. At rest, PaO_2 is approximately 100 mmHg and $PaCO_2$ is approximately 45 mmHG. At canter, PaO_2 decreases to 80–85 mmHg and during strenuous exercise to 65–70 mmHg and $PaCO_2$ increases to 50–55 mmHg. Still, normal horses become hypoxaemic and hypercapnic during strenuous exercise (Allen et al. 2016).

Upper Airway Tract (URT) Obstruction. During strenuous exercise there are dramatic increases in airflow coupled with large changes in airway pressures generated primarily by diaphragmatic movement. In other athletic species (man, greyhound) a switch to oral breathing during exercise helps overcome the high resistance from the nasal passages. The horse is an obligate nasal breather, so the prevalence of URT obstructions is higher (Franklin and Allen 2017). During exercise, URT resistance becomes more than 80% of the total airflow resistance (Janicek and Ketzner 2008). Choanal stenosis, a

potentially catastrophic ball valve obstruction due to the Bernoulli effect, can be a cause of exercise-induced pulmonary haemorrhage (EIPH) (Cook 2014b).

Computational models have shown that during inhalation the most negative pressures and highest airflow turbulence occur at the floor of the rostral aspect of the nasopharynx and within the larynx, which requires muscle activity to maintain patency. Dynamic collapse of the URT is thought to occur either as a result of neuromuscular pathology or fatigue or conformational change of the URT musculature (Franklin and Allen 2017). An URT obstruction can result in more severe hypoxaemia (Davidson et al. 2011b; Franklin and Allen 2017) and hypercapnia, lower maximal oxygen consumption, higher blood lactate and greater degree of respiratory muscle fatigue (Franklin and Allen 2017).

Furthermore, URT obstructions may lead to premature diaphragmatic fatigue, which affects exercise tolerance by reducing ventilation, changing breathing mechanics, causing increased sensation of dyspnoea and respiratory muscle fatigue-induced metaboreflex (Romer and Polkey 2008). The respiratory muscle metaboreflex is a cardiovascular reflex, originating within the fatigued respiratory muscles (diaphragm and abdominal muscles), which induces a sympathetically-mediated vasoconstriction of limb locomotor muscle vasculature, this being combined with increased competition for blood flow with limb locomotor muscles, exacerbating peripheral fatigue and leading to a performance reduction (Romer and Polkey 2008; Franklin and Allen 2017).

Ridden Head and Neck Positions Influence on the URT. In feral horses, nasal plane angle positively correlates with gait (and/or speed), but no such correlation is seen in the ridden horse (McGreevy et al. 2010). Riding head and neck position can be accurately quantified in the sagittal plane from angles and distances based on standard anatomical landmarks and home-video captured images (Elgersma et al. 2010) and can be described, according to Go et al. (2014a), by the ground angle (GA), between the ridge of the nose and the horizontal ground plane, and by the withers angle (WA), between the ridge of the nose and the line connecting the neck and the withers.

During exercising endoscopy, head and neck position has been shown to be an important predisposing factor for several forms of URT collapse, due to rostral advancement of the larynx and/or more compliant airway walls (McCluskie et al. 2008; Franklin and Allen 2017), The pharynx, having no bones or cartilaginous support, is especially prone to changes in pressure and airflow during exercise. In fact, the majority of findings indicate that a hyperflexed position, together with a certain degree of stress and interaction with the rider, like factors relating to the bit and bridle, can induce and exacerbate upper airway, pharyngeal and laryngeal, obstruction (Strand et al. 2009; Allen et al. 2011; Davidson et al. 2011a; van Erk 2011; Gerber 2014; Go et al. 2014a, b, c; Zebisch et al. 2014; Parente 2018) due to increased inspiratory negative pressure (Strand et al. 2009; Sleutjens et al. 2012).

This hyperflexed position is not new. It can be found in pictures painted in some ancient Greek Chalcidian kraters from ca. 540 BC. In modern times, it was reintroduced by the Schockemöhles in the 1960s in show jumping and controversy about the use of this position started in the 1990s (van Weeren 2013). Kienapfel et al. (2014) describes "Rollkur" as the ridden nasal plane of the horse being behind the vertical more than 10°. Becker-Birck et al. (2012) found no negative effect of the hyperflexed position

alone in the lunged horse, but could not exclude a stressful experience of the position when achieved by active intervention of the rider. Sloet van Oldruitenborgh-Oosterbaan et al. (2006) measured blood lactate concentration and HR immediately after a trot and canter in hyperflexion and reported that they were higher than in the same horses ridden with light rein contact. Nevertheless, van Breda (2006) refers that HR variability has not yet been proven as an established measure for chronic pain. Sleutjens et al. (2012) investigated the effect of head and neck position on airway resistance using a balloon catheter in the oesophagus (intrathoracic pressure – ΔIP-) and it appeared to increase in all the positions that were different from the natural head carriage and mostly in the hyperflexed position. Tilley et al. (2018) found a significantly higher intrathoracic pressure difference (ΔIP) in horses ridden with 100° ground angle position when compared to 85°.

Cehak et al. (2010) took lateral radiographs of the pharynx and measured the pharyngeal diameter (pharyngoepiglottic distance), the shortest distance between the dorsum of the epiglottis and the roof of the pharynx. For Go et al. (2014a) the pharyngeal diameter in a neutral head and neck position was 34,6–76,8 mm (51,3 ± 8,87 mm) and in an extended head and neck position it was 38,4–79,1 (55,6 ±8,9 mm). For Cehak et al. (2010) the smallest pharyngeal diameter occurred at the dorsal flexed position (29,6 ± 11,3 mm). For Allen et al. (2011), pharyngeal diameter was 14,4-36,4 mm in the flexed position, and for Go et al. (2014a) it was 28,5 ± 9,6 mm, reaching the smallest diameter of 7 mm with the head 10° behind the vertical. According to Zebisch et al. (2014), hyperflexion originates a significant reduction (5%) in the laryngeal opening area. Tilley et al. (2018) found a significantly smaller pharyngeal diameter in horses ridden with 100° ground angle position when compared to 85°.

The equine airway is a high-resistance, low-capacity ventilatory passage and, being a cylindric tube, resistance can be calculated using the formula $R = (8xnL)/r^4$ (N = gas viscosity (constant); L = length of trachea; r = radius of trachea). Because the radius is to the 4th power, a small difference in airway radius has a major impact on airway resistance (Janicek and Ketzner 2008).

Ridden Head and Neck Positions Influence on Psychological Factors. This relates to the effects of head and neck positions on measures of stress, anxiety and discomfort for the horse. Association of the lowered head positions with submissive behavioural patterns and inability to escape pressure, introduces the concept of "learned helplessness" (Gerber 2014; ISES 2018). In fact, increases in rein tension are used as a "stop" sign. So, the simultaneous increase in the use of a whip and spurs, which are mainly used as "go" signs, are likely to induce a state of conflict in the horse (Kienapfel et al. 2014). Also, head position can be readily moderated by bit pressure, but doing so mixes 2 responses (deceleration and neck-flexing) with 1 signal (bit pressure) and as such, it is a violation of the principles of learning theory (McGreevy et al. 2010). All pressure-based training places the horse in a discriminatory dilemma: he must respond to some pressures (*e.g.* bit) but he must habituate to others (*e.g.* girth) (McGreevy et al. 2011). Maximum flexion of the cranial neck and head creates delayed nerve conduction as well as increased lactate dehydrogenase activity, 4, 6 and 24 h after exercise in this position (Zsoldos and Licka 2015). Psychological factors like excitement, anxiety, fear and pulling against the rider

may result in elevated lactate and HR values, particularly if an unfamiliar place or unfamiliar exercise is used (Davie and Evans 2000; Allen et al. 2016a). The flexed position also compromises forward vision, which can contribute for anxiety and fear (McGreevy et al. 2010; ISES 2018), manifested by conflict behaviour. In a study by Smiet et al. (2014), behaviour results, HR variability and cortisol analysis revealed changes that could be interpreted as exercise stress in artificially induced head-neck positions. Tilley et al. (2018) referred a significantly higher blood lactate level at the end of 100° ground angle exercise test when compared to 85°. As to HR and RR, both were significantly higher at the end of the 100° ground angle exercise test when compared to the end of the 85°. Borstel et al. (2009) demonstrated that a coercively obtained rollkur position may be uncomfortable for horses and that it makes them more fearful and therefore potentially more dangerous to ride. Horses moved slower and showed more often behavioural signs of discomfort, such as tail-swishing, head-tossing or attempted bucks and, when given the choice, 14 out of 15 horses chose significantly more often a maze-arm associated with the normal rather than the rollkur position.

Pain in horses is difficult to assess as horses potentially suppress the exhibition of obvious signs of pain in the presence of possible predators as is suggested with other prey species which have evolved the ability to mask obvious signs of pain under specific circumstances (i.e. presence of a predator such as humans) (Dalla Costa et al. 2014). However, horses show conflict behaviour, which is the response exhibited by animals that experience difficulty coping with mental or physical discomfort, most often demonstrated as some form of resistance to handling or training cues and/or equipment. Examples of conflict behaviour are head shaking, pulling the reins out of the rider's hands, mouth opening, tail swishing and pinning the ears backwards (Smiet et al. 2014; Górecka-Bruzda et al. 2015). In a study by Górecka-Bruzda et al. (2015), pulling the reins out of the rider's hands was most frequent in show jumping and tail swishing in dressage. Tail swishing in horses at liberty occurs in just two situations, as a strategy to displace insects and during social encounters (e.g., a nonestrous mare refusing breeding approaches by the stallion; mare refusing the foal's attempts to suckle). Tilley et al. (2018) reported that the occurrence of various conflict behaviours was significantly higher with a 100° ground angle position, when compared to an 85°. Rider encouragement (whip/kicking/noise) was also significantly more common with the 100° position. On the contrary, relaxation behaviours, occurred significantly more often with the 85° position.

Furthermore, it was verified that in horses shown at dressage competitions, the ones in higher classes showed more conflict behaviour. The prevalence of different head-neck positions was evaluated and it was verified that a head behind the vertical was penalised with lower marks in the lower but not in the higher competition levels (Kienapfel et al. 2014; Lashley et al. 2014). Also, head position is considered when awarding the collective scores, especially those for submission. So, in recent years competitors have been rewarded for meeting criteria other than the horse's head being on the vertical (Lashley et al. 2014). This appeal of flexed necks is further supported by recent studies of dressage judges, who focus their visual attention preferentially on the cranial half of the horse (head, neck and chest). Yet, cave paintings already despicted heavily crested equidae and crested necks can be further accentuated by flexion of the neck (McGreevy et al. 2010; Casper et al. 2015). Therefore, the use of modern objective mobile gait analysis

tools, such as Inertial Measurement Units, should be considered as part of future visual attention training sessions for judges (Lashley et al. 2014).

Nevertheless, as a function of their anatomical conformation, different horses may bend their neck and poll with more or less ease (Gerber 2014), so conformation largely determines the severity of welfare impacts of head and neck flexions (ISIS 2018). Still, additional research is necessary to assess the relevance of neck conformation for different equestrian disciplines (Zsoldos and Licka 2015).

Influence of the Use of the Bit on the URT and on Psychological Factors. Bits, fitted within the mouth, were introduced in about 2300 BC (Mellor and Beausoleil 2017). Fluoroscopic studies show that the bit rests on the tongue, rather than on the bars of the mouth, as was originally believed and sometimes can be associated with a reduction in swallowing frequency by restricting the movement of the tongue (McGreevy et al. 2011).

Wild horses walk, trot and gallop with their mouths always closed, sealed lips and no drooling. When healthy bit-free horses begin to walk or move at a faster pace they swallow. After swallowing, the generated negative pressure holds the soft palate firmly against the immobile root of the tongue and prevents it from being sucked dorsally into the nasopharyngeal airway during inspiration (Cook 2014a; Mellor and Beausoleil 2017). Oral subatmospheric pressure is documented in mammals, as it is used for suckling. Also, to drink a horse depends on subatmospheric pressure in its oral compartment. At a critical intake, deglutition occurs, subatmospheric pressure is re-established and the cycle continues. The ability to generate an oral subatmospheric pressure by swallowing also facilitates airflow when running (Cook 2014a).

Most horses exhibit clear behavioural evidence of aversion to a bit in their mouths, but the most important is the potential for bits to disrupt the maintenance of negative pressure generated by swallowing in the oropharynx, which apparently acts to prevent the soft palate from rising and obstructing the nasopharynx, because there is a loss of the airtight lip seal and/or the laryngeal-pharyngeal seal. The seal can be broken during vigorous exercise with low jowel angle or when aversive bit sensations cause sufficient mouth opening to break the airtight seal (Cook 2014a; Mellor and Beausoleil 2017).

Repeated swallowing in DDSP and palatal instability (PI) might represent attempts to reinstate negative pressure in the oropharynx after palatal displacement has occurred or may be necessary to prevent inhalation of bit-induced saliva (Cook 2014a; Mellor and Beausoleil 2017). So, some possible reasons why the bit may be the cause of DDSP during exercise are mouth opening, tongue withdrawal and increased salivation (Allen et al. 2011). By triggering instability and DDSP, the bit may also be incriminated as a cause of asphyxia. The bitted rein pressure, kinking the airway and rendering parts of it flaccid is a further cause of asphyxia and negative pressure pulmonary oedema is one of its signs. Therefore, a more precise and scientifically useful name for EIPH would be negative pressure pulmonary oedema (NPPE), analogous to a rare but life-threatening disease in man known as NPPE. EIPH is not confined to racehorses, it also occurs in the hyperflexed dressage horse. Bit-induced obstruction of the nasopharynx, leading to negative pressure pulmonary oedema with cardiac failure, can be a cause of sudden death in the horse (Cook 2014a; Cook 2014b; Cook 2016).

A principal of gas flow in an obstructed tube is that negative pressure strengthens as distance from the point of obstruction increases. So, the negative pressure generated in the nasopharynx will be stronger in the larynx and stronger still in the cervical trachea and most severe in the lung, where negative pressure will peak in the alveoli of the caudal lobe, causing the typical EIPH pulmonary lesions. Hypoxia also causes pulmonary vasoconstriction, another cause of pulmonary oedema (Cook 2016).

Dynamic Upper Respiratory Tract (URT) Abnormalities. Various URT abnormalities occur in the horse. McGivney et al. (2017) recently published a thorough grading scale booklet for these abnormalities, recorded both at rest and during overground endoscopy.

The primary respiratory function of the palatal musculature of the horse is to facilitate obligatory nasal breathing (Allen et al. 2007). DDSP is the most common dynamic upper respiratory tract obstruction in horses and the gold standard diagnostic modality is exercise endoscopy using a high-speed treadmill or an overground equipment as, in many cases, it only occurs when the horse fatigues. The high air flows that occur during exercise cause the displaced soft palate to vibrate, creating the typical "gurgling/choking/snoring" expiratory noise. There is strong evidence that head and neck position influences DDSP. Palatal instability (PI) and DDSP are manifestations of the same condition, but PI may or may not progress to DDSP (Barakzai and Dixon 2011; Allen 2015; Chesen and Whitfield-Cargile 2015; Franklin and Allen 2017).

In a study by van Erck (2011) both lower airway inflammation and pharyngeal lymphoid hyperplasia were associated with pharyngeal instability (PI or nasopharyngeal collapse), but not with any other forms of dynamic upper airway obstruction. The cases of PI were generally associated with grade 3 pharyngeal lymphoid hyperplasia (grades 1 to 4). Woody (2011) refers that severe lymphoid hyperplasia does not directly cause abnormal respiratory noise, but the severe inflammation may lead to pharyngeal collapse or DDSP, which causes abnormal respiratory noise. In a study by Durando et al. (2002) pharyngeal collapse was the most commonly associated situation with abnormal exercising blood gases. Franklin and Allen (2017) also refer that severe dorsal and circumferential pharyngeal wall collapse was shown to decrease minute ventilation by 23%. According to Boyle et al. (2006), dynamic pharyngeal collapse occurred more in males (intact or gelded) than females, like in Man. It is characterized by ventral displacement of the dorsal pharyngeal wall with resultant covering of the arytenoid cartilages and/or axial displacement of the lateral walls of the pharynx at the end of expiration and early inspiration and it is a precursor of DDSP.

Dynamically flaccid epiglottis is a medial collapse of the lateral margins of the epiglottis induced or exacerbated by poll flexion (McCarrel and Woodie 2015).

Bilateral dynamic laryngeal collapse is defined as collapse of both arytenoid cartilages and vocal folds during exercise and is only manifested during exercising endoscopy with forced poll flexion. The aetiology is unknown, but affected horses have a more rostral position of the larynx, which may result in physical compression of the larynx and also have more negative tracheal peak inspiratory pressures, when exercising with the poll flexed (McCarrel and Woodie 2015).

Recurrent laryngeal neuropathy (RLN) involves submaximal arytenoid abduction and inspiratory noise. Clinical recommendations range from 60 to 80% of maximal

abduction in non-performance horses to 80–90% in racehorses (McCarrel and Woodie 2015). The most often used grading system for RLN is a 4-grade classification system for the assessment of laryngeal respiratory function in unsedated horses examined at rest and a 3-grade classification system for examination during exercise. Resting grades III and IV are likely to experience clinical disease during exercise whereas resting Grades I and II have normal laryngeal function (Ducharme 2003; Chalmers et al. 2012). Lindegaard et al. (2007) proposed that sedation during resting endoscopy could avoid false negative diagnosis, based on the speculation that endoscopy excites most horses making them able to abduct more.

Tilley et al. (2018) verified that, in the overground endoscopy, multiple upper airway tract abnormalities were more commonly associated with a 100° ground angle position, when compared to the 85°.

Some horses with disorders of the upper and/or lower respiratory tract, instead of a pattern of one breath every one stride, adopt a 2:1 (each breath is taken over 2 strides) (Fitzharris et al. 2015).

Based on the substantial number of scientific studies on the impact of hyperflexed head and neck postures on horse welfare, the International Society for Equitation Science (ISES 2018) position statement suggested that further research may be warranted on the physiological and psychological effects of lesser degrees of poll flexion and extension. In this line of thought, Tilley et al. (2018) evaluated horses ridden in two very close head and neck positions and were able to identify significant differences for various parameters.

2 Lower Airway Tract

Inflammatory airway disease (IAD) could be the effect (not the cause) of repeated episodes of nasopharyngeal asphyxia and its sequel, pulmonary bleeding or EIPH (Cook 2014a). Horses with DDSP have a higher prevalence of IAD but we cannot determine whether IAD is a risk factor for DDSP, or the other way round (Mazan 2018).

IAD, EIPH and Severe Equine Asthma Syndrome (sEAS). EIPH and IAD are the two most important lower airway diseases of the athletic horse. EIPH may be considered, at least at the onset, as a problem of physiology rather than a disease, and IAD is a disease primarily of domestication. Both account for a wide number of horses that fail to perform to their potential (Mazan 2018).

Although EIPH is the most publicly recognized lower airway disease of sport horses, IAD has a more pervasive and broad impact on the sport horse population, as it affects all groups of horses, including those, like endurance or dressage horses, that do not perform under conditions that produce high pulmonary artery pressure (Mazan 2018).

RAO and IAD represent a spectrum of chronic inflammatory disease of the airways in horses resembling human asthma in many respects. Therefore, the American College of Veterinary Internal Medicine (ACVIM) consensus statement chose to use the term equine asthma syndrome (EAS) to describe horses with mild or moderate (IAD) to severe (RAO) airway disease, referring that individuals with mild respiratory clinical signs have an increased risk of developing RAO (Couëtil et al. 2016).

RAO affects horses over 7 years of age, whereas IAD can affect horses of all ages. Also, in IAD there is occasional coughing and poor performance, but no increased respiratory efforts at rest are seen. In RAO there is regular to frequent coughing and exercise intolerance, and there are increased respiratory efforts at rest. Diagnostic confirmation can be done by bronchoalveolar lavage fluid (BAL) fluid cytology and lung function tests, like indirect pleural pressure measurement using the oesophageal balloon catheter technique. In IAD there is mild increase of neutrophils (5–20%), eosinophils and/or metachromatic cells in BAL fluid, and there is no evidence of airflow limitation as DPmax is lower than 10 cm H_2O. In RAO there is moderate to severe increase in neutrophils (> 20%) in BAL fluid, and there's moderate to severe airflow limitation during disease exacerbation as DPmax is higher than 15 cm H_2O (Couëtil et al. 2016).

Severe Equine Asthma Syndrome (sEAS) – RAO. sEAS is a widely recognised airway disorder, characterised by hypersensitivity-mediated neutrophilic airway inflammation and lower airway obstruction in a subpopulation of horses when exposed to suboptimal environments high in airborne organic dust. It is the most common cause of chronic coughing in horses in temperate countries, showing a disease phenotype of varying severity ranging from exercise intolerance to coughing and severe expiratory dyspnoea. It is largely reversible, so avoidance of the inciting airborne agents results in significant disease remission over time (Thompson and McPherson 1984; Pirie 2014). The main features of the disease include airway neutrophilic influx, mucus accumulation, bronchospasm, bronchial hyper-reactivity and airway remodelling (Pirie 2014).

Epidemiology and Genetics. The epidemiological studies conducted on severe EAS provide useful information on prevalence and disease associated risk factors. Hotchkiss et al. used a validated questionnaire and reported an estimated disease prevalence in the UK of 14%. They described horses as being kept under a great variety of conditions with some potentially exposed to high concentrations of organic dusts associated with stabling (Hotchkiss et al. 2006; Hotchkiss et al. 2007a, b). Ireland et al. (2015) estimated a prevalence of 20% in the UK and incidences as high as 54% were found in horses housed in traditional stables (Bracher et al. 1991). There are reports of a significant RAO risk increase with gender (females), age (especially ≥ 7 years of age), breed (Thoroughbred), season (spring and winter), exposure to an urbanised environment, and exposure to hay and respiratory infection in early life (Couëtil and Ward 2003; Hotchkiss et al. 2007b).

Genetic effects are likely to be due to variations in several genes, as they are polygenetic. It is, therefore, unlikely that single gene tests can be diagnostically useful in these disorders and Gerber et al. (2015) pointed out that much work was still needed to uncover diagnostically useful genetic markers or even causative genetic variants that could influence the risk of developing RAO. A study by Schnider et al. (2017) revealed a suggestive new quantitative trait locus on chromosome 13 and described TXNDC11 as a probable functional candidate gene.

In the future, genetic profiling panels, based on multiple genetic markers combined with an assessment of environmental risk factors (such as hay feeding in RAO), may allow "at risk" matings to be avoided and may help to identify individuals with an increased genetic risk before the disorder manifests itself (Gerber et al. 2015). However, selective breeding with the goal of reducing the prevalence of a disease must not necessarily be

based on genetic tests. Once it is established that a disease has a strong genetic basis, identification of affected individuals and their removal from the breeding pool can reduce the risk of producing affected offspring. This practice is already established for stallions affected with RLN and even RAO in some European breeding associations (Nikolic 2009).

Some studies have been published on certain high prevalence families, having revealed an association between RAO and both decreased shedding of strongylid parasite eggs and an increased risk for allergic skin disease (Pirie 2014; Gerber et al. 2015).

Neuhaus et al. (2010), Bründler et al. (2011) and Schleuniger et al. (2011) concluded that RAO-affected horses may be more resistant to strongylid nematodes than unrelated unaffected pasture mates, but family history of RAO does not necessarily confer protection against helminth infections. Furthermore, the status of "shedding of strongyle eggs" has a genetic background, although they could not prove whether "egg shedding" and RAO shared common genetic components.

Simões *et al*. (a) also identified a lower shedding of strongyle eggs in Lusitano horses with severe asthma in a Mediterranean climate, compared to healthy control mates, which may indicate these horses also present a genetic resistance to gastrointestinal parasite infection.

Also, insect bite hypersensitivity (IBH) has been associated with the presence of airway hyperreactivity, suggesting a probable link with equine asthma (Kehrli et al. 2015; Lanz et al. 2017).

Multiple hypersensitivities (MHS) have been described in humans, cats, dogs and now also in horses. MHS include RAO, IBH and urticaria. Specifically, an increased risk for IBH is to be expected in RAO-affected horses, and MHS is significantly associated with the absence of nematode eggs in the faeces (Kehrli et al. 2015). Lanz et al. (2017) studied 22 healthy controls, 24 horses with IBH alone and 23 horses with IBH and EAS. The PaO_2 was measured and flowmetric plethysmography was used to assess airway reactivity to increasing doses of inhaled histamine. The airways of horses with IBH alone showed hyperreactvity similar to the airways of horses with EAS, so it was concluded that IBH is associated with airway hyperreactivity and decreased PaO_2, even in the absence of overt respiratory clinical signs. Therefore, horses suffering from IBH have a higher risk for airway hyperreactivity and might be predisposed to develop EAS in the future.

In a recent study, Pessoa *et al*. (a, b) evaluated various Lusitano horse stud farms, each having at least five horses showing evidence of IBH, and were able to identify the Culicoides species most frequently found near horses in Portugal. A questionnaire was filled for all the 60 horses included in the study (30 IBH and 30 control) and the IBH lesions of each horse were scored a grade (1 to 5). Peripheral leukocyte stimulation was evaluated through quantification of sulfidoleukotriene generation by CAST ELISA technique and sera reactivity was evaluated by quantification of specific IgE to recombinant Culicoides nubeculosus allergens. In vivo relevance of these allergens was evaluated through Intradermal Skin Tests and Skin Prick Tests.

Aetiology. As in all allergic diseases, severe EAS depends on the exposure of genetically susceptible horses to offending environmental allergens (Marti et al. 1991), mainly

from stables, including fungal spores (> 50 species identified, like *Aspergillus fumigatus*, *Faenia rectivirgula* and *Thermoactinomyces vulgaris*), bacterial endotoxins, forage and storage mites, microbial toxins, peptidoglycan, proteases, pollen, plant debris and inorganic dust (Clarke 1987; McGorum and Pirie 2008; Seguin et al. 2012; Niedzwiedz et al. 2015; Klier et al. 2018). Exposure to high levels of ammonia and other pollutants may further aggravate airway inflammation, which is why a good hygiene and ventilation are fundamental (Pirie 2014). Exacerbation at times occurs also with exposure to pasture environment – pasture-associated asthma, where pollen and other outdoor allergens may be involved (Swiderski et al. 2017). Environmental high temperatures and humidity have also been found to worsen clinical signs (Bullone et al. 2016).

Pathogenesis. The complex nature of the immunological mechanisms involved in severe EAS is noticeable in the numerous cytokines, chemokines, genes and cells involved (Art et al. 2008).

Even though there is still some controversy (Deaton et al. 2007), a number of studies suggest that IgE-mediated immediate type allergic reactions are associated with RAO. TH2-type cytokine-mediated chronic airway pathology (increased expression of IL-4 mRNA and decreased expression of IL-2 mRNA) is involved. This is consistent with the hypothesis that RAO is an allergic condition similar to human asthma (Lavoie et al. 2001; Cordeau et al. 2004; Anton et al. 2005). Nonetheless, a delayed response has been vastly described in this disease, leading to neutrophil recruitment into the airways and increase of CD4 + T cells in the BALF of affected horses (McGorum et al. 1993; Kleiber et al. 1999). The cytokines IL-8, IL-17 and especially IL-4 appear to play an important part in this neutrophil chemotaxis (Franchini et al. 2000; Debrue et al. 2005; Ainsworth et al. 2006; Lavoie-Lamoureux et al. 2010), which is a hallmark of severe equine asthma (Bullone et al. 2018). Contradicting evidences have been reported regarding interleukins (IL) 4, 5, 13, and interferon (IFN) γ expression, and while some studies support a Th2 response based on the cytokine profile, others found no evidence of a specific cellular response (Th1 or Th2) (Giguere et al. 2002; Ainsworth et al. 2003; Cordeau et al. 2004; Horohov et al. 2005; Kleiber et al. 2005). Recently new evidence of a Th17/Th2 immunological response for the pathogenesis of the disease has been suggested (Pacholewska et al. 2017).

Another important feature is mucus accumulation in the airway lumen (Gerber et al. 2003; Gerber et al. 2004a), which occurs due to increased viscolelasticity with decreased mucus clearance (Gerber et al. 2000; Jefcoat et al. 2001; Gerber et al. 2004a). Also, increased mucus production may be related to overexpression of the mucin gene EqMUC5AC and an increased number of mucus cells (Gerber et al. 2003; Lugo et al. 2006; Gerber et al. 2009)

Bronchoconstriction and bronchial smooth muscle remodelling result from airway inflammation, mucus accumulation and also a defective inhibitory nonadrenergic-noncholinergic (NANC) function, as well as α_2 and β_2 adrenoreceptors and muscarinic-receptor dysfunction (Yu et al. 1994; Olszewski et al. 1997; Zhang et al. 1999; Abraham et al. 2006; Abraham et al. 2007; Venugopal et al. 2009). Endothelin may also contribute to bronchoconstriction (Fluvio et al. 2012a, b).

Clinical Signs. Exercise intolerance, cough and increased expiratory effort at rest characterise severe EAS-affected animals, although clinical signs vary with disease severity. However, cough is usually the first sign reported (Allen and Franklin 2007; Tilley et al. 2012b; Bosshard and Gerber 2014). Nasal discharge, nasal flaring, abnormal breathing pattern and a 'heave' line may also be observed (Robinson et al. 2001; Tilley et al. 2012b; Bosshard and Gerber 2014) as well as significant body mass loss or even cachexia (Mazan et al. 2004). It's important to remember that all horses that cough do not necessarily have IAD or RAO, as coughing can be caused by DDSP, epiglottic entrapment, rhinitis, and foreign bodies among other things (Mazan 2018). A study by Tilley et al. (2012a) intended to characterize severe EAS differential diagnosis (DD) in a group of 59 horses referred for respiratory investigation due to long term cough, and to establish a possible parallel between the DD in severe EAS and in human asthma. 27/59 (45.8%) horses were included in a DD group, based on medical history, physical exam, respiratory endoscopy, thoracic radiography and bronchoalveolar lavage (BAL) fluid evaluation. These horses were found not to have severe EAS but various upper airways abnormalities, like DDSP, palatopharyngeal arch oedema/inflammation, RLN, dorsal collapse of the laryngopharynx, pharyngeal lymphoid hyperplasia, tracheal collapse and small pharyngeal/laryngeal cysts. These results stress the importance of a thorough diagnosis, including BAL and respiratory endoscopy, since, similarly to what is referred in human asthma guidelines, various upper airways abnormalities can clinically mimic RAO, producing long term cough.

3 Diagnostic

Medical History and Clinical Examination. Because clinical signs of severe EAS are quite typical, a tentative diagnosis can be made based on clinical history and examination. Severe asthmatic horses present with a history of chronic and persistent cough, which can be seasonal or associated with stabling and hay feeding (Robinson et al. 2001). However, clinical history and signs alone do not identify asthmatic horses in remission nor sometimes with low-grade inflammation (Severe EAS grades 1 and 2, Tilley et al. 2012a) (Couëtil et al. 2001; Laumen et al. 2010).

Thoracic Radiography. Radiography is the diagnostic method of choice for evaluating the pulmonary parenchyma, especially in diseases affecting the deep lung and the mediastinum (Barton et al. 2018) and radiographic imaging of the equine asthmatic lung may reveal changes in lung pattern and thickening of smaller bronchi bifurcations (Koch et al. 2007; Bakos, 2008). In chronic cases, the x-rays may reflect irreversible changes in the lung parenchyma, such as bronchiectasis (Allen and Franklin, 2007). Tilley et al. 2012b showed a positive correlation (0.67–0.78) of radiographs with staging of RAO, especially increases in interstitial and bronchial patterns, as well as tracheal thickening.

Respiratory Endoscopy. Endoscopy is important in ruling out contributions from upper airway disease and is also the best method for assessing mucus accumulation in the trachea (Mazan 2018).

Endoscopic evaluation of severe EAS horses is characterised by the presence of mucus or mucopurulent secretions in the tracheal and bronchial lumen (Gerber et al. 2000; Robinson et al. 2003; Gerber 2004a, b). Several endoscopic scoring systems have been used to determine disease severity based on mucus quantity and appearance (Gerber et al. 2004b; Koch et al. 2007; Tilley et al. 2012b), having correlated well (0.61-0.84) with other features of the disease (Tilley et al. 2012b).

However, mucus accumulation has also been observed in cases of mild and moderate EAS (IAD) (Couëtil et al. 2016) and in severe EAS horses in remission little differences were found in endoscopic mucus scores compared to healthy horses (Rettmer et al. 2015). Therefore, mucus grading systems should not be used as a sole indicator of severe EAS (Pirie 2014; Couëtil et al. 2016).

Bronchoalveolar Lavage (BAL). BAL fluid cytology is considered a reference technique for the diagnosis and monitoring of generalised lung disease, enabling the assessment of lower airway inflammation characteristic of severe EAS (Jean et al. 2011; Pirie 2014). It can be easily performed in the standing lightly sedated horse even in field conditions (Hoffman 1999; Mazan and Hoffman 2003) using a flexible endoscope or 'blindly' with an equine BAL catheter equipped with an inflatable cuff (Couëtil and Hawkins 2013).

BAL fluid examination is considered superior to tracheal wash in terms of specificity and sensitivity for confirming the diagnosis of RAO. In BAL fluid cytology, nonseptic inflammation is evidenced by the presence of inflammatory cells without any proof of an aethiological agent. Besides the increase of nondegenerate neutrophils (> 20%) in horses with RAO, there is an associated reduction in macrophage and lymphocyte percentages, which may be accompanied by an increased amount of mucus and by the presence of Curschmann's spirals. An increased number of eosinophils may also be occasionally observed (Hoffman 2008; Tilley et al. 2012a, b; Couëtil and Hawkins 2013; Pirie 2014; Cian et al. 2015).

It has now been suggested that, like in human asthma, different cytological phenotypes may characterise severe EAS and in addition to the classical neutrophilic phenotype, associated with the incidence of cough, horses may also exhibit a paucigranulocytic phenotype with moderate (5–20%) or absent increase in neutrophil percentage (Leclere et al. 2011; Bullone and Lavoie 2017). It is still not fully understood how these different phenotypes may dictate disease evolution, but the paucigranulocytic cases are associated with more severe mucostasis and peripheral airway lesions and it is hypothesised that a neutrophilic phenotypic switching may occur (Lavoie-Lamoureux et al. 2010; Brujinzeel et al. 2015).

Nonetheless, BAL fluid differential cell counts correlate well with airway obstruction and airway responsiveness (Hoffman 2008) and it has been shown that an increase in neutrophil percentage is associated with increased disease severity (Tilley et al. 2012b; Bullone et al. 2018).

Slightly increased mast cells (>2%) and eosinophils (>0,1% or >0,5%) can also be observed (Cian et al. 2015; Mazan 2018). In one study, horses with mastocytosis were more likely to have airway hyperresponsiveness when assessed with histamine bronchoprovocation (Benedice et al, 2008). A study by Nolen-Walston et al. (2013) showed that Thoroughbred racehorses were more likely to have mastocytosis-eosinophilia with

increased mucus in the BALF, whereas Standardbred of the same age were more likely to have neutrophilic inflammation, but both groups had similar exercising hypoxemia, heart rate and blood lactate levels.

A cytological overlap may be seen between horses with IAD and RAO cases in remission. This acquires even greater importance for complicated cases where multiple disorders may be present. Association between IAD and EIPH and between infectious processes and RAO, have been reported in horses (Cian et al. 2015; Mazan 2018).

Arterial Blood Gas (ABG) Analysis. ABG analysis provides information on the efficiency of lung gas exchange, allowing the assessment of alveolar ventilation (Chevalier and Divers 2003).

Horses affected with severe EAS will usually present with lower values of PO_2, sO_2 and pH and increased values of PCO_2, however hypoxemia is the most common finding (Stopyra et al. 2012). Gas exchange is impaired (ventilation perfusion mismatch and dead space ventilation) and animals in exacerbation may present values of $PO_2 < 80$ mmHg (Nyman et al. 1991; Stopyra et al. 2012). However, asthmatic horses may present normal PO_2 values while in disease remission and in early stages of inflammation (Ferro et al. 2002).

Simões et al. (2019) found PO_2 to be the most significant parameter in the arterial blood gas analysis in its contribution to RAO staging.

Conventional Lung Mechanics. Conventional lung mechanics are considered the gold standard for pulmonary function testing, having been used in research for more than fifty years.

It is an invasive technique that relies on the measurement of estimated changes in pleural pressure (ΔPpl), using an oesophageal balloon catheter placed in the thoracic oesophagus, and the measurement of airflow at the nostrils with a pneumotachograph.

The measured values can be interpreted on their own, with $\Delta Ppl > 15$ cm H_2O being considered a positive indicator of severe EAS, or can be used to calculate dynamic compliance (C_{dyn}), pulmonary resistance (R_L), and work of breathing (W) (Robinson 2001; Couëtil et al. 2016). Severely affected asthmatic horses usually present increased R_L, W and ΔPpl, and a decreased C_{dyn} (Robinson et al. 1999; Robinson et al. 2000).

The study carried out by Simões et al. (2019) in RAO horses showed an increase in ΔPpl as the disease progressed.

Flowmetric Plethysmography and Histamine Bronchoprovocation. It was developed for equines and derives from the boxless plethysmography technique (Hoffman et al. 2001), combining the use of respiratory inductance plethysmography (RIP) and pneumotachography (Hoffman 2002). It is a non-invasive technique, not requiring sedation, unless it is associated with histamine bronchoprovocation (Hoffman 2002) and it is one of the few pulmonary function tests available for field assessment of asthmatic horses (Nolen-Walston et al. 2009).

The difference of flow measured by the RIP bands and measured at peak expiration by the pneumotachograph is called delta flow (Δflow) (Hoffman 2002). In cases of bronchoconstriction, such as in severe EAS, the airflow measured at the nostrils is less

than that of the bands, reflecting an increased Δflow value (Hoffman 2002; Nolen-Walston et al. 2009). Also, thoracoabdominal asynchrony determined by RIP is usually present in severe EAS-affected horses (Mazan and Hoffman 2003).

The association of histamine bronchoprovocation to assess airway hyperreactivity (AHR) increases the sensitivity of flowmetrics, allowing detection of severe EAS-affected horses in remission (Rettmer et al. 2015; Wichtel et al. 2016). This test assesses the reversible exaggerated narrowing of the airways (sensitivity) exhibited by some individuals with a higher sensitivity, in response to a bronchoconstrictor agonist stimulus of a certain magnitude (Hoffman 2002; Mazan and Hoffman 2003).

Severe asthmatic horses in exacerbation will invariably present AHR (Vandenput et al. 1998; Couëtil et al. 2016) and in a study involving the different RAO stages (Tilley et al. 2012b) and control horses, Simões et al. (2019) found that the maximum histamine concentration tolerated by the horses during bronchoprovocation presented significant differences between groups ($P = 0.00$). Although in a lesser magnitude, AHR is also observed in horses in disease remission (Mazan et al. 1999; Nolen-Walston et al. 2009; Rettmer et al. 2015; Wichtel et al. 2016) and in horses with IAD, proving very useful in these situations. Still, like in people, some horses without IAD display AHR which may be tied into the recent finding of signs of IAD being strong risk factors for eventual development of RAO (Gerber et al. 2015; Mazan 2018). Bosshard and Gerber (2014) identified early phenotypic indicators, like occasional coughing and mucus nasal discharge, for the risk of transition from IAD to RAO.

Immunological Testing. There is still some controversy surrounding immunological testing in severe EAS diagnosis. Tahon et al. (2009) found that IgE immunological testing and intradermal testing (IDT) did not contribute to the diagnosis of severe EAS, although IDT could identify sensitisation to allergens. Niedzwiedz et al. (2015) found that severe EAS horses had significantly higher concentrations of specific IgE against mites.

Tilley et al. (2010) and Tilley et al. (2012c) reported for the first time the use of skin prick tests (SPT) in a group of 40 horses (30 Severe EAS and 10 controls), which proved to be more reliable than *in vitro* IgE tests in identifying allergen hypersensitivity in severe EAS horses. Clinical confirmation of symptom reduction after allergen avoidance from the environment highlighted the relevance of the allergens identified by SPT in the aetiology of severe EAS in this group of horses. However, owner compliance with the recommended environmental changes is fundamental to ensure remission of clinical signs and in a recent study by Simões et al. (b) most owners were reticent on implementing all the necessary changes, due to socioeconomic constraints and lack of time. This ultimately lead to an incomplete recovery of pulmonary function and persistence of airway inflammation.

Staging Methods for Severe EAS. Because severe EAS is a chronic disease with a significant impact on the equine population the development of staging methods becomes essential to optimise equine medical care. One such method, the Horse Owner Assessed Respiratory Signs Index (HOARSI), relied solely on information reported by horse owners to identify the presence of respiratory disease. (Ramseyer et al. 2007; Gerber et al. 2009; Laumen et al. 2010; Rettmer et al. 2015). However, it does not provide clinical

information on the degree of severity of each disease stage and may misdiagnose horses in disease remission or in early inflammation stages of severe EAS.

Alternatively, the RAO (severe EAS) clinical staging method published by Tilley et al. (2012b) encompasses clinical history reported by the owners and clinical signs observed during clinical examination, namely cough frequency, nasal flare and abdominal lift. It also uses ancillary diagnostic tests to quantify airway inflammation and remodelling, such as thoracic x-ray, endoscopy and BALF cytology. This method allows not only the identification but also the staging of severe EAS and has been used since then as a method for staging severe EAS in equine hospitals and by various research groups which produced papers such as Haltmayer et al. (2013), Niedzwiedz and Jaworsky (2014a, b), Niedzwiedz et al. (2014a, b), Niedzwiedz et al. (2016), Decloedt et al. (2017) and Barton et al. (2018). It has also been included in two major revision papers (Pirie 2014; Mazan 2015) and in a book chapter (Piviani 2014).

Recently, our team adapted the staging method for severe EAS (Tilley et al. 2012a) so that it can be used in the field and the new staging method showed good results in identifying not only horses in disease exacerbation, but also those that presented low-grade airway obstruction. This staging method relies on clinical history and examination, percentage of neutrophils in BALF, pleural pressure measurement, flowmetric plethysmography and histamine bronchoprovocation (Simões et al. 2019).

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The Role of Uteropathogenic *Escherichia Coli* in the Development of Canine Pyometra

E. Silva[1], M. F. Silva[1], S. Henriques[1], P. Diniz[1], C. Carneiro[2], L. Lopes-da-Costa[1], and L. Mateus[1(✉)]

[1] Reproduction and Development Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
lmateus@fmv.ulisboa.pt

[2] Microbiology and Immunology, Laboratory Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal

Abstract. Pyometra is a common dioestral uterine disease in middle aged female dogs that often leads to a loss of breeding potential and, in some cases, can be life threatening. Hormonal and bacterial factors are central in the pathogenesis of the disease. Most pyometra cases are caused by specialized *Escherichia coli* (*E. coli*) strains that have similar characteristics to the uropathogenic strains. Uteropathogenic *E. coli* (UtPEC) strains express a multitude of virulence factors to break the inertia of the mucosal barrier. In response to the breach by UtPEC, host inflammatory responses are triggered leading to cytokine production, neutrophil influx, and local tissue destruction. In this Review, we discuss current concepts on the pathogenesis of UtPEC, with particular emphasis on the bacteria virulence factors and natural defenses within the uterus. We will survey the current understanding of host responses to UtPEC-mediated pyometra, as well as the consequences of these interactions for the pathophysiology of pyometra.

Keywords: *Escherichia coli* · Canine pyometra · Host-bacteria interaction

1 Introduction

Pyometra is a common uterine disease, especially in countries where elective spaying is not performed. In two Swedish studies enrolling more than 200,000 insured dogs, the overall proportion of bitches that developed the disease by 10 years of age was between 19% and 25% (Egenvall et al. 2001; Jitpean et al. 2012), with a trend to increase with aging. Reported mean age at time of development of pyometra is 6.5–10.4 years, with an age range of one to 19 years (Niskanen and Thrusfield, 1998; Egenvall et al. 2001; Jitpean et al. 2012, 2014; Gibson et al. 2013; Machado et al. 2017). Breed influences the risk of developing pyometra, which suggests that genetic factors may be involved in the pathogenesis of pyometra (Niskanen and Thrusfield 1998; Jitpean et al. 2012; Gibson et al. 2013). The disease often causes subtle changes in the early stages; therefore, the diagnosis is often made late in the disease process (Smith, 2006). Clinical signs depend

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on the severity of the disease (Jitpean et al. 2014; Jitpean et al. 2017). The diagnosis of pyometra is based on case history, physical examination and laboratory analysis, combined with radiography and/or ultrasonography of the uterus and ovaries (Mateus and Eilts 2010). Despite being a potentially life-threatening illness, the mortality rate is as low as 1% following surgical treatment, and around 3–10%, when euthanasia cases are included (Egenvall et al. 2001; Gibson et al. 2013; Jitpean et al. 2014). Ovariohysterectomy is considered the safest and most effective treatment, but medical alternatives can be used in selected cases (Fieni et al. 2014).

Pyometra develops as a result of a complex interaction of etiological factors. These factors include changes within the endometrium, the hormonal influence on uterine environment, the virulence and type of the bacteria, and the individual defence mechanisms (Dow 1959; De Bosschere et al. 2001; Chen et al. 2003; Sugiura et al. 2004; Tsumagari et al. 2005; Mateus et al. 2013; Gabriel et al. 2016; Gultiken et al. 2016; Moxon et al. 2016). The bacteria most commonly isolated from the uterus of affected bitches are *E. coli* (up to 90% of the cases) (Wadås et al. 1996; Fransson et al. 1997; Dhaliwal et al. 1998; Chen et al. 2003; Coggan et al. 2008; Mateus et al. 2013; Machado et al. 2017). Its presence is normally associated with severe systemic signs and a potentially life-threatening situation. If left untreated it is lethal and patients may develop endotoxemia, sepsis or septic shock (Fransson et al. 1997, Jitpean et al. 2014; Jitpean et al. 2017). However, other bacteria isolated include *Streptococcus* spp, *Klebsiella* spp, *Staphylococcus aureus*, *Pasteurella* spp, *Proteus* spp and *Pseudomonas* spp. (Wadås et al. 1996; Fransson et al. 1997; Dhaliwal et al. 1998; Coggan et al. 2008). These organisms are also those most commonly isolated from the vagina of normal bitches (Watts et al. 1996; Root Kustritz 2006) and can ascend to the uterus during pro-estrus and estrus (Root Kustritz 2006). In this Review, we discuss current concepts on the pathogenesis of UtPEC with emphasis on the bacteria virulence factors and natural defenses within the uterus. We will survey the current understanding of host responses to UtPEC-mediated pyometra as well as the consequences of these interactions for the pathophysiology of pyometra.

2 Uteropathogenic *E. Coli*

Pyometra *E. coli* isolates derive from the host's fecal and perineal flora (Wadås et al. 1996; Mateus et al. 2013; Agostinho et al. 2014). However, it is controversial whether the pathogenesis is due to its higher prevalence within fecal microbiota or to the acquisition of virulence factor (VF) genes. A high genomic diversity was observed among *E. coli* isolates, which indicates that the isolates do not originate from a specific clone that is epidemically spread between animals (Hagman and Kühn 2002; Mateus et al. 2013). Although no VF genes or virulence traits could be specifically associated with *E. coli* pyometra isolates, these isolates are mainly from the highly virulent phylogenetic group B2 (Mateus et al. 2013; Machado et al. 2017). The prevalence of phylogenetic group B2 was significantly higher in pyometra (94%) than in cystitis (48%) and fecal (39%) isolates. In addition, *E. coli* pyometra isolates are characterized by a high number of UPEC VF genes and pathogenicity-associated islands (PAIs) markers (Mateus et al. 2013). Thus, these results can be seen in the light of the "*special pathogenicity theory*". However, a similar virulence profile was also found in a subset of cystitis and fecal

isolates, leading to the suggestion that they may be potentially able to induce pyometra in receptive hosts (Mateus et al. 2013). In cases of *E. coli* pyometra with a concurrent subclinical urinary tract infection, the urinary tract and the uterus are likely to be infected with the same bacterial strain (Wadås et al. 1996; Hagman and Kühn 2002). Among healthy female dogs, the fecal *E. coli* population varies considerably with respect to both clonal diversity and phylogenetic distribution. However, *E. coli* clones belonging to phylogenetic group B2 tend to behave as dominant clones (Lourenço et al. 2017), which leads to the hypothesis that prior to the occurrence of a pyometra, bitches may experience an important increase in vaginal colonization with *E. coli* strains of phylogenetic group B2 (Silva et al. 2018). Overall, the available data suggest that the prevalence and the special-pathogenicity hypothesis can be complementary. More studies are needed to assess the relative importance of clonal dominance versus intrinsic virulence potential for the pathogenesis of *E. coli* pyometra.

2.1 Uteropathogenic *E. Coli* Virulence Factors

Uteropathogenic *E. coli* have a range of VF that promote colonization and infection of the genital tract. The *E. coli* VF that have been potentially implicated in the establishment of pyometra can be associated with the surface of the bacterial cell and/or secreted and exported to the site of action.

2.1.1 Surface Virulence Factors

Surface VF include different types of adhesive organelles (fimbriae), which promote bacterial attachment to the cells of the uterus. Adherence is a key event in the initiation of pyometra pathogenesis. After vaginal contamination and colonization, *E. coli* migrate to the uterus, an event that requires appendages such as flagella and pili. In the uterus, the complex host-pathogen interactions ultimately determine whether *E. coli* is successful in colonization or is eliminated (Fig. 1).

Adhesins are classified as fimbrial or afimbrial. The most common types of pili are Types 1, P and S. Type 1 pili are also referred to as mannose sensitive pili and are commonly expressed in pathogenic and non-pathogenic strains of *E. coli*. Genes encoding for Type 1 fimbriae were detected in 91–100% of pyometra *E. coli* isolates (Chen et al. 2003; Mateus et al. 2013; Maluta et al. 2014). The role of the type 1 fimbriae in pyometra is difficult to ascertain because 95.7–100% and 88.5–100% of *E. coli* from urinary tract infection (UTI) and fecal origin, respectively, expressed this gene (Johnson et al. 2003; Mateus et al. 2013). In addition, fimH expression is regulated by environmental conditions and mannose receptors availability (Schwan 2011). *E. coli* pyometra isolates have the ability to transcribe the *fimA* gene (encodes for the major sub-unit of the fimbria), which mediates the reversible switch between the fimbriated (Phase-On) and non-fimbriated (Phase-OFF) states (Diniz et al. 2014). This allows *E. coli* to express Type 1 fimbriae under favorable conditions and to evade humoral responses targeting this organelle (Eisenstein 1981). Although adherence to canine endometrial cells was shown to be mediated at least in part by Type 1 fimbriae (Krekeler et al. 2012) targeted deletion of specific adhesin genes in a canine pyometra strain was compensated by the presence of other adhesins (P fimbrae and F1C fimbrae), which may indicate functional

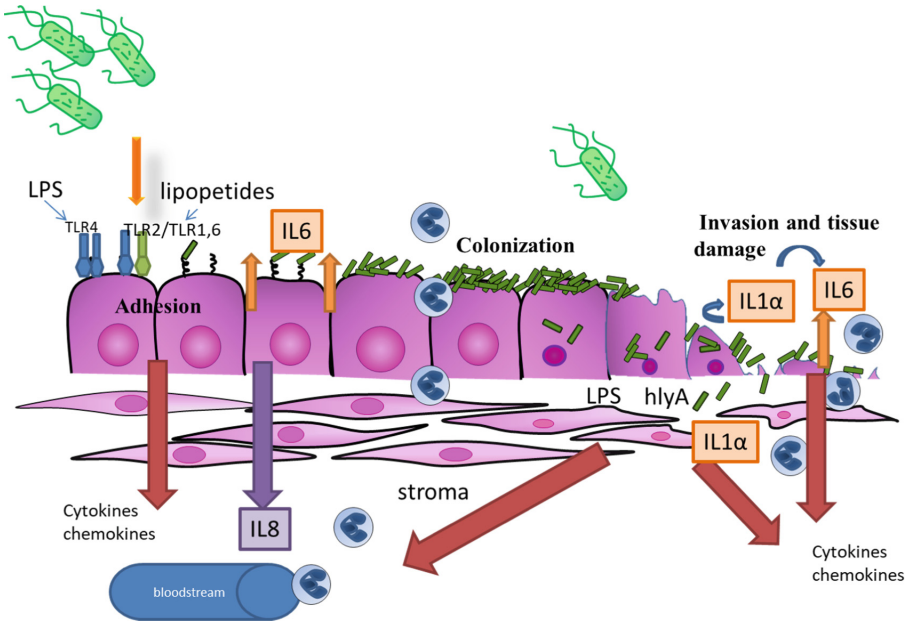

Fig. 1. Development of pyometra. During pro-estrus and estrus, *E. coli* have access to the uterus. If *E. coli* manages to sidestep the constitutive host defenses and contacts the epithelium, its continued presence can trigger the activation of additional host defense mechanisms. After the occurrence of a breach in the epithelium, endometrial stromal cell damage by HlyA may facilitate progression of *E. coli* into the uterine tissue.

redundancy among adhesins (Krekeler et al. 2013). In accordance, no differences in adhesion levels were observed when endometrial cells were incubated with *E. coli* with different adhesins encoding gene profiles. In addition, internalization was poor, which may indicate that cell invasion is not the principal mechanism responsible for cell and tissue damage (Henriques et al. 2016). The ability of *E. coli* to adhere to the uterine endometrium was higher under the influence of progesterone (Sandholm et al. 1975; Ishiguro et al. 2007), but this effect was not corroborated in a later study (Krekeler et al. 2012). Differential expression of epithelial and glandular receptors may also enhance bacterial attachment (Leitner et al. 2003; Ververidis et al. 2004; Yasunaga et al. 2013; Gabriel et al. 2016; Payan-Carreira et al. 2016).

Besides their role in adhesion, Type 1 fimbriae and P fimbrae are involved in innate immune response (Fischer et al. 2006). P fimbriae gene was present in 30% to 57% *E. coli* pyometra isolates (Chen et al. 2003; Coggan et al. 2008; Siqueira et al. 2009; Ghanbarpour and Akhtardanesh 2012; Mateus et al. 2013; Maluta et al. 2014), similar to cystitis strains (23.5% to 39.1%) and higher than in fecal strains (18 to 19%) (Siqueira et al. 2009; Mateus et al. 2013). P fimbrae may play an important role in the pathogenesis of pyometra as described in lower UTIs and pyelonephritis in humans and dogs (Johnson et al. 2000; 2003; Lane and Mobley 2007; Vaisanen et al. 1981; Féria et al. 2001). *papGIII* gene allele was detected in 26% to 50% of pap-containing pyometra isolates (Chen et al. 2003; Siqueira et al. 2009; Maluta et al. 2014), whereas *papGII* gene, usually

associated with pyelonephritis cases, was detected in 5.8% of pap-containing pyometra isolates (Siqueira et al. 2009), and *papGI* allele was not detected (Chen et al. 2003; Maluta et al. 2014). F1C fimbriae encoding gene (focG) was detected in 61.3% of the pyometra strains, similar to cystitis strains (47.8%) and higher than fecal strains (34.6%). S fimbrae encoding gene was only detected in cystitis strains (17.4%) and fecal strains (11.5%) (Mateus et al. 2013). These two types of fimbriae show binding to epithelial and endothelial cell lines derived from the lower human urinary tract and kidney (Parkkinen et al. 1989; Virkola et al. 1988).

Virulence factors located on the bacterial surface also include the capsule and the lipopolysaccharide (LPS). The capsule provides protection against phagocytic engulfment and complement-mediated bactericidal effect. *kpsMT-II* encoding gene was found in 41.4% of canine *E. coli* pyometra strains (Henriques et al. 2014). LPS is an integral component of the cell wall of Gram-negative bacteria and is thought to be responsible for the systemic signs of pyometra in bitches. High plasma concentrations of endotoxin in pyometra bitches were related to poor prognosis (Okano et al. 1998), and to high plasma levels of PGFM (Hagman et al. 2006). Endotoxins strongly stimulate prostaglandin E2 synthesis, which might further contribute to the suppressed activity of cellular immunity during diestrus (Silva et al. 2012).

2.1.2 Secreted Virulence Factors

Production of toxins by colonizing *E. coli* may cause an inflammatory response, a possible pathway for pyometra symptoms. The most important secreted virulence factor of UtPEC is α-haemolysin (HlyA). β-hemolytic *E. coli* was found in 30% to 52% of *E. coli* pyometra cases (Chen et al. 2003; Coggan et al. 2008; Siqueira et al. 2009; Mateus et al. 2013; Maluta et al. 2014) suggesting that hemolysin contributes to the virulence of *E. coli* strains during canine uterine infection. Cystitis isolates had similar prevalence of *HlyA*, but this was only 12–19% in fecal *E. coli* isolates (Siqueira et al. 2009; Mateus et al. 2013). The HlyA is a pore-forming toxin, which belongs to the family of RTX (repeats in toxin) toxins. Depending on its concentration and on the type of targeted cells, HlyA displays cytolytic activity on erythrocytes and nucleated host cells, induces apoptosis of target host cells, including neutrophils, T lymphocytes and epithelial cells, or induces innate immune signaling pathways, via Ca^{2+} oscillations in epithelial cells (reviewed by Wiles and Mulvey 2013). Recent studies suggest that α-hemolysin can be associated to uterine tissue damage and to a compromised uterine early immune response in cases of pyometra (Henriques et al. 2016). The known cytotoxic effect of hemolysin in granulocyte and lymphocytes, as well as the enhancement of access to cell nutrients and iron storages (reviewed by Bien et al. 2012) might also enable hemolytic *E. coli* to better survive and induce a higher level of uterine tissue damage.

The cytotoxic necrotizing factor 1 (CNF1) was detected in 26–57% of *E. coli* pyometra isolates, a higher prevalence than in fecal isolates (12–19%) (Johnson et al. 2003; Coggan et al. 2008; Siqueira et al. 2009; Mateus et al. 2009; Maluta et al. 2014). This cytotoxin may facilitate bacterial invasion of the bloodstream due to interference with polymorphonuclear phagocytosis and evokes apoptotic death of epithelial cells in the bladder (Emödy et al. 2003). However, the detailed role of CNF1 in the pathogenesis of pyometra remains unclear.

Iron is an essential nutrient for bacterial viability within the host. The detection of multiple iron acquisition genes (fyuA, iucD, iroN, fepA, sitA and chuA) in the majority of the pyometra *E. coli* isolates (≥84%) suggests that the uterus is an iron limiting environment (Mateus et al. 2013; Maluta et al. 2014). During infection, the amount of iron available to bacteria is even lower due to the production of host proteins, like lactoferrin, that interact with iron metabolism. The average number of iron acquisition related genes per isolate was similar in pyometra, cystitis and fecal isolates (4.7, 4.5 and 3.9 genes per isolate, respectively). However, the higher prevalence of fyuA (Yersinibactin system) and chuA (Heme receptor) genes in pyometra isolates may indicate a more relevant contribution of these factors (Mateus et al. 2013). Besides its role in iron uptake, Yersinibactin system and ChuA may interfere with the host response and innate immune system, being characteristic of bacteriemic *E. coli* (Johnson and Stell 2000). These genes may represent a relevant component of the virulence of canine pyometra *E. coli* strains, contributing to the persistence and load of bacteria.

VF-genes *traT* protectin, *ompT*, *ibeA*, *usp*, *iss* and *tcpC* had a variable prevalence in canine *E. coli* pyometra strains (Siqueira et al. 2009; Agostinho et al. 2014; Henriques et al. 2014; Maluta et al. 2014). These VF-genes may have relevant roles in the virulence potential of the bacterium and in the pathogenesis of pyometra, by promoting endothelial cell invasion and interfering and/or avoiding the host defense mechanisms (Johnson and Stell, 2000; Yadav et al. 2010).

3 Host Defenses Against *E. Coli* Colonization of the Uterus

The uterus is not a sterile environment, and infection is prevented by several host defense mechanisms to prevent bacterial colonization and survival. Endometrial epithelial adherence is critical for establishment of uterine infection. Uteropathogenic *E. coli* strains possess different types of adhesins that enable the bacteria to aggregate and adhere to the cellular surfaces. Consequently, the first line of host defense against uterine infection is centered on preventing *E. coli* adherence to the uterine mucosa.

3.1 Primary Uterine Defenses

The uterus has several specialized defenses against bacterial colonization. The mucus layer on the apical surface of the epithelia provides an obstacle to microbes. The transmembrane cell surface mucin, MUC1, is a major component of the epithelial glycocalyx, and acts as an anti-adhesive molecule on the uterine surface (King et al. 2003; Brayman et al. 2004), being under ovarian steroid control. Reduction of MUC1 expression is associated with increased *E. coli* adherence in the canine uterus at the early stage of dioestrus (Ishiguro et al. 2007). Defensins (a group of small, highly cationic antimicrobial peptides) have the capacity to kill bacteria, fungi, and some encapsulated viruses. These peptides attach to the anionic phospholipids on the cell wall of pathogens and disrupt their cell membrane function, increasing cell permeability and causing cell death (Ganz, 2003). Diestrous bitches had a 100-fold increase in β-defensin 1 mRNA expression in the endometrium, when compared to anestrous or estrous bitches (Krekeler et al.

2012). However, pyometra uteri had no upregulation expression of defensin family member genes, suggesting a constitutive expression (Hagman et al. 2009). The chemokine CXCL10, which has antimicrobial properties similar to those of defensins, had a significant up-regulation expression in canine endometrial epithelial cells after a 4 h incubation with *E. coli* (Henriques et al. 2016).

Endometrial epithelia also express additional natural antimicrobials. For example, the iron-binding protein lactoferrin was shown to be oestrogen regulated, showing maximal expression in the proliferative phase (Kida et al. 2006). Lactoferrin impairs LPS ligation to Toll-like receptors (TLR), thus also attenuating the subsequent activation of the inflammatory response (Machnicki et al. 1993). Therefore, lactoferrin expression in pyometra uteri may counterbalance an excessive inflammatory response. However, lactoferrin also activates TLR4 on the surface of phagocytes and epithelial cells, and the dramatic reduction in lactoferrin expression observed at early diestrus may impair the antimicrobial defense. Paradoxically, lactoferrin is also stored in granules of neutrophils, and an enhanced endometrial expression of lactoferrin is associated with neutrophil invasion into the pyometra uterus (Kida et al. 2006).

A natural consequence of the uterine infection is the upregulated expression of a wide range of antimicrobial peptides and proteins (AMPs). Upregulation of bactericidal/permeability-increasing protein (BPI), secretory leukocyte protease inhibitor (SLPI), and acyloxyacyl hydrolase genes was identified in pyometra uteri (Hagman et al. 2009; Voorwald et al. 2015). The BPI and SLPI proteins have LPS-neutralizing activity, impairing the innate immune activation by TLRs (Greene et al. 2004; Balakrishnan et al. 2012). This way, upregulation of SPLI and BPI in pyometra may be endometrium-protective, against both microorganisms and the immune-mediated tissue damage. Acyloxyacyl hydrolase is a lipase that partially deacylates bacterial LPS (Hall and Munford 1983).

The next line of endometrial defense is the columnar epithelium. Epithelial cells are more resistant than stromal cells to cytolysis caused by α-hemolysin (Fig. 2). Sublytic concentrations of HlyA may disrupt the epithelial layer due to epithelial cell apoptosis (Smith et al. 2006) and/or degradation of proteins involved in cell–cell and cell–matrix interactions (Dhakal and Mulvey 2012). Following a breach in the epithelium, endometrial stromal cell damage by HlyA may facilitate progression of *E. coli* into the uterine tissue. In fact, β-hemolytic *E. coli* infection was more associated with the occurrence of metritis and higher uterine tissue damage than non-hemolytic *E. coli* infection (Henriques et al. 2016) (Fig. 3).

3.2 Pattern Recognition Receptors

If a microbe manages to overcome the constitutive host defenses and contacts the epithelium, its continued presence can trigger the activation of additional host defense mechanisms (Bien et al. 2012). Uterine infection elicits both innate and adaptive immune responses, although an efficient host defense depends upon an early activation of the innate immune system (Hickey et al. 2012). Innate immunity encompasses multiple host resistance mechanisms ranging from antimicrobial peptides and acute phase proteins to cytokines (Cray et al. 2009; Horne et al. 2008). The peripheral plasma concentrations of acute phase proteins, such as C-reactive protein, serum amyloid A and Haptoglobin

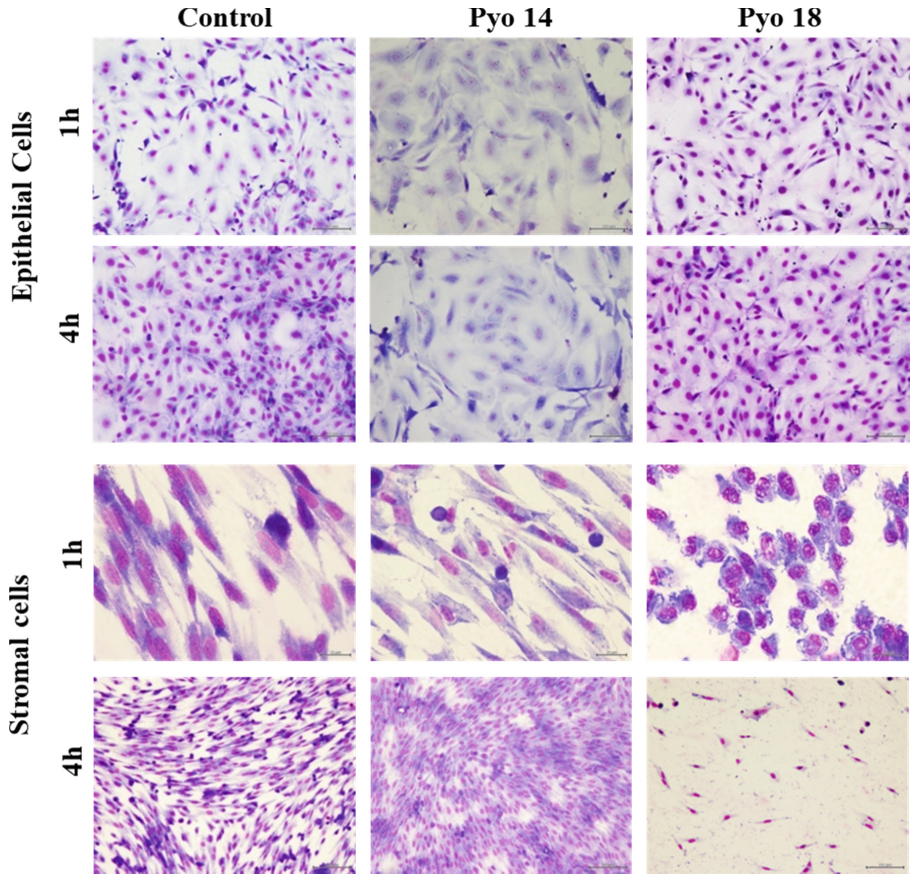

Fig. 2. Epithelial cells are more resistant than stromal cells to cytolysis caused by α-hemolysin. Morphology of endometrial epithelial (**A**–**F**) and stromal (**G**–**L**) cell cultures stained with Giemsa after 1 and 4 h of incubation: unstimulated cells (**A**, **D**, **G**, **J**); cells incubated with a non-hemolytic *E. coli* (Pyo 14) (**B**, **E**, **H**, **K**) and with a β-hemolytic *E. coli* (Pyo 18) (**C**, **F**, **I**, **L**). (Henriques et al. 2016).

are associated with the severity of the uterine infection and high C-reactive protein concentrations were associated with SIRS in pyometra cases (Fransson et al. 2007). The postoperative concentrations of these acute phase proteins may be used as routine diagnostic markers of early postoperative complications, as their concentrations normally decrease during an uncomplicated postoperative course (Dabrowski et al. 2009; Dabrowski et al. 2013).

The innate immune response depends on cellular pattern recognition receptors (PRR) that bind pathogen-associated molecular patterns (PAMPs), which are found in microbes but not eukaryotic cells (Horne et al. 2008). Examples of PAMPs are LPS, lipopeptides, flagellin, and microbial RNA and DNA. The PRRs include three families, the TLRs being the most well characterized in the female reproductive tract (Kannaki et al. 2011).

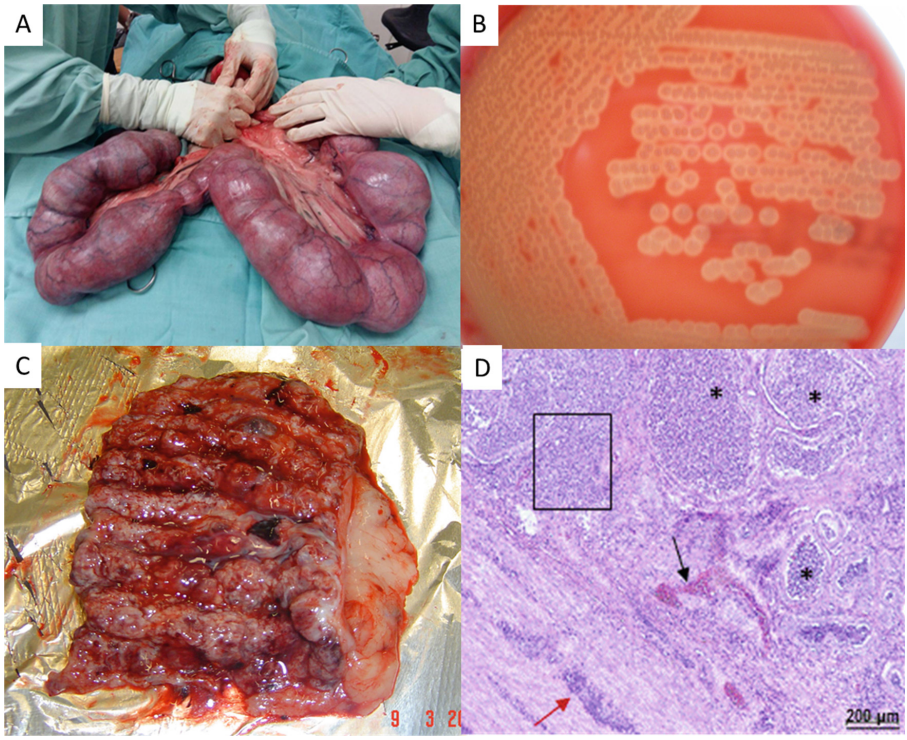

Fig. 3. Closed hyperplasic pyometra in a 7 years old bitch due to β-hemolytic *E. coli.* A. Uterus during ovariohysterectomy; B. β-hemolytic *E. coli* colonies; C. Macroscopic endometrial lesions, with hemorrhage and abscess as major features; D. Histological section evidencing extensive infiltration of neutrophils in basal glands (*), metritis (red arrow) and areas of hemorrhage (black arrow) and necrosis (box). Staining by H&E.

These receptors are expressed in many effector cells of the immune system - including macrophages, neutrophils, dendritic cells, and lymphocytes - and in endometrial epithelial and stromal cells (Davies et al. 2008; Silva et al. 2012; Henriques et al. 2016).

TLR-mediated immune surveillance is an important component of the defense mechanisms within the canine uterus. Transcription of *TLR*1-7 and 9 was detected in the canine endometrium in all phases of the oestrous cycle (Silva et al. 2012). This observation indicates that canine endometrium can recognize a large variety of PAMPs and orchestrate an innate immune response against bacterial and viral pathogens. In association with the accessory molecules MD-2 and CD14, TLR4 is the signal transduction receptor for Gram-negative bacteria lipopolysaccharide (LPS) and heat shock proteins (Pioli et al. 2004). In contrast, a broader range of microbial products activates the immune response through engagement of TLR2, including lipoteichoic acid (LTA) from Gram-positive bacteria and bacterial lipoproteins/ lipopeptides from Gram-negative and Gram-positive bacteria (Zähringer et al. 2008). Recognition of some microbial products by TLR2 also appears to be dependent upon the formation of heterodimers with either TLR1 or TLR6.

TLR3 binds to viral dsRNA, TLR5 binds to bacterial flagelin, TLR8 recognises ssRNA and TLR9 binds to bacterial CpG DNA (Takeda and Akira 2004).

Differential endometrial *TLR* transcription and expression occurred during the oestrous cycle, indicating a regulatory role of ovarian steroids (Silva et al. 2012). The high expression of TLR2 and TLR4 during the follicular phase (Silva et al. 2012) may be related with the uterine contamination normally occurring during proestrus and oestrus, resulting from ascending bacteria from the vagina (Root Kustritz 2006). The fact that TLR2 and 4 transcription and expression are at the lowest levels during early dioestrus (Silva et al. 2012), in association with the low numbers of endometrial leucocytes observed in non-pathological dioestrous uteri (Henriques et al. 2016), probably reflects the suppression of the immune system by progesterone (Faldyna et al. 2001; Kida et al. 2006, Silva et al. 2012).

3.3 Host Signaling in Response to Uteropathogenic *E. Coli* Recognition

Within TLRs, TLR4 is the most studied in the context of the mechanisms of innate immune defense in the uterus. Stimulation by LPS or Type 1 or P fimbriae, triggers a TLR4 signaling cascade that culminates in the activation of NFkB and IRF3 (Fischer et al. 2006; Iwanaszko and Kimmel 2015) and production of inflammatory mediators. Displaying multiple signaling pathways for production of cytokines is advantageous because bacteria have the ability to suppress certain signaling events important for cytokine and chemokine production. The fact that TLR2 and 4 signaling components are constitutively transcribed in diestrous uteri prompts the uterus to sense and rapidly mount an immune response following a pathogen insult (Henriques et al. 2016). In fact, up-regulation of TLR 2 and 4 transcription (Hagman et al. 2009; Silva et al. 2010) and expression (Silva et al. 2012) was shown in *E. coli* pyometra endometrium. Recently, microarray screening allowed the identification of pyometra specific differential gene transcription. Many of these genes are associated with chemokines, cytokines, inflammatory cell extravasations, anti-bacterial action, proteases and innate immune response (Hagman et al. 2009; Voorwald et al. 2015). This up-regulation reflects the infiltration of inflammatory cells and the endometrial cells' response. Like bovine endometrial cells (Healy et al. 2014), canine uterine epithelial and stromal cells appear to be a major source of interleukin-6 (IL-6) and interleukin-8 (IL-8) after infection with UtPEC (Karlsson et al. 2015; Henrique et al. 2016). A powerful pyrogen, IL-6 has stimulatory effects upon leukocyte activation and myeloid progenitor cell proliferation and regulates the synthesis of acute phase proteins (Scheller et al. 2011). High serum levels of IL-8, a potent neutrophil chemotactic molecule, were observed in dogs with pyometra, especially in those that developed SIRS, making IL-8 a useful early biomarker of uterine infection and, possibly, of sepsis in dogs (Karlsson et al. 2012). Neutrophil recruitment to the site of infection is critical for bacterial clearance, being the presence of neutrophils in the uterine content a hallmark of pyometra. However, their action may also lead to local tissue damage (Silva et al. 2014; Henriques et al. 2016).

Recently, it was shown that hemolytic *E. coli* down-regulates IL-10, TNFα and IL-1β gene transcription in stromal cells (Henriques et al. 2016). Inhibition of cytokine production by α-hemolysin may occur during early stages of infection, allowing the bacteria to establish a niche prior to the activation of an adequate innate immune response.

This may compromise the chemotaxis and activation of immune cells, leading to a precocious higher level of cell and tissue damage, as stromal cells are more sensitive to the cytolytic effect of α-hemolysin bearing *E. coli*. On the other hand, delay in IL-10 production might be associated with an excessive inflammatory reaction.

Damage-associated molecular patterns (DAMPs, intracellular molecules) are released following cell death and can be recognized by the innate immune system, signaling tissue damage. Several S100 proteins, including S100A8 and S100A9, identified as DAMPs, stimulate the production of the pro-inflammatory cytokines TNFα, IL-6, IL-1B and IL-8. Genes encoding these pro-inflammatory cytokines genes are overexpressed in pyometra uteri (Voorwald et al. 2015; Henriques et al. 2016).

The activation of TLR2 and TLR4 also triggers an increase in prostaglandin (PG) synthesis (Helliwell et al. 2004). In pyometra cases, prostaglandin synthesis genes were up-regulated and endometrial concentrations of PGE_2 and $PGF_{2\alpha}$, were high, which could further regulate the local inflammatory response (Silva et al. 2010). The higher ratio of $PGF_{2\alpha}$ to PGE_2 concentrations may be associated to the early luteolysis observed in *E. coli* pyometra cases. Around 60% of pyometra cases are associated with serum P4 concentrations lower than 1 ng mL−1 at the time of diagnosis (Fieni, 2006; England et al. 2007; Machado et al. 2017), Blood $PGF_{2\alpha}$ metabolite (PGFM) concentrations are a good indicator of endotoxin release, and helps in the differentiation between pyometra and CEH (Hagman et al. 2006).

4 Neutrophil Extracellular Traps (NETs)

Polymorphonuclear neutrophils (PMNs) are the first line of innate immune defense against invading bacteria. Following bacteria phagocytosis, the main bacteria killing mechanism, PMNs death causes the release of intracellular components, mainly DNA, histone and enzymes such as elastase, cathepsin G, and myeloperoxidase (Brinkmann et al. 2004). These structures, called neutrophil extracellular traps (NETs), may mediate antimicrobial and pro-inflammatory responses (Brinkmann et al. 2004). The *in vivo* presence of NETs in *E. coli* pyometra uteri was recently reported (Rebordão et al. 2015). Excessive uterine NETs formation and/or impaired clearance might be associated with the level of inflammation and tissue damage observed at diagnosis. Further work is needed to evaluate if the level of NETs' bacteria trapping, NETs disruption, or bacteria ability to resist to NETs, determines the progression of uterine bacterial contamination from proestrus/estrus to diestrous pyometra (Rebordão et al. 2015).

5 UtPEC and UPEC as a Zoonotic Risk

Sharing of *E. coli* strains within household humans and dogs was demonstrated (Jonhson et al. 2008; Stenske et al. 2009; Stokholm et al. 2012), suggesting a clinically relevant transmission pathway of pathogenic bacteria, with potential long term health effects, especially in pregnant women, children, older people and immune-incompetent patients. A role for dogs as zoonotic reservoirs of ExPEC strains is supported by the following facts: (1) ExPEC strains are the most frequently isolated pathogens in UTI and pyometra cases of female dogs; (2) canine uterine *E. coli* strains mainly belong to the highly

virulent phylogenetic group B2; (3) humans may acquire canine pathogenic bacteria; and (4) dogs represent a potential source of spread of antimicrobial resistance due to the extensive use of antimicrobial agents in these animals.

6 Conclusions

This review focuses on the latest insights towards the understanding of pathogen-specific modulation of host immunity during pyometra, which may influence the severity of disease and clinical outcomes. In a breeding context, the previous screening of phylogenetic B2 *E. coli* in the bitch fecal flora will require the implementation of strict hygiene measures during breeding management. Future studies should assess how different strains influence disease progression and outcome. Novel approaches to the prevention of pyometra in bitches should include blockage of hormonal action and bacterial adhesion, or development of a vaccine against specific bacterial virulence factors (Sivick and Mobley 2010; Hartmann et al. 2012; Nesta and Pizza 2018). Development of molecular markers is central for the early diagnosis of uterine infection and therapeutic intervention (Fontaine et al. 2009).

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What Goes Wrong from a Mare Healthy Endometrium to Endometrosis?

G. Ferreira-Dias[1(✉)], M. R. Rebordão[1,2], A. M. Galvão[1,3], R. Roberto-da-Costa[2], A. Amaral[1], C. Fernandes[1], P. Pinto-Bravo[2], S. Morazzo[1], G. Alexandre-Pires[1], K. Lukasik[3], A. Szóstek-Mioduchowska[3], and D. J. Skarzynski[3]

[1] Physiology Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
gmlfdias@fmv.ulisboa.pt
[2] Polytechnic of Coimbra, Coimbra Agriculture School, Coimbra, Portugal
[3] Institute of Animal Reproduction and Food Research of PAS, Olsztyn, Poland

Abstract. Mare endometrial function involves innumerous mediators as hormones (progesterone, estradiol, oxytocin, prostaglandins), cytokines, nitric oxide, enzymes and others. When this complex interaction is disrupted reproductive function is impaired. While some mares carry on a pregnancy to term, others develop recurrent post-breeding endometritis and chronic degenerative fibrosis (endometrosis), leading to infertility. Endometrosis is also linked to oviduct fibrosis, which exacerbates infertility. A thorough etiology, diagnosis and pathogenesis of equine endometrosis is still incomplete. In the uterus, equine neutrophils may release neutrophil extracellular traps (NETs). Besides holding bacteria *in loco* and kill them, they have a deleterious effect by inducing collagen formation. Our *in vitro* studies have shown that NETs persistence might be linked to endometrosis, due to NETs proteases action, mainly elastase and cathepsin G. These NETs proteases increase $PGF_{2\alpha}$ synthesis or $PGF_{2\alpha}$ receptor transcripts. Also impaired PGE_2 or PGE_2 receptor 2 transcripts are associated to higher collagen type I production, characteristic of fibrosis. In order to fight NETs fibrotic effect, the use of an elastase inhibitor stimulated the *in vitro* production of anti-fibrotic PGE_2 and inhibited the pro-fibrotic $PGF_{2\alpha}$. Since these are promising results, further studies should be performed to reduce the establishment of mare endometrial fibrosis in susceptible mares.

Keywords: Mare · Endometrium · Endometrosis · Prostaglandins · Cytokines · NETs

1 Introduction

In the breeding season, the mare endometrium, which is a very complex tissue, undergoes physiological cyclic changes. The endometrium cyclic pattern is mainly regulated in the mare by the ovarian steroid hormones estradiol (E_2) and progesterone (P_4), as well as endometrial prostaglandins (PG) (Stout and Allen 2002; Roberto da Costa *et al.*

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2007a). Throughout the estrous cycle, the mare endometrium grows and regresses, which involve angiogenic changes coordinated with cellular proliferation/apoptosis to prepare the uterus for pregnancy (Ferreira-Dias *et al*. 2001; Roberto da Costa *et al*. 2007a; 2007b; 2008). The physiological process of apoptosis occurs throughout the estrous cycle and is caspase-3 dependent (Roberto da Costa *et al*. 2007a). To meet the physiological needs for pregnancy maintenance in the mare, endometrial cells undergo hyperplasia and protein synthesis increases under the influence of progesterone, at the time the histotroph is crucial for equine embryo nourishment, in case of pregnancy (Roberto da Costa *et al*. 2007a).

Considerable knowledge lacks on the physiopathological mechanisms leading from a healthy endometrium to an endometrium that develops chronic degenerative changes (endometrosis). It is well established that in the mare, breeding induces a transient physiological endometritis, as an essential inflammatory response for the removal of excessive spermatozoa, cellular debris, and contaminating microorganisms introduced into the uterus (Troedsson 2006). This may induce fibrosis (collagen deposition) in the stroma, leading to a degenerative chronic condition named endometrosis (Kenney 1992, Hoffmann *et al*. 2009; Rebordão *et al*. 2014a). Actually, endometrosis, that is found in Kenney and Doig (1986) categories IIB and III, may also be classified as an active or inactive fibrosis process that develops around the endometrial glands and/or in the stroma (Hoffmann *et al*. 2009).

2 Endocrine Environment in the Endometrium

The complex interaction among the different hormones and their receptors on mare endometrium plays a role on estrous cycle and early pregnancy regulation. It has been long established that ovarian function regulation is strongly dependent on endometrium influence. In particular, at the time of luteolysis, the connection between the ovaries and the endometrium is quite evident. Both epithelial and stromal cells from mare endometrium have the ability to respond to *in vitro* stimulation of oxytocin (OXT) or ovarian steroids (estradiol, progesterone) by producing prostaglandin E_2 (PGE_2) and $F_{2\alpha}$ ($PGF_{2\alpha}$) (Szóstek *et al*. 2012a, 2014a). They mediate their action by involving specific receptors on mare endometrium, such as progesterone receptor (PGR), estrogen receptors 1 (ESR1) and 2 (ESR2), as well as oxytocin receptor (OXTR) (Rebordão *et al*. 2017a). Interestingly, in the mare oviduct these receptors are also present and are estrous cycle phase dependent. Whereas ESR1, ESR2 and PGR, were expressed in the oviduct epithelial cells, OXTR was shown in the stroma (Pinto-Bravo *et al*. 2017). The prostaglandin production by equine endometrial cells depends on prostaglandin endoperoxide synthase 2 (PTGS2), synthases of prostaglandin $F_{2\alpha}$ (PTGES, AKR1C3), PGE_2 (PTGES) and PGI2 (PTGIS) expression upregulation (Szóstek *et al*. 2014a; Rebordão *et al*. 2017a). Likewise, mare oviduct explants produced $PGF_{2\alpha}$ and PGE_2 when stimulated by OXT or ovarian steroids in all estrous cycle phases, even though to different extent (Pinto-Bravo *et al*. 2017). Moreover, we have found high expression of AKR1C3 and PTGES in mare oviduct epithelial cells (Pinto-Bravo *et al*. 2017). These data suggest coordinated physiological actions and mechanisms of steroid hormones and OXT in the equine oviduct and endometrium, which may be involved in the regulation of the estrous cycle and early pregnancy (Szóstek *et al*. 2012a, 2014a, Pinto-Bravo *et al*. 2017).

The endometrial endocrine environment by modifying the cross-talk between the different cells can modulate the type of response of this tissue to the same type of stimulus. An example is OXT that has a pro-luteolytic action during late-luteal phase, but induces luteostasis during mid-luteal phase (Rebordão *et al.* 2016; Vanderwall *et al.* 2016; Rebordão *et al.* 2017a). This later effect is the base for its use as a method to prolong luteal function in the mare (Vanderwall *et al.* 2016). Different endometrial spatial expression of OXTR and PGR and reduction of ESR2 may be a mechanism by which chronic OXT administration to mares in the mid-luteal phase enables luteal maintenance (Rebordão *et al.* 2017a).

Besides ovarian steroids and OXT, nitric oxide (NO) may modulate the well-developed regenerative capacities of the equine endometrium and prostaglandin secretion throughout the estrous cycle (Roberto da Costa *et al.* 2007b). There is considerable evidence that NO is an important regulatory molecule of a plethora of physiological functions in many cells including those in the reproductive system (Jaroszewski *et al.* 2001; Pinto *et al.* 2002). It is a powerful vasodilator and an essential mediator of angiogenesis, and contributes for the preservation of vascular tone, vessel integrity and function (Palmer *et al.* 1987; Snyder 1995; Murohara *et al.* 1999; Jaroszewski *et al.* 2001). The enzymes involved in NO synthesis, namely eNOS and iNOS, have been detected in the endometrium of cyclic and pregnant mares (Welter et al. 2004; Roberto da Costa *et al.* 2007b). Even though *in vitro* NO production by equine endometrial explants occurred in both follicular and luteal phases, it was in the luteal phase when it increased (Roberto da Costa *et al.* 2007b). This may suggest that NO plays some roles in both follicular and luteal phases in mare endometrium. Most likely, the increase in endometrial explant *in vitro* NO production in the luteal phase may be ascribed to the regulatory role of NO on endometrial prostaglandin production and blood flow (Roberto da Costa *et al.* 2007b).

3 Cytokines and Prostaglandins in Healthy Mare Endometrium

Cytokines play an essential role on physiological processes, such as cell renewal and tissue homeostasis. Specifically, in the mare reproductive tract, cytokines interferon gamma (IFNγ), Fas ligand (FASL), tumor necrosis factor alpha (TNFα), transforming growth factor beta (TGFβ), and interleukins (IL) have been related to luteal function (Galvão *et al.* 2013a); and interleukins (IL)-1β and 6 and TNFα also to the endometrial response to semen (Palm *et al.* 2008). In addition, TGFβ-1 plays a role on endometrial and/or trophoblast growth and differentiation during placentation (Lennard *et al.* 1995), while fibroblast growth factor-2 (FGF2) is expressed in the equine conceptus and endometrium of pregnant and cyclic mares (Ruijter-Villani *et al.* 2013).

We have shown that in mare endometrium, besides the coordinated action of ovarian steroid hormones, cytokines, such as IFNγ, TNFα, and FasL may regulate proliferative, angiogenic and secretory functions in mare endometrium (Galvão *et al.* 2013b). Cytokines TNFα, FASL and IFNγ, and their receptors are present in mare endometrium, and expressed differently according to estrous cycle phases. Thus, transcription of *TNFα* and *FasL* was mostly expressed in the early and late luteal phase endometrium, while *IFNγ* mRNA was up-regulated in the late luteal phase (Galvão *et al.* 2013b). With

regard to both T*NFα* receptors mRNA levels, they were increased in the mid luteal phase. Protein expression of TNFα and its receptor TNFRSF1A was up-regulated under the estrogen influence (follicular phase), but also under the progesterone influence (mid luteal phase). In the mid luteal phase, IFNγ and its receptor IFNR1 were also expressed, while in the late luteal phase the highest expression of FASL and FAS was noted (Galvão *et al*. 2013b). Immunohistochemistry studies have shown that all the cytokines studied and their receptors were expressed in endometrium stroma, as well as in surface and glandular epithelium (Galvão *et al*. 2013b).

In another *in vitro* study, TNFα stimulated PGE_2 production by equine endometrial explants to a larger extent than PGF2α secretion, which was mediated by up-regulation of PG synthases transcripts (Szóstek *et al*. 2014b). Also, TNFα stimulated NO production by equine endometrium explants *in vitro* (Galvão *et al*. 2013b). In the mare endometrium, a close cross-talk between TNFα and ovarian steroids, as well as its interaction with PG regulate physiological processes involved in reproduction (Szóstek *et al*. 2014b). Similarly, in the mare oviduct, TNFα also stimulates PGF2α and/or PGE_2 production from different portions of the oviduct (Pinto-Bravo *et al*. 2017). This cytokine has shown that mRNA levels and protein localization varied between the infundibulum, ampulla and isthmus of mare oviduct throughout the estrous cycle. This study suggests TNFα also plays a physiological role in the equine oviduct (Pinto-Bravo *et al*. 2017).

4 Cytokines and Prostaglandins in the Unhealthy Endometrium

In spite of the involvement of cytokines on a number of physiological processes, pro-inflammatory cytokines were reported in the equine unhealthy endometrium, from 24 h to day 7 after artificial insemination (Fumuso *et al*. 2003). In this study, the mRNA expression of pro-inflammatory cytokines *IL-1β, IL-6* and *TNFα* was up-regulated in mares susceptible to endometritis compared to resistant mares (Fumuso *et al*. 2003). However, in the initial immune response to semen, evaluated in the first 24 h after insemination, mRNA levels of *IL-6, IL-1 receptor antagonist* (*IL1RN*), and *IL10* were more expressed in the endometrium of resistant mares than in susceptible mares (Woodward *et al.* 2013). Therefore, the contrasting differences in the immune responses mounted in the endometrium of resistant and susceptible mares after insemination, either as an initial response (before 24 h) or as a later response (after 24 h until 7 days), may explain the pathogenesis of persistent post-breeding endometritis in susceptible mares (Fumuso *et al*. 2003; Woodward *et al.* 2013). In fact, there is a transient nature of inflammation in resistant mares, either in response to breeding (Fumuso *et al*. 2003; Woodward *et al*. 2013) or to endometritis, whereas susceptible mares develop a sustained inflammatory response with a prolonged gene transcription of pro-inflammatory cytokines *IL-1β, IL-8, IL-1 receptor antagonist* (*IL-1ra*) and *IL-1β:IL-1rα* ratio (Christoffersen *et al*. 2012). Also, experimental closure of the cervix in mares to induce a delayed uterine clearance had a profound effect on uterine inflammation, by stimulating IL-1β, IL-6, IL-10 and TNF-α accumulation in the uterine fluids (Reilas *et al*. 2016). In the endometrium of post-partum mares with retained fetal membranes, *IL-1β* mRNA transcription also increased, even though no change in the low expression of TNFα mRNA or protein was found (Jaworska and Janowski 2019). Therefore, these studies support the hypothesis

that an unbalanced endometrial gene expression of inflammatory cytokines might play an important role in the pathogenesis of persistent endometritis (Fumuso *et al*. 2003; Christoffersen *et al*. 2012; Woodward *et al*. 2013).

Since cytokines and prostaglandins appear to be differently modulated in equine endometritis, we have addressed their involvement in the course of endometrial fibrosis (endometrosis). In spite of dissimilar etiology and clinical symptoms, most chronic fibrotic conditions are triggered by a persistent noxious agent. It sustains the production of pro-fibrotic cytokines, angiogenic factors, growth factors and proteolytic enzymes, which modulate the destruction of normal tissue architecture and the deposition of connective tissue components (Tomasek *et al*. 2002). We have shown that in the course of endometrosis, mRNA transcripts of *PG synthases* and PG production are altered in mare endometrium, which might disrupt the estrous cycle and lead to early embryo loss (Szóstek *et al*. 2012b). Specifically, in category II endometrium, when endometrosis is moderate and insipient, and in category III endometrium, when endometrosis is fully established, *PTGS-2, PGFS* and *PGES* mRNA levels and PG production were altered when matched to the respective estrous cycle phases in category I healthy endometrium (Szóstek *et al*. 2012b). We have also shown that various degrees of inflammation and fibrosis differently influence gene expression of the pro-inflammatory cytokines *IL-1α* and *IL-1β* and their receptors (*IL-1RI, IL-1RII*) in the equine endometrium (Szóstek *et al*. 2013). In addition, interleukins appear to regulate prostaglandin secretion in mare endometrium, through prostaglandin synthases pathways. The alterations in endometrial secretory function mediated by interleukins in endometrosis, may suggest serious disturbance in endometrial microenvironment and be closely related to impaired endometrial processes responsible for subfertility or infertility in endometrosis (Szóstek *et al.* 2013).

The involvement of the cytokine Nodal, a member of TGFβ superfamily, was also evaluated on mare endometrosis (Morazzo *et al*. 2017; Morazzo *et al.* 2018). Our data suggest that Nodal may contribute for the establishment of endometrosis in the mare, by impairing the production of anti-fibrotic PGE_2 and the pro-fibrotic TGFβ1 signaling pathways and by increasing $PGF_{2\alpha}$ endometrial production (Morazzo *et al*. 2017). It appears that this pro-fibrotic effect of Nodal in mare endometrium is mainly noted in the luteal phase, but its increase in the follicular phase in category IIA endometria, mostly characterized by inflammatory lesions, may also contribute for fibrosis development (Morazzo *et al.* 2018). Therefore, in endometrosis, the affected areas of the endometrium might give up responding in a physiological manner to the control mechanisms acquiring specific pathogenic differentiation dynamics (Lehmann *et al*. 2011). Consequently, endometrosis makes the endometrium incapable of supporting the survival of the embryo, and/or implantation mechanisms leading ultimately to early pregnancy loss. In fact, this pathological condition has been considered as the main cause of infertility in the mare.

5 Fibrosis in Mare Endometrium and Oviduct

Endometrosis is the result of diverse insults that lead to degenerative changes in the uterine glands and the surrounding stroma, with deposition of extracellular matrix (Kenney and Doig 1986; Hoffmann *et al.* 2009). While there seems to be a relationship

between endometrial fibrosis and the fertility rate, the association between the development of endometrosis and chronic inflammation of the endometrium remains controversial (Hoffmann *et al.* 2009, Aresu *et al.* 2012; Reilas *et al.* 2016). Pro-fibrotic chemokines, cytokines and growth factors secreted by injured and inflammatory cells activate resident fibroblast leading to fibrogenesis. This paracrine form of cell signalling links inflammation to fibrosis (Liu Y 2011). Presence of bacteria or sperm in the mare uterus induces an inflow of neutrophils into the uterine lumen and the release of a number of inflammatory by-products (LeBlanc and Causey 2009). Inflammation persistence, due to deficient uterine clearance, increases the chance of cell damage (LeBlanc and Causey 2009). In response to specific bacteria that cause endometritis in the mare (Fig. 1) and in the dog, neutrophils are able to form neutrophil extracellular traps (NETs) and trap bacteria (Rebordão *et al*. 2014b; Rebordão *et al*. 2017b). NETs are DNA strands surrounded by various cytoplasmic and nuclear proteins that trap and/or kill bacteria and parasites (Brinkmann *et al.* 2004). In addition to neutrophil role as a first line of defense, the infiltration of these inflammatory cells in tissues affected by chronic inflammation, caused by either pathogens or environmental agents, may perpetuate tissue injury through NETs release, leading to fibroblast activation and fibrogenesis (Lögters *et al.* 2009; Chrysanthopoulou *et al*. 2014). Thus, we have hypothesized that NETs persistence can provoke a detrimental effect on the endometrium, acting as potential mediators of endometrial fibrosis and leading to collagen deposition. Through *in vitro* studies in which endometrial explants were incubated with different doses of proteases found in NETs (elastase-ELA, myeloperoxidase- MPO and cathepsin- CAT), an increase in the production of collagen type I (COL1), characteristic of fibrosis, was observed in the presence of ELA, regardless of endometrial category or estrous cycle phase (Rebordão *et al*. 2018a). However, the response to each specific protease depended on the estrous cycle phase and/or Kenney´s category of the endometrium. All proteases found in NETs enhanced the production of COL1 in the endometria obtained in the follicular phase, despite the presence or absence of pathological alterations. In contrast, in mid-luteal phase, only the highest doses of ELA and CAT caused an increase in collagen in tissues with minimum or mild lesions (Rebordão *et al*. 2018a). Increased collagen production by lung fibroblasts *in vitro* challenged with NETs has also been referred (Chrysanthopoulou *et al*. 2014).

Prostaglandins pathways are among the multiple possible mechanisms implicated in the extremely complex pathogenesis of fibrogenesis in several organs other than the uterus, in human species. Signaling through its receptors 2 (EP2) and 4 (EP4), PGE_2 is considered an anti-fibrotic mediator (Huang *et al.* 2010; Wang *et al.* 2017). In contrast, $PGF_{2\alpha}$ upon signaling through their respective receptor (FP) mediates fibrotic actions (Olman 2009). Since the endometrium is an organ where PGs are profusely produced, also using *in vitro* studies with proteases found in NETs, prostaglandin pathways were considered as potential mediators of the development of endometrial fibrosis in the mare, due to the persistence of NETs. The uterine response to proteases found in NETs varied with the stage of the estrous cycle or endometrial type. Our data suggest that PGE_2 may protect against mare endometrosis, through its receptor EP2, but not EP4. Impairment of PGE_2 production or EP2 was associated with increased endometrial collagen deposition in tissues obtained in the follicular phase and with moderate to intense degenerative lesions after challenge with proteases found in NETs. In tissues

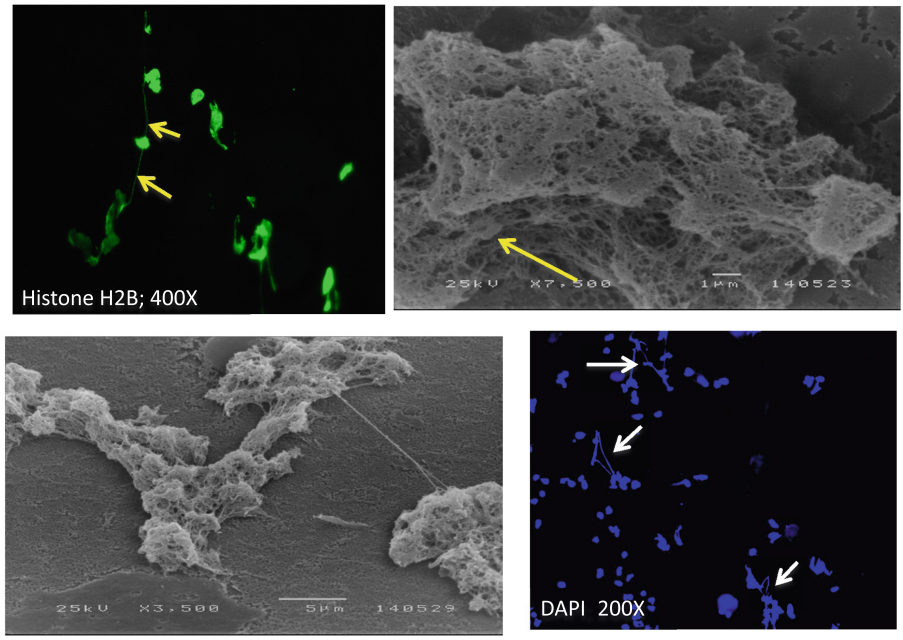

Fig. 1. Ex vivo NETs visualized in the uterus of mares with *E. coli* and *S. equi* spp *zooepidemicus* endometritis.

obtained in mid-luteal phase, decreased PGE_2 production was also observed, regardless of endometrial category. By decreasing the levels of either PGE_2 or EP2 in equine endometrial tissues, PGE_2 can no longer exert its anti-fibrotic functions (Rebordão *et al.* 2019). Increased $PGF_{2\alpha}$ and COL1 production by equine endometrium challenged with ELA or CAT was only detected in tissues obtained in the follicular phase and without or with slight pathological lesions. However, enhanced production of COL1 and $PGF_{2\alpha}$-FP receptor *(FP)* transcripts was observed in all endometrial categories obtained in the follicular phase after CAT treatment. It was also noted an increase in *FP* transcripts in endometria with moderate to intense lesions obtained in the mid-luteal phase. Both conditions may stimulate the development of fibrosis in the endometrium of the mare (Rebordão *et al.* 2018b). In addition, mare endometrial explants obtained in follicular phase challenged with ELA *in vitro*, showed enhanced *COL1* transcripts and $PGF_{2\alpha}$ production. Sivelestat, an ELA inhibitor, was able to reduce *COL1* transcripts and $PGF_{2\alpha}$ production and enhance PGE_2 output. Thus, inhibition of proteases found in NETs may be a putative therapeutic means to inhibit fibrotic changes in mares with persistent endometritis (Amaral *et al.* 2018).

Once mare infertility has been associated to recurrent endometritis and endometrosis, we have investigated collagen gene expression and deposition in the oviduct with respect to aging and endometrial fibrosis (Pinto-Bravo *et al.* 2018). Even though to different extents, COL1 and COL3 were present in all portions of the oviduct (infundibulum, ampulla, isthmus) and also in the endometrium in all mares, regardless of their age (Fig. 2) (Pinto-Bravo *et al.* 2018). However, in the youngest mares, from 4 to 8 years of

age, transcription of *COL3* was increased, when compared to older mares (10 years old and over). This up-regulation of *COL3* transcripts and COL3 protein in young mares oviduct occurred mainly in the infundibulum and isthmus. The fact that with aging, COL1 and COL3 increase both in the equine endometrium and oviduct, potential information on oviduct fibrosis may be inferred from endometrium biopsy histopathological evaluation, since oviduct biopsy for diagnostic purposes is not feasible (Pinto-Bravo *et al.* 2018).

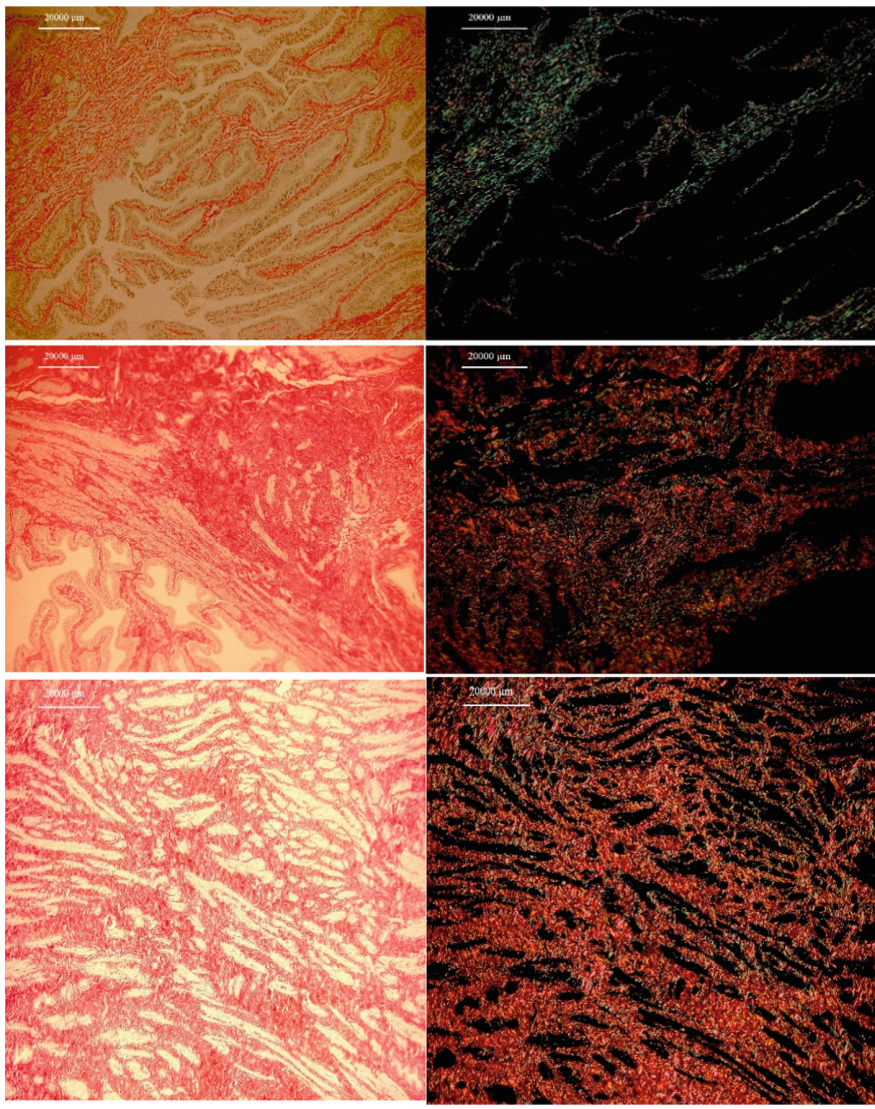

Fig. 2. Mare oviduct (a – infundibulum; b – ampulla; c – isthmus) stained with picrosirius red and observed and photographed under light microscopy (on the left) and by polarized light microscopy (Leica Leitz DMRD; Mag = 100X). COL1 stained red, while COL3 fibers stained green.

Identifying the cellular and molecular mechanisms behind the healthy equine endometrium is essential to understand the processes that lead to the impairment of mare's reproductive function. Thus, it is important to unravel the complex interactions between the different mediators (hormones, cytokines, growth factors, enzymes) not only produced by the cells within each reproductive organ, but also the crosstalk between the ovaries, oviducts and the uterus. This novel knowledge will allow the discovery of potential molecules to be used as prophylaxis and/or therapeutic measures to overcome endometrial fibrosis in the mare.

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Pregnancy Toxaemia in Small Ruminants

P. B. A. Simões, R. Bexiga, L. P. Lamas, and M. S. Lima(✉)

Clinical Research Laboratory, Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal
mlmslima@fmv.ulisboa.pt

Abstract. Pregnancy toxaemia (PT) is a condition occurring in ewes and does during the last month of gestation. It is characterized by anorexia, ruminal atony, depression, and reluctance to stand or walk. This condition carries a poor prognosis with a reported case fatality rate above 80% even when a caesarean section is performed or after the induction of kidding. Metabolically, PT leads to ketonemia, metabolic acidosis, hypokalaemia and hypo, normo or hyperglycaemia. This condition is caused by negative energy balance resulting from increased energy demands for rapid foetal growth during late gestation. In pregnant ewes low quality roughage is a particular risk because not enough can be consumed to meet requirements when the rumen volume is reduced by the presence of an enlarged uterus in the abdomen. PT occurs commonly in dairy goats especially in specific breeds, like Saanen and Alpine, which are genetically more prone to become pregnant with multiple foetuses. Goats are considered to be tropical/sub-tropical animals and their body fat stores are laid down in intraabdominal fat rather than in the subcutaneous tissues as occurs in cows and ewes. Because of high mortality rate characteristic of PT, the strategy to deal with this condition should be based on prevention.

Keywords: Pregnancy toxaemia · Small ruminants · Metabolic acidosis · Ketonemia · High mortality rate · Prevention

1 Definition and Aetiology

Pregnancy toxaemia, also known as ketosis or twin-lamb disease is a condition occurring in ewes and does in the last two to four weeks of gestation. It is characterized by anorexia, ruminal atony, preference for recumbency with reluctance to stand or walk, depression and poor prognosis with a case fatality rate above 80% even when a caesarean section is performed or after the induction of parturition (Smith and Sherman 2009; Constable *et al.* 2017). The condition is caused by negative energy balance resulting from increased energy demands for rapid foetal growth in late gestation and insufficient intake (Pearson and Maas 1996). In pregnant females low quality roughage is a particular risk because not enough can be consumed to meet requirements when the volume of the rumen is reduced by the presence of an enlarged uterus in the abdomen (Smith and Sherman 2009; Constable *et al*. 2017). Pregnancy toxaemia occurs more commonly in specific dairy

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breeds of goats, such as Saanen and Alpine, which are genetically more prone to become pregnant with multiple foetuses (Smith and Sherman 2009). Similar to tropical/subtropical animals, goat's body fat stores are laid down not in subcutaneous tissue but in the omentum, mesentery and around the kidneys (Harwood 2006). So, obese pregnant goats might not be clearly detectable and are at greatest risk of developing pregnancy toxaemia when pregnant with twins or triplets.

2 Pathophysiology

The central metabolic events of pregnancy toxaemia are fat mobilization and the availability of glucose (Smith and Sherman 2009). The blood sugar level is fairly constant in a given animal when variations due to circadian rhythm, breed, sex, nutrition and stress of handling are taken into account (Clarenburg 1992), but large differences exist between different animal species. Whereas in humans and other monogastrics, glucose levels of about 90 mg/dL are common (rising after a meal to 120 to 145 mg/dL and falling between meals to 80 mg/dL), in ruminants blood glucose levels are usually much lower, approximately 36 to 60 mg/dL (Clarenburg 1992; Constable *et al*. 2017). Regulating the blood glucose level is a complex function, under the general control of the neuroendocrine system, in which the liver plays a key role (Fig. 1). Nearly 80% of the foetal growth occurs during the last six weeks of gestation (Rook 2000). During the final month of gestation, the energy requirement of a pregnant ewe or doe carrying twins or triplets is 180% or 240% greater, respectively, than that of an ewe/doe with a single foetus (Navarre *et al*. 2012).

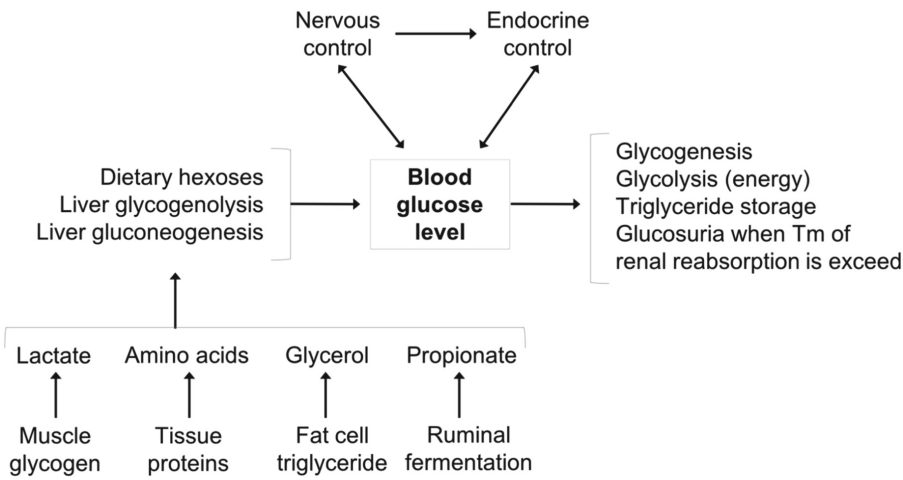

Fig. 1. Regulation of the blood glucose in ruminants (adapted from Clarenburgh 1992).

The ovine/caprine foetus has two mechanisms to insure survival and continued growth at the expense of the dam. First, the placenta can transfer glucose to the foetus, even in the presence of very low maternal blood glucose concentrations. This is

because the foetus maintains a very low plasma glucose concentration (about 8 mg/dL), which facilitates transfer of glucose from maternal plasma. Secondly, the foetus maintains a relatively high plasma fructose concentration (80–100 mg/dL), which functions as a carbohydrate reserve for foetal tissues. This fructose is synthesized entirely from glucose by the placenta and cannot readily pass back through the placenta into the maternal circulation. However, even though fructose is more abundant than glucose in the plasma of foetuses, the foetal tissues use (approximately) twice as much glucose (Bergman 1993).

In obese pregnant females, namely goats, the intra-abdominal cavity becomes filled with accumulated fat and an ever-expanding uterus during late gestation. This reduces space available for ruminal expansion, therefore these animals are unable to consume sufficient quantities of feedstuffs to satisfy energy requirements, aggravating the negative energy balance. During these periods of negative energy balance, blood glucose may drop slightly, the insulin:glucagon ratio drops and these and other hormones (catecholamines, growth hormone) activate hormone sensitive lipases that convert tissue fat to non-esterified fatty acids (NEFAs) and glycerol. In the liver the glycerol may be used to produce glucose or be recombined with NEFAs to make triglycerides. In addition to being recombined with glycerol to make triglycerides, the NEFAs may be degraded through beta-oxidation, and the two carbon fatty acids converted to acetyl-CoA. The acetyl-CoA combines with oxaloacetate to enter the tricarboxylic acid cycle for the production of energy. If there is not enough oxaloacetate available, the acetyl-CoA is converted to ketone bodies, which in high concentration can reduce appetite and perpetuate the negative energy balance (Pearson and Maas 1996).

When the liver is overwhelmed with mobilized NEFAs, greater amounts of triglycerides are deposited within the hepatocytes. These triglycerides eventually leave the liver as Very Low Density Lipoproteins (VLDLs), which are plasma soluble complexes of phospholipid, cholesterol, triglycerides and apolipoprotein A. Hepatic lipidosis results when the rate of hepatic triglyceride formation exceeds oxidation of fatty acids, formation and release of VLDLs into the peripheral circulation (Pearson and Maas 1996). Ruminants have a decreased ability to secrete triglycerides as VLDLs from the liver, particularly in animals with hepatic lipidosis (Pearson and Maas 1996). Fatty liver, increased abdominal fat and a small rumen are consistent necropsy findings in does that died or were euthanized due to pregnancy toxaemia (Lima *et al*. unpublished data).

Many studies involving pregnancy toxaemia in both, ewes and goats, are based on a starvation model (González *et al*. 2012; Cal-Pereyra *et al.* 2015). This model is not comparable to that of spontaneous pregnancy toxaemia, because in spontaneous cases a lack of energy and feed intake is not necessarily the predominant pathogenic factor (Henze *et al*. 1998). Therefore the conclusions drawn from these studies should be interpreted with caution as they may not be directly applicable to field conditions.

3 Clinical Signs

The early signs of pregnancy toxaemia are usually mild. In goats (but not ewes) there is often a noticeable subcutaneous oedema of the hindlimbs (which can also occur in the forelimbs) (Fig. 2). The most common clinical signs are anorexia, ruminal atony,

polypnea, and a preference for recumbency. In the most severe cases, animals can exhibit neurologic signs and drooped ears. Blindness is a common sign in ewes but not in goats. In the authors' experience in over 20 years only one goat with pregnancy toxaemia presented blindness (Lima *et al*. 2012a). The most frequently observed neurologic clinical sign in goats is stargazing (Fig. 3). Some of the goats vocalize. In goats, as the condition progresses, faeces become reduced and more fluid (Lima *et al*. 2012a). A recent study tried to correlate the clinical signs with the outcome. In this study 100% of the goats with neurologic signs or drooped ears died. In addition, 94% of the goats with anorexia, 90% of the recumbent goats, 78% of the goats showing polypnea and 72% of the goats with swollen limbs also died (Lima *et al*. 2016b). In ewes the course of the untreated disease varies from 12 h to one week (Smith and Sherman 2009), while in goats 50% of the females have been shown to die in the first 24 h even with very aggressive treatments (Lima *et al.* 2012a).

Fig. 2. Goat with pregnancy toxaemia showing subcutaneous oedema of the forelimbs.

In sheep the earliest signs are separation from the group, failure to come up for feeding in pastoral animals, or standing near the trough with the remainder of the flock but not eating, while in housed animals, altered mental state and apparent blindness which is manifested by an alert bearing but a disinclination to move (Smith and Sherman 2009). In later stages, marked drowsiness develops and episodes of more severe nervous signs occur but they may be infrequent and are easily missed. Convulsions are frequently observed. Affected ewes usually become recumbent in 3–4 days and remain in a state

Fig. 3. Goat with pregnancy toxaemia showing stargazing.

of profound depression or coma for a further 3–4 days, although the clinical course is shorter in fat ewes with pregnancy toxaemia (Smith and Sherman 2009). Affected ewes commonly have difficulty in lambing (Constable *et al*. 2017). In both ewes and does the rectal temperature is within the reference range but the heart rate and respiratory rate are above the reference range (Smith and Sherman 2009; Lima *et al*. 2012a).

Goats with pregnancy toxaemia that do not die tend to have dystocia and higher kid mortality (Smith and Sherman 2009). In pregnant goats, maternal ketoacidosis due to pregnancy toxaemia has a negative impact on the survival rate of the offspring. It has been observed that the percentage of kids alive from natural kidding was 100% (14 out of 14) while the number of kids that survived after a caesarean surgery being performed in pregnancy toxaemia goats and remained alive after seven days was only 64% (16 out of 25) (Andrade *et al*. 2017).

4 Clinical Pathology

Pregnancy toxaemia is characterized metabolically by ketonemia, metabolic acidosis, hypokalaemia and hypo, normo or hyperglycaemia (Marteniuk and Herdt 1988; Constable *et al.* 2017). In the field an easy way to confirm a suspicion of pregnancy toxaemia is to collect urine and measure the ketone bodies with a dipstick. Ketones are detectable in urine with the nitroprusside test (Rothera's test). The nitroprusside test is highly sensitive to acetoacetate, slightly sensitive to acetone and insensitive to beta-hydroxybutyrate

(BHB). In ruminants the ratio of BHB to acetoacetate is normally greater than 10:1, so the concentration of ketone bodies determined by the urine dipstick is always under-valued (Clarenburgh 1992). A more correct strategy consists in measuring BHB in the blood, which in pregnancy toxaemia goats should be above 3 mmol/L (Smith and Sherman, 2009; Lima *et al*. 2016a). Sheep can be induced to urinate by temporarily closing off their nostrils. A doe that has been down often urinates when forced to stand up (East 1983). Ketone bodies (BHB and acetoacetate) are strong acids (Andrews 1997; Navarre *et al*. 2012), and their accumulation in the blood leads to metabolic acidosis (ketoacidosis), indicated by decreased blood pH, HCO_3^- and base excess (BE). Ketonuria, aciduria and metabolic acidosis are associated with an increased severity of the condition (Lima *et al*. 2012a).

Changes in the pH of the extracellular fluid produces reciprocal H^+ and K^+ shifts between the cells and the extracellular fluid. As a result, K^+ tends to move into the cells with alkalemia and out of the cells with acidemia. These pH-induced effects, however, are transient and frequently overridden by concurrent variations in other mechanisms that influence K^+ transport (Rose and Post 2001). Hypokalaemia has been described in humans with liver failure, including acute fatty liver during pregnancy, and attributed to the marked loss of K^+ in the urine of patients with ketoacidosis and ketonuria (Rose and Post 2001). Hypokalaemia has also been observed in some goats (Lima *et al*. 2012a), which can be partially explained by inadequate feed intake.

Glucose blood levels in pregnant goats affected with pregnancy toxaemia varies dramatically (Lima *et al*. 2012b). Hypoglycaemia might indicate that the foetuses are alive and hyperglycaemia that the foetuses are dead (Lima *et al*. 2012b). Therefore, the assessment of glucose levels in field conditions using a glucometer would be an useful quick test for practitioners in order to evaluate the status of the foetuses. The situation can progress to an irreversible stage, where there is dehydration and increased blood urea nitrogen (BUN) values (Navarre *et al*. 2012). According to some authors, this increase in BUN can be caused by increased protein catabolism, by decomposing foetuses or by terminal kidney failure (Andrews 1997; Constable *et al*. 2017). As previously referred, does with pregnancy toxaemia tend to have higher kid mortality (Smith and Sherman 2009). This negative impact on offspring survival rate is due to maternal ketoacidosis and appears to be associated to metabolic acidosis (lactic acidosis) (Gomez *et al*. 2013). Recently, Andrade *et al*. (2017) compared the short-term survival rate of newborn kids born from pregnancy toxaemia goats following caesarean section with kids born by natural delivery from healthy goats. Blood parameters were measured in the immediate post-partum period in order to understand differences between these two groups of kids and identify those that could be used as prognostic indicators of survival. Kids delivered by natural kidding ($n = 14$) and kids delivered by caesarean section ($n = 25$) presented significant differences in blood levels of Na^+ (140 vs. 137 mmol/L), HCO_3^- (24 vs. 19 mmol/L), BUN (17 vs. 20 mg/dl) and L-Lactate (3.3 vs. 5.8 mmol/L), as well as different BE (-2 vs. -10 mmol/L) and pH (7.30 vs. 7.18) (Andrade *et al*. 2017).

5 Prognosis

Once pregnancy toxaemia is diagnosed the prognosis is often poor (Lima *et al*. 2012a; Zobe *et al*. 2015). Case fatality is very high in both sheep and goats (Smith and Sherman

2009; Constable *et al*. 2017). Lima *et al.* (2012a) reported a case fatality rate of 86% in goats and a correlation between clinical signs and blood parameters with the prognosis. The clinical signs most indicative of a poor prognosis are drooped ears, neurologic signs, namely stargazing, anorexia with absence of ruminal motility and recumbency (Lima *et al*. 2016b). The prognosis for goats with polypnea and swollen limbs is more favourable (Lima *et al.* 2016b). From blood parameters, hypokalaemia and metabolic acidosis are the most relevant, i.e. mortality rate of pregnancy toxaemia goats with a blood pH below 7.12 was 100% (Lima *et al*. 2012a). However, the interpretation of the blood values requires some caution because the condition of a pregnancy toxaemia goat can deteriorate very fast.

In Lima *et al*. (2012a) blood glucose in pregnancy toxaemia goats was generally below the reference range (50–75 mg/dL). However, it was detected a dramatic increase of glucose levels after the death of the foetuses, *e.g.* glucose levels of 215 mg/dL. The mortality rate for these goats was 100% (Lima *et al*. 2012b).

Serum BHB concentrations above 3 mmol/L have been associated with a poor prognosis in sheep (Scott and Woodman 1993), in contrast with the authors' experience, where no relevance of blood values for the prognosis of pregnancy toxaemia in goats was found (Lima *et al*. 2016b). The unreliability of a BHB cut-off value (>2.9 mmol/L) as indicator of a likely worse prognosis was suggested by the lack of statistically significance of the correlation coefficient, of both blood BHB and pH (Lima *et al*. 2016b) (Fig. 4). It remains to be investigated what other factors contribute to the metabolic acidosis (besides BHB).

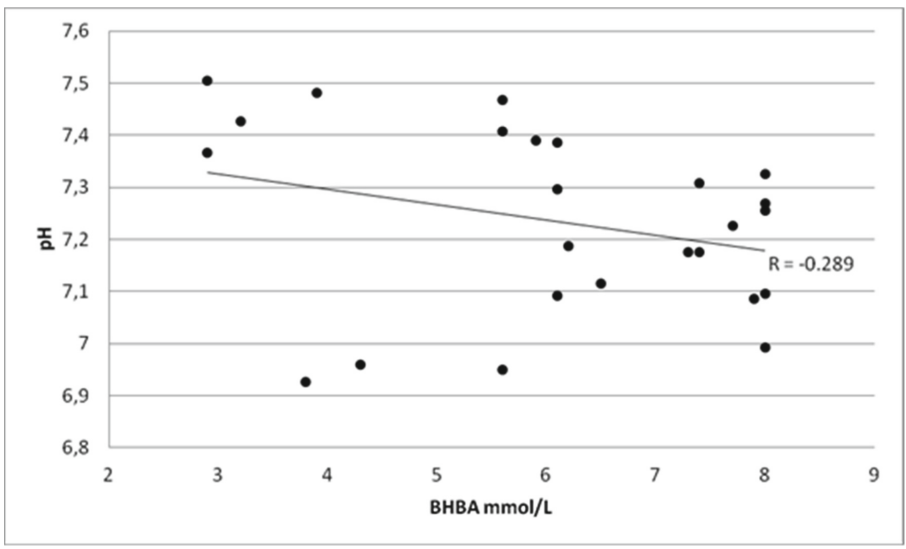

Fig. 4. Blood pH and beta hydroxybutyrate acid (BHB) values in 25 does with clinical signs of pregnancy toxaemia and BHB values > 2.9 mmol/L (Lima et al., 2016b).

6 Treatment

The diagnosis of pregnancy toxaemia in ewes or goats requires a prompt decision regarding the type of intervention - medical treatment, induction of kidding or caesarean section in the most severe cases (Lima *et al*. 2012a). That decision should be made on the assumption that the rate of mortality is always very high, above 80%, unless establishment of the treatment is initiated very early (Lima *et al*. 2012a).

Early intervention may have a favourable result but the response to treatment is usually poor, as the disease becomes irreversible in the later stages. To achieve the best results, blood chemistry and electrolyte analysis should be performed and the animal treated accordingly (Bulgin 2005). The analysis should be performed as soon as possible in the field (point of care laboratory tests), with reliable, fast and affordable tests. In the authors' experience they are critical in situations of management of pregnancy toxaemia.

Treatment of pregnancy toxaemia involves increasing the energy and glucose supply to the animal and correcting secondary abnormalities such as acidosis and dehydration (Marteniuk and Herdt 1988).

It is important to encourage the goat to continue eating (Clarkson 2000). Goats that continue eating may survive, totally anorexic animals will generally die (Lima *et al*. 2016a). In the authors' experience, palatable food, such as green grass, good quality hay and concentrate in pellets yield the best results, as referred by Clarkson (2000). Force feeding could be an option (Smith and Sherman 2009), however, in the authors' experience it is relatively easy in ewes but much more difficult in goats, as they tend to discard the food introduced in their mouth.

There are several supportive medical therapy protocols described in the literature. Lima *et al*. (2012a) study aimed to establish the efficacy of six treatment options for goats' pregnancy toxaemia:

Treatment No. 1: 30 g of a dry, commercial oral electrolyte product containing 70% glucose, 15% sodium chloride, 12% sodium bicarbonate (and vitamins C and E) dissolved in 1.5 to 2.0 L of water and administered orally, at 12 to 24 h intervals.

Treatment No. 2: Propylene glycol administered orally, in a dose of 50 ml, and repeated at 12-h intervals. (its use was limited only to PT does in which ruminal contractions were present).

Treatment No. 3: 5% glucose in water administered intravenously in a dose of 0.5 L (0.53 quarts), repeated at 12-h intervals.

Treatment No. 4: 20% calcium borogluconate solution administered subcutaneously in a dose of 50 ml, once daily.

Treatment No. 5: Flunixin meglumine administered intravenously in a dose of 2.5 mg/kg, once daily (its use was limited only to pregnancy toxaemia does that had swollen limbs).

Treatment No. 6: Isotonic sodium bicarbonate solution administered intravenously, accordingly to the base deficit.

However, 19 of the 22 clinical cases reported in that study died. Therefore, the relative efficacy of these treatments could not be established.

The administration of isotonic or hypertonic glucose has been recommended by several authors (Constable *et al*. 2017; Mathews 2016). Van Saun (2000) reported a good clinical response to treatment of pregnancy toxaemia ewes after a calcium, phosphorus and dextrose solution (30 to 50 ml, one single treatment) administered intravenously. A constant drip of 5% intravenous glucose is ideal but may not be practical (Bulgin 2005). Yet, the therapeutic effect of an intravenous infusion has been criticized by other authors (Henze *et al*. 1998; Duehlmeier *et al.* 2013; Lima *et al.* 2016aa). The main criticism to the therapy with glucose relies on the fact that the increase in glycaemia resultant from the infusion disappears one hour after the intravenous administration, being insufficient to suppress the demand that can continue for a few days or weeks. Furthermore, glucose intolerance has been shown to occur in animals with pregnancy toxaemia (Fig. 5) either in ewes (Henze *et al*. 1998; Duehlmeier *et al.* 2013) or goats (Lima *et al.* 2016b) which can be associated with fatty liver (Lima *et al*. 2016b). If the blood glucose level is not measured before treatment, a glucose infusion may even provoke death by hyperglycaemic shock in already hyperglycaemic ketotic ewes (Henze *et al*. 1998).

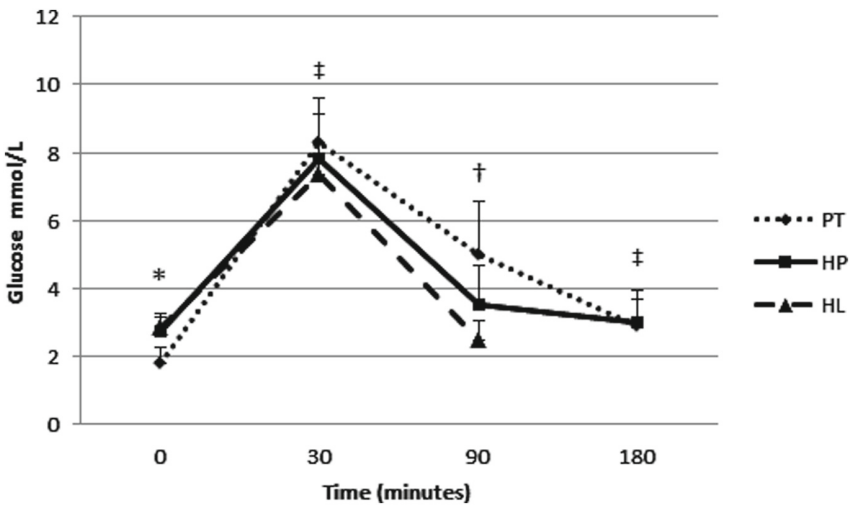

Fig. 5. Blood glucose concentrations (mmol/L) in 8 Pregnancy Toxaemia Does (PT), 8 Healthy Pregnant Non-lactating Does (HP) and 8 Healthy Lactating Non-pregnant Does (HL), before and after IV administration of 0.5 L of a 5% glucose solution (Lima et al. 2016b) (* differences between groups $p < 0.01$ (0 min); † differences between groups $p < 0.05$ (90 min, PT vs HP), $p < 0.001$ (90 min, PT vs HL); ‡ differences within the groups when compared with the baseline $p < 0.05$ (0 min))

Propylene glycol administered orally has been advised by many authors as one of the treatments of choice to treat both ewes and does with pregnancy toxaemia (Smith

and Sherman 2009; Lima *et al*. 2012a). This compound is partially converted into propionate in the rumen (Nielsen and Ingvartson 2004). The remaining propylene glycol is absorbed directly from the rumen without alteration (Nielsen and Ingvartson 2004) and is metabolized like propionate or lactate in the liver because of the similarity in molecular structure (Fig. 6). Thus, propylene glycol would provide additional 3 - carbon units for hepatic gluconeogenesis (Bergman 1993). Smith and Sherman (2009) suggest the dosage of 60 ml, two or three times daily, whereas (Mathews 2016) suggests as much as 200 ml of propylene glycol twice a day. This dosage seems excessive and likely to overwhelm the liver capacity to metabolize it. Overdoses of propylene glycol can be fatal, creating plasma hyperosmolarity that impairs neurologic function (Smith and Sherman 2009). In situations in which ewes or goats have absence of rumen motility or fatty liver (both clinical signs occur in pregnancy toxaemia), the administration of propylene glycol should be avoided.

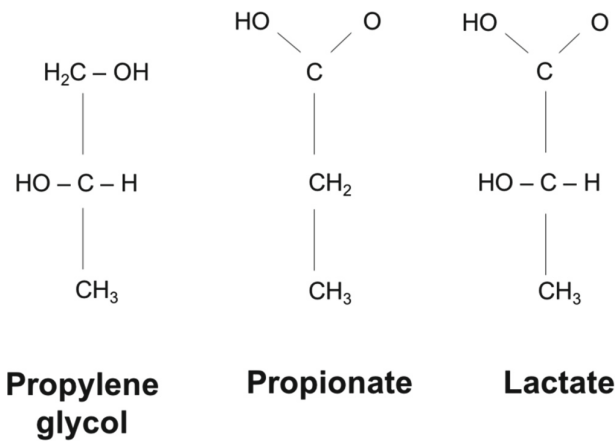

Fig. 6. Similarities of glucose precursors.

The administration of glycerol, 60 ml in warm water orally, twice daily for 4 or 5 days, can be a good alternative to propylene glycol (Mathews 2016). Glycerol is more palatable than propylene glycol (Mathews 2016) and is fermented in the rumen to volatile fatty acids, namely propionate (Kay 1983).

Some authors recommend the administration of bicarbonate intravenously to correct the acidosis (Smith and Sherman 2009; Mathews 2016). In the authors' experience in goats this practice has some risks. The administration of bicarbonate to these goats can cause a very dramatic hypokalaemia which can accelerate the death of the goat (Lima, unpublished data).

Zamir *et al*. (2009) recommend the administration of flunixin meglumine to ewes affected with pregnancy toxaemia. Ewes that had been depressed and reluctant to move, walked and showed almost normal eating behaviour. In the authors' studies with does the improvement was not observed (Lima *et al*. 2012a).

Insulin has been used in conjunction with other treatments to reduce ketogenesis and fatty acid mobilization (Marteniuk and Herdt 1998). However, its administration to small ruminant females with pregnancy toxaemia is uneconomic (Sargison 2007).

The decision adopted by the authors regarding whether to perform a caesarean section or induce parturition is based on clinical signs and blood pH. In goats with clinical signs, like drooped ears or neurologic signs, caesarean section is the option. When blood pH is greater than 7.15 the option is to induce parturition; when blood pH is less than 7.15, caesarean section is the option (Lima *et al*. 2012a). This strategy has some risks, because pH can drop very fast especially in goats that carry triplets or even quadruples in which case the doe may die before parturition occurs (Lima *et al*. 2012a). In the authors' experience the rate of metritis in goats in which kidding was induced using dexamethasone (1 mg/10 kg BW, IM) and dexcloprostenol (125 μL, IM) is very high, leading to a mortality rate also very high (Lima, unpublished data).

The decision taken by the veterinarian to perform a caesarean section or induce parturition has to take into account the advantages and disadvantages of both methods. The viability of the neonate is thought to be dangerously reduced at a point under 95% of the length of normal gestation. In a gestation of 156 days, induction of kidding or performing a caesarean operation earlier than 8 days before the expected parturition date may result in reduced kid viability. Unless the exact day in which the doe was bred is known it is impossible to determine the age of the foetus(es) (Smith and Sherman 2009). Performing a caesarean section has a cost which the farmer is not always willing to pay (Lima *et al*. 2012a). As a rule, the authors always measure the glycaemia of the pregnant female with pregnancy toxaemia to assess the chances of the foetuses being alive. As mentioned before, when the glycaemia is low (20 to 45 mg/dL) there is a very good chance that the foetus(es) are alive; if the blood glucose levels go up, most likely the foetus(es) are already dead (Lima *et al*. 2012b).

Transabdominal ultrasound examination of the uterus for evidence of foetal movement or heartbeat is an option to verify that the foetuses are still alive (Smith and Sherman 2009). In many cases, humane euthanasia should be considered to prevent further suffering whenever the prognosis is poor. In the authors' experience with goats this should be the option when blood pH of the pregnancy toxaemia doe is below 7.15 or when the blood glucose is well above reference range values (Lima *et al*. 2012a, b).

To sum up, treatment is often not successful (Marteniuk and Herdt 1998). Many different therapeutic approaches have been taken, often with confusing unpredictable results (Marteniuk and Herdt 1998).

7 Prevention

When pregnancy toxaemia is diagnosed, the emphasis of the veterinarian should be on prevention of further cases in the flock. According to Marteniuk and Herdt (1998), preventive measures are fairly simple and can be very effective but require an understanding of the nutrient requirements of ewes and does throughout the cycle of gestation and lactation

The success of controlling an outbreak of pregnancy toxaemia in dairy goats is based on two principles:

a) avoid obese goats, this occurs mainly with low producing goats
b) decrease mobilization of fat in the last month of gestation (Chamberlain 2015)

Animals carrying multiple foetuses cannot meet their nutrient requirements even on the best quality rations and so some weight loss will occur and should be rationed. As an example, a doe in late pregnancy in the last week to term and carrying two kids can loose 140 g a day and a doe carrying 3 kids can loose 180 g per day (Chamberlain 2015).

Body condition score (BCS) can be an important indicator of does nutritional status. Ideally, does should be dried-off and freshen with a body condition score of 3.5 to 4 (on a 5-point scale). Late gestational does with minimal body stores (2.5 or below) are at increased risk of clinical ketosis due to insufficient energy reserves to meet the late gestational mismatch between energy demands and dry matter intake. Does with excessive body fat (4.5 or greater) have increased risk of compromise from hepatic lipidosis (fatty liver) as well as the reduced abdominal capacity (limiting feed intake) from accumulation of omental fat (Rowe 2014). However, according to some other authors (Harwood 2006; Chamberlain 2015), body condition scoring can be misleading in goats. Goats are considered to be tropical/sub-tropical animals and body fat stores that develop are laid down not in subcutaneous tissue but in the omentum, mesentery and around the kidneys (Fig. 7) (Harwood 2006; Chamberlain 2015).

Fig. 7. Abdominal fat observed at necropsy in a goat that died from pregnancy toxaemia.

The latter perspective seems to corroborate the observation of one of the authors that have conducted a study involving 90 obese does in the dry period (BCS > 4) in order to identify the ones at risk based on BCS, in which only three goats developed pregnancy toxaemia (Lima, unpublished data). Because it is difficult to control the amount of fat in the abdominal cavity, it would be more efficient to separate the pregnant females into different feeding groups based on the number of foetuses carried (Sargison 2007). The utilization of ultrasound pregnancy diagnosis to confirm pregnancy and identify the number of foetuses that a pregnant female carries can be an important tool. Foetal counting is more accurate when performed early in gestation (40 to 75 days) (Rowe 2014).

Exercise in a daily basis should be encouraged (Mathews 2016). Forced exercise by enlarging the distance between feed and water, or making the goats walk to the milking parlour every day in the dry period will utilize some of the excess NEFAs and thus may be helpful (Bulgin 2005). Timid does and slow eaters should be housed separate from dominant, aggressive animals that might drive them away from the feeder (Smith and Sherman 2009)

In the authors' experience with milking goats most of the clinical cases of pregnancy toxaemia occurs with goats that are dried off. The prevalence of pregnancy toxaemia among goats that are milked until they give birth is much lower. In dairy cows it was postulated that a possible explanation for this is the fact that NEFA are eliminated in the milk and do not accumulate in the liver (Drackley 1999). Zobel *et al.* (2015) study showed that when goats were managed without dry period, more animals remained healthy compared with those that were dried off. Thus, reducing or eliminating dry periods in dairy goats can be a promising management practice in order to reduce the incidence and prevalence of pregnancy toxaemia.

Rumen protected choline added to rations during the transition period appears beneficial in preventing and treating fatty liver in dairy cows. This compound seems to reduce the accumulation of triglycerides and clear mobilized fat in the liver. They may also have a role to play in the dairy goat and there is some experimental support for the ability of rumen protected choline to reduce plasma BHB levels and hepatocellular accumulation in the peripartum period and in increasing milk production, fat and protein yield but further work is required. One problem is that this compound is expensive and most likely many farmers are not willing to pay for it (Mathews 2016).

Feeding niacin daily has also been recommended to reduce the risk of pregnancy toxaemia by reducing fatty acid mobilization (Smith and Sherman 2009; Mathews 2016). Practical research documenting a beneficial effect of feeding niacin appears to be lacking in goats, but the practice does not appear to carry any risk (Smith and Sherman 2009).

At the moment, educating farmers to recognize management factors that contribute to pregnancy toxaemia, and helping them with strategies that optimize observations and practices that minimize risk factors, seems to be the best approach to the disease and can significantly reduce the incidence and mortality from this economically important disease (Rowe 2014).

8 Conclusions

Pregnancy toxaemia is one of the most common diseases in sheep and goats occurring in the last month of gestation. In dairy goats, the prognosis is very poor unless the diagnosis is established in the early phases of the disease. However, the condition of the affected animals can deteriorate very fast, especially in pregnant females carrying multiple foetuses. Therefore, these animals should be monitored carefully (clinical signs and blood parameters such as pH, BHB and glucose), and medical treatments should be implemented early. Induction of parturition should be the option for the least severe cases, while a caesarean section should be performed in the most severe cases.

Because of the high mortality due to pregnancy toxaemia the strategy to deal with this condition should be based on prevention.

There appears to be differences between goats and ewes with pregnancy toxaemia concerning the pathophysiology, clinical signs and the response to several treatments. These differences should be investigated.

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Historical Note

CIISA: A 25 Year Old Young Adult… or… A Dream on Veterinary Science

Luis Tavares(✉) and All Researchers Working in CIISA 1992–2018

Microbiology and Immunology Lab, Faculty of Veterinary Medicine, Centre for Interdisciplinary Research in Animal Health (CIISA), University of Lisbon, Lisbon, Portugal
ltavares@fmv.ulisboa.pt

Brief History of CIISA: A Fairy Tale

Let me introduce you someone I know very well, whom I met still in the womb, assisted in his birth, 25 years ago, and had the privilege to guide through childhood.

Mother was an old and traditional 160 year old lady and therefore, life for this child was not easy. This child was quite irreverent, full of energy and scientific curiosity, always struggling to occupy his legitimate place in the family and yet, the family did not recognize his value and qualities at first. Too proud of her ancestry the mother did not want, at first, to give the newcomer any credit or room to strive. However, the child insisted, kept growing and struggling and with the help of a few young friends, became a mature young identity that got even to help guiding the mother to grow younger and stronger and to represent wonderfully the family in society.

You might have guessed that this child name is CIISA. It was baptized with this designation to have a broad spectrum of representation of scientific interests in the veterinary field and to establish interdisciplinary connections between the different areas of health research.

Now, that we are celebrating his 25th birthday and Jubilee, I want to wish that this child, young adult, never grows old. I know he won't because it keeps in a great company. All of you, young researchers, full of energy, scientific curiosity and irreverence. The main qualities that, together with freedom to choose your one ways, are the most important characteristics needed to accomplish great Research!

Facts: Main Marks Time-Line

1991 – JNICT launched "Programa Ciência" and grant proposals were prepared at FMV.
1992 – Results became available and the project of CIISA was highly rated (2nd) in the main area of Agriculture and the center was created and funded near 6.5 million €.
1993 – Purchase of equipment and establishment of the research labs.
1994 – Pluriannual Funding program started and maintenance funding was obtained. CIISA's structure and statutes were defined.
1996 – First international evaluation. Classification was "Very Good" and, as a consequence, Pluriannual funding doubled.
1999 – Second international evaluation. FMV and CIISA moved to new facilities in "Alto da Ajuda".

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2000 – Evaluation results were revealed, classification was "Good" and re-organization in four main research areas occurred.
2001 – CIISA organized the 1st International Symposium on Animal Health.
2005–2006 – Preparation of the third international evaluation.
2007 – Evaluation visit. Results rated CIISA as "Very Good".
2013 – Preparation of the 4th international evaluation. Re-structure of the center.
2014 – Election of the new Coordinator. Evaluation visit occurred and results rated CIISA again as "Very Good".
2017–2018 – Preparation of the 5th international evaluation.
2018 – Visit by the Evaluation panel. CIISA's Congress 2018 and celebration of the 25th anniversary and Jubillee.

Now, back to origin … let's take a quick look at the very begining…!

A View from Inside…

We were in the year 1991 and a few of us had recently returned from abroad after completing PhD degrees in well internationally reputed universities. At that time, only a few research labs operated FMV in just a few scientific areas and the facilities and equipment were very scarce. Therefore, although the academic positions at FMV were warranted, the chances to continue the research lines started abroad, during the PhD training work, were low, not to say unattainable.

At the national level, however, research at the universities was just starting to be considered essential and the institution responsible to consolidate research, "Junta Nacional de Investigação Científica e Tecnológica (JNICT)", launched the first important National Research Funding program called "Programa Ciência". This was the opportunity the newcomers, that did not have conditions or labs to pursue their research lines, were waiting for.

Based on experience acquired overseas we immediately started writing a grant proposal to apply for funding to equip the labs, most of which were only used then for practical classes and education purposes. To be successful this proposal should involve most, if not all, the sectors of activity in Veterinary Sciences. In a very narrow time frame we would have to collect CVs, put together consistent plans to start research lines, lists of equipment needed, and contact research partners at both national and international levels. Most of all, the school had to act fast and unite to achieve this goal. However, some of the older professors responsible for these sectors were quite reluctant to support the demanding efforts necessary to put together such a proposal. While trying to motivate everybody to collaborate in the proposal, we were accused of "getting on our toes". We have to remember that, at this time, the departments had only recently finished organizing themselves and there were strong personalities heading them. Therefore, the school departments decided that there should be not one, but two independent proposals, one from the Department of Technology and Animal Health (DETSA), and one from the Department of Morphology and Clinics (DEMOC). The application was submitted, in the last deadline day, after a sleepless night, running against the time, harms full of dossiers, finally delivered at JNICT, in the main research area of Agriculture, since the area of Veterinary Sciences did not exist at that point.

Then came the usually long evaluation period and, while waiting, only a few of us kept hope of success, when faced with the high number of proposals that entered the program. When the results were finally revealed, we learned that DETSA's grant proposal for creation of the center for interdisciplinary research in Animal Health (CIISA) was rated second best in the area of agriculture, thus entitled for funding, and the evaluators proposed merging the two FMV's proposals. The total amount of funding attributed to CIISA was near 6.5 million euros.

The objectives of CIISA, as clearly stated in the original grant proposal are reproduced here (in italic).

The Centre for Interdisciplinary Research in Animal Health (CIISA) was formed (in 1991/1992), to develop, integrate and articulate R&D activities conducted at the Faculdade de Medicina Veterinária (FMV). The main goal to be achieved with the establishment of this center (CIISA) was to improve the existing conditions to pursue research activities, framed within the following main areas or fundamental objectives:

a) *To promote the study of ethio-pathogenesis, epidemiology, diagnosis and therapeutics of animal diseases with scientific, economic and social impact.*

   i. *To promote the best knowledge of the interactions involving animals, humans and environment to optimise the economic and ecological efficiency of the animal farms and to avoid its negative aspects.*
   ii. *To study the strategies for quality control of the products of animal origin.*
   iii. *To create polyvalent infrastructures to be used in the future by other research areas within the scope of the proposed main objectives.*
   iv. *To develop and test new technologies in basic science to serve the previously mentioned objectives.*

b) *To provide services to the community thus participating in the national development by celebrating research contracts with public institutions and private companies operating in agricultural, pharmaceutical and food industry.*
c) *To enhance the technical and scientific interchange programs and international cooperation between our researchers and their counterparts in the Portuguese speaking countries, in the EU and in the USA.*
d) *To open FMV to the outside providing the professional community with education activities that include the organisation of specialised courses and updates in animal health.*

Next step was taken much more confidently but it was even a more difficult one to accomplish. It was now necessary to select which equipment to acquire, with the available 6.5 million in funds, from a very long list of units previously proposed by the various laboratories that had applied. For greater efficiency of the process, the decision was made to establish priority criteria. The first was the shared use of equipment between laboratories, aiming to try to tear down the walls between sectors and to stimulate a true spirit of interdisciplinarity, while avoiding unnecessary and expensive duplications. The second was demonstration that equipment was indispensable to achieve the objective of establishing the research lines proposed. This phase took place with a certain degree

of dispute and some uneasiness, already foreseeable. However, the greater challenge came unexpectedly when, decided what to purchase, it was time to start the acquisition process, following the strict rules of the public administration, which were in certain cases quite restrictive as far as the choice of the supplier based in quality and specificity of the equipment were concerned. In addition, according to JNICT rules, the funds would only be reimbursed after completion of the whole acquisition process, invoice and receipt included, which made it virtually impossible to continue, due to the scarcity of resources the institution could make available to pay expensive pieces of equipment up front. Finally, the solution was found and, after long hours of negotiation and an exercise of mutual trust between the school and the suppliers, the paraphernalia of scientific tools were delivered, distributed and accommodated in the different labs.

The first important phase of CIISA's birth process, the materialistic one, had been accomplished. Of course, by then the first reports on funding application had to be produced and delivered in due time, which required a careful administrative organization and the choice of a coordinator and a coordination team. All researchers involved had the opportunity to participate in the coordinator´s election process and the democratic choice fall on my shoulders, most probably reflecting the success of the previous steps.

Once equipped the labs now needed funding for consumables in order to operate. That started the second important phase of consolidation of the center, and the insistent request for pluriannual funding. Finally, in 1994 the first Pluriannual Funding program launched by JNICT took place and CIISA, now more confidently, once again did not miss the opportunity to apply, and once again it was funded. Another grant application had to be organized, now describing in more detail how the centre planned to develop further and how a true cooperation between labs to achieve common goals was underway. We can consider that 1994 and the start of the Pluriannual Funding achieved then, mark the transition of CIISA from baby to childhood.

By that time, although most school members, professors and researchers, were already convinced of the advantages of unifying all research efforts within the scope of CIISA, it was still common to hear expressions like "my research is independent from CIISA", from members and labs that obtained individual project funding. It took very many years before all the walls between labs became bridges to bring together growth perspectives.

Originally, CIISA integrated two main research units: Technology and Animal Health and Morphology and Clinics, mirroring the two initial proposals from the departments that had merged. A third Unit of Tropical Veterinary Sciences was included later to accommodate the researchers from the tropical Veterinary and Zootechnic center (CVZ-INII) also hosted by FMV at the time. These units comprised several research nuclei, which were corresponding to the various teaching and research sections pre-existing at FMV.

A. Technology and Animal Health Unit

- Nucleus of Retrovirology and Immunology
- Nucleus of Infectious Diseases
- Nucleus of Economics, Epidemiology and Public Health
- Nucleus of Reproduction and Physiology

- Nucleus of Genetics
- Nucleus of Animal Products Technology
- Nucleus of Biochemistry
- Nucleus of Sanitary Inspection of Food Products of Animal Origin
- Nucleus of Animal Nutrition
- Nucleus of Parasitology and Parasitic Diseases
- Nucleus of Wildlife Research

B. Unit of Morphology and Clinics

- Nucleus of Surgery
- Nucleus of Special Compared Pathology and Clinics
- Nucleus of Pharmacology and Toxicology
- Nucleus of Anatomy
- Nucleus of Electron Microscopy
- Teaching Hospital

C. Unit of Tropical Veterinary Sciences

- Nucleus of Tropical Veterinary Sciences

CIISA's evolution, the constant search for interdisciplinarity and the advise of multiple evaluation teams and advisory boards that visited the center (see below), lead to re-organization in four main research areas:

A. Animal Health and Prevention
In which the following nuclei found expression:

- Epidemiology and Veterinary Public Health
- Infectious Diseases
- Virology and Immunology.
- Bacteriology and Mucosal Immunology
- Parasitology, and Wild, Feral and Zoo Animals

B. Food Safety and Technology
Including the following nuclei:

- Food Inspection
- Food Biochemistry
- Pharmacology and Toxicology
- Technology of Products of Animal Origin

C. Patology and Medicine
Including the following nuclei:

- Anatomy.

- Patology and Histology
- Teaching Hospital and specialty clinics: Surgery, Medicine, Imagiology. Large Animal Medicine

D. Biotechnology and Animal Production
Including the nuclei of:

- Physiology and developmental biology
- Reproduction
- Nutrition and Biotechnology
- Animal Production
- Tropical Animal Science

According to CIISA's statutes the organization and decision making had the following structure: **Scientific Board** - integrated all researchers.

Executive Board: formed by:

CIISA's Coordinator (elected every 3 years) and 6-8 representatives of the nuclei working in: Animal Health; Clinics and Pathology; Animal Production;

The administrative services had one secretary that assisted the coordinator and the boards and interacted with the financial services for the management of funding.

The scientific board elected the coordinator and approved the criteria, proposed by the Coordination Board, to allocate funding obtained from JNICT's Pluriannual Funding programs. Therefore, priority was given to the following items:

- **Publications and journals acquisition -** to promote publication of scientific papers in international journals by supporting publication costs and to purchase scientific references.
- **Equipment maintenance and repair -** to pay equipment maintenance and repairs not considered within the projects.
- **Active participation in scientific meetings -** to support travel, congress registration and other expenses related with the reporting of research work in national and international meetings.
- **Preliminary and progression projects -** to support new projects and research ideas mainly aiming junior researchers and FMV assistants depending on these projects for academic progression.
- **Promising research areas -** to support and stimulate new research areas allowing preliminary studies to be initiated before external funding can be found.
- **Post-Docs -** to complement Post-docs FCT scholarships, absorbing recent postgraduates.
- **Researchers and lab technicians -** to complement FCT's BIC and PBIC scholarships.
- **Projects for MSc or PhD students -** to finance projects that involve students awarded with MSc or PhD FCT's scholarships. The funding of these projects was subjected to internal evaluation.
- **Graduating or training courses -** not applicable in 1997

- **Development of community supporting services -** to implement the exchange technical resources of CIISA and the Community, aiming at developing future financing sources.

More recently, when preparing a very thorough self-evaluation report in 2013, CIISA executive board proposed a considerable change in the structure of the center reflecting the recommendations issued by the previous evaluation panels, and a re-organization in two main research groups:

1- Animal Health and Veterinary Medicine – focused on prevention, diagnosis and therapeutics of diseases of different animals species (domestic, wild, exotic, zoological, laboratory), including zoonosis and involving aspects of Comparative Medicine.
2- Animal Science and Food Safety – concerning mainly animal production, animal protection and welfare as well as the quality and safety of their products to the consumers.

The two groups proposed conducting four major interdisciplinary thematic lines for the next 5 year period, as follows:

a) ***Disease Surveillance, Prevention and Control Towards a Sustainable Animal Health –*** aiming to advance veterinary sciences and related public health through discovery of the biological principles of animal health, animal disease and related biomedicine with the goal to develop novel prevention and intervention strategies (the 'One Health' concept).
b) ***Clinical Research Towards Novel Diagnosis and Therapeutic Strategies -*** aiming the integration of research activities across the broad field of research including infectious, parasitological and genetic diseases of animals and Man.
c) ***A sustainable Animal Production For The 21st Century –*** aiming to implement an integrated approach to study animal production systems and their profitability, obtained by a more efficient utilization of feed, genetic improvement, reproductive success and animal welfare. Environmental and quality issues are key elements to sustain the development of highly efficient and socially acceptable animal production systems.
d) ***Advanced food processing, quality and safety: new challenges -*** aiming to assess and manage emergent technologies allowing for a chemical and biological hazard control, eventually present in traditional processes. This line deals with: residues of drugs in food, antimicrobial resistance of bacteria found in food matrix; surviving and ecological behavior of micro-organisms in food processing; environmental contaminants of food chain and new generated compounds by physical and chemical treatments applied to new ingredients and new food.

With this current structure, CIISA aims to enhance chances for scientific innovation at the interface between disciplines and to create a stimulating and attractive scientific environment. In addition, CIISA is committed to establish rational mechanisms of sharing research facilities and to enlarge its available instrumentation to allow extending its

research capabilities. Overall, we expect to strengthen our position for the acquisition of external research funds.

Common **objectives** of these lines are:

a) To **serve the society** by creating a challenging environment with cutting-edge multi-disciplinary expertise and research facilities that is attractive for a variety of stakeholders;
b) To offer a **broad spectrum of training** for MSc and PhD students – under this strategic program CIISA wishes to recruit internationally, highly motivated graduates, with excellent scientific potential, and with a solid background in biomedicine, biology and related scientific areas.

CIISA has developed a comprehensive strategic plan that is structured to stimulate future research in the various thematic lines:

a) Develop dynamic, innovative research programs that are globally competitive and have a local impact;
b) Ensure a continuous high-quality output so that its researchers are able to attract sustainable funding to further develop its programs;
c) Facilitate the development of multi-disciplinary teams aligned to various research themes;
d) Foster strategic alliances with other local and international institutions;
e) Attract increasing numbers of local and international postgraduate students.

CIISA looks at itself as the scientific floor for innovation and debate that benefits veterinary practice, animal production, public health, economy and the Society as a whole. To implement this strategy CIISA has established a representative, flexible and transparent structure, able to communicate efficiently with its members and to encourage group work in a participative way.

View from the Outside…

It is rather interesting to review the reports and main recommendations of the evaluation panels, as they document how CIISA is viewed from the outside. Although the results from this assessment exercises were sometimes received inside as not very fair, mainly because the evaluation panels almost never included veterinarians, they were in fact determinant for the progression and development of the center.

CIISA was first evaluated in 1996 in the scope of activities carried between 1994 (year when pluriannual funding started) and 1996. Since then, international panels periodically evaluated it in 1999, 2007, 2013 and 2018.

As a result of the 1996 first evaluation in which CIISA was rated "very good", pluriannual funding was raised significantly and almost doubled, allowing for a very expressive implementation of the internally funded projects

It was evaluated again in 1998–99. The international panel composed by 6 members visited the center in the 5th of November 1999. In the year 2000 we received the panel's report (reproduced here in italic) and the overall classification was GOOD:

The panel was positively impressed by the good feeling among people working at the centre. The staff is motivated and willing to improve the quality of the research in the excellent new facilities. The opportunity created by the moving from "Gomes Freire" to "Ajuda" should be used to reorganise the centre as is suggested below.

The scientific productivity of the centre is acceptable but could be improved, especially in terms of articles in international journals. There is an excessive number of articles in proceedings in comparison with those in well-known journals. The ratio of the number of articles per money spent in projects is also below the international standard.

There is a noticeable difference in the quality of the research between the various nuclei. The teams working on infectious diseases and in animal nutrition seem to be at a higher level in comparison with the other teams.

The gap of knowledge between nuclei may increase due to a lack of co-operational work within the centre. The panel sees the need for reorganising the centre by merging some nuclei into larger units identified by a common research scope or objective. The new facilities can be a powerful tool for encouraging the synergies between research teams. Besides the increased co-operation between nuclei the centre should also improve co–operative research with foreign research institutions e.g. through EU projects.

The clinic unit is making little research. It will be difficult with the present staff to do teaching, to have the new hospital running, and to do research simultaneously.

The panel noticed a lack of information on research management. Also some emphasis should be put on information acquisition. As a result of this isolation in scientific terms the panel noticed a lack of contact with other foreign institutions or groups working in the same or close related subject matters e.g. African swine fever.

The research work on food science is rather poor and should be completely restructured since food quality and safety has become a very important area of research.

Research on economics is also weak and does not involve policy e.g. evaluating the cost of eradicating a disease.

Recommendations: The panel sees no purpose in the work of the tropical nucleus. There is also no apparent goal for the studies in tropical animal nutrition. However, they could be continued in collaboration with the nucleus of animal nutrition.

The panel had the impression that there are too many research objectives, the efforts are too dispersed, and therefore the research does not go deep enough into each subject matter. There is a need to concentrate efforts and to increase the critical mass of the various research teams. Sometimes research seems to be confused with teaching needs and not addressed to problem solving.

There is very little active research on animal production and the little research existing requires closer interaction with agriculture. There is also a need to integrate the chain from the farm to the slaughtering house including the environmental impact of the activity. One possible way to develop the perspective could be to have joint projects with institutions more devoted to agriculture. The panel noticed that there is no research on swine and poultry production despite the enormous importance these activities have in the country.

The centre has made a large effort on outside funding since about 90% of the projects are externally financed. The amount of money for research is at an acceptable level and seems to be sufficient to support an acceptable research production.

The panel considers that the centre has a large potential for improving the quality of the research by a rational use of the research staff and the new facilities. The centre could develop a few areas of excellence starting with those nuclei that are already more advanced and have an higher level of internationalisation.

Next evaluation started in 2003, again by an international panel of 8 members covering the *Agricultural Sciences* research units of which 6 were French researchers from the Institut National de la Recherche Agronomique (INRA) and one from Euragri Agricultural Research Department, from Holland. Like in 1996 and 1999, evaluations were based on written reports prepared in advance by the centres, on-site visits and informal discussions with research leaders and individual scientists.

The panel issued a global report on the 23 institutions in the Agricultural area, visited by the panel throughout the country. There was no individual report. However, extracts that refer to CIISA were:

(…) In several areas, the research units evaluated by the Panel are at a good level and have some potential for reaching a level of excellence, under certain conditions (…)

– *Animal health and production: the Panel was impressed by the efficient coordination of original research on numerous topics at CIISA –Veterinary School in Lisbon. The level of research is high and the publications are of particular interest. However, it seems that this Centre should move towards two directions. A first one is to divide CIISA into 2 or 3 sub-centres, around one thematic. A second one is to give to CIISA a new mission of coordination of veterinary science in Portugal, in order to improve the quality of research in other centres (especially CECA at Vairão, in Porto, CECAV at Vila Real and IISA at ISA-Lisbon), to better link this research with INIA, and to avoid double lines of research between teams…*

In the 2007 evaluation, another panel, again rated CIISA as "Very Good" and commented:

CIISA is one of the leading units in veterinary medicine area in Portugal. …The ratio between the veterinarians and the other specialists is almost equal which is considered to be a positive trend. It seems that the structure of the unit with the four groups: Animal Health and Prevention; Pathology and Medicine Biotechnology and Animal Production and Food Safety with a few exceptions is very well and multidisciplinary composed in order to be able to accomplish the respective objectives as: Control of economically important diseases; animal models for important human and animal diseases and safety, quality and control of food production. This role has been shown to be performed with close cooperation with other national (26) and international (25) Universities; official services (34); private enterprises (27); professional associations (8) and other national and international institutions (61). Thus, it is very relevant to the national priorities of the country. To great extent it deals also with the European and African animal pathology and epidemiology problems. The organization of CIISA is realized by Scientific Board

which integrates all researchers. The funding in the period 2003 – 2006 was over 5 mln € for every year (trances between 5 – 5, 5 mln €). During this period the FCT and Government support has not been increased. However recognizing the importance of CIISA to become a Center of Excellence the FMU allocate plural funding for more than 82 projects like: preliminary studies for promising research areas; academic programs – MS; PhD and Post docs; funding 'in – between' projects; active participation in scientific meeting; equipment maintenance and repair publications; organization of training courses, workshops and congresses and hiring young researcher. Because of the strong activities of the Animal health and Prevention group almost 50% of income of the unit is due to funds coming from FP of EC.

The publications have shown significant and impressive increased ratio especially the articles in international journals with 65% (216). The ISI index as a whole has been also improved very much. Most of the publications demonstrate a good nature of fundamental and applied research. The unit is located in relatively new building. The panel however got the impression that most of the recent facilities and the equipment slowly are getting out of date and need renovation. As a whole the unit has one of the most well combined and modern infrastructure in Portugal.

The extension service is very good. The hospitalization is done in place with small and big animals. It helps the unit to get a good image in the society as well. Training of young researchers and students is very satisfactory. Organization of workshops is regular. Interdisciplinary activities are very good as a whole. However, more integration between the groups is definitely required in order to be more successful in the future.

Additionally, the panel recognizes that the research which deals with drought tolerance in maize and biofuel studies in Biotechnology and Production group could be much better developed if they were integrated into the research of ICAM unit N 115 in Evora or the Centro de Botânica Aplicada à Agricultura AGR – LVT – Lisboa 240. Interactions with other national and international research units and companies. The tendency to improve essentially the cooperation and the integration into national and international programs and initiatives should continue.

Some priorities should be identified for the better internationalization in order to avoid the dilution. Participation in international research programmes (EU etc.) The good examples which already exist in some of the groups like Animal Health and Prevention should be followed from the rest in order to achieve the goal of excellence. Knowledge and technology transfer are well developed. Outreach activities are welll developed. As far as attitude and work environment, the leadership of the unit and the groups showed clear organizational and motivation capacity. The groups and the unit meet regularly.

The researchers are committed. They respect each other and the leadership as well. Most pertinent comments and recommendations: The Panel has observed some improvements from the last evaluation exercise and thinks that the unit developed sufficient potential to continue to improve its entire R&D work. The Panel could make the following recommendations:

- *To develop more strategic vision and partnership in both national and international level.*
- *To develop External Advisory Board and on the base of this more strategic leadership.*

- *To apply more actively into the EC framework programs and Ministry of Health care in more coherent approach.*
- *Better exploitation of the equipment and the facilities.*
- *To strengthen the socioeconomical and socioethical research and policy.*
- *Integration into LEAF is highly desirable.*

The last evaluation, of which results are available, occurred in 2013 although the evaluation panel only visited CIISA in 2014. Again, the classification was "Very Good" and the panel issued the following report:

CIISA is a unique center providing very good veterinary research, with interesting applications also in biotechnology and food science and industry. Emphasis on societal and agricultural impact of animal science has been appreciated. The center features impressive facilities that are of outmost usefulness for carrying state-of-art veterinary research, from basic science to field applications and services to the public. The center might have a central role not only at regional but also at international level. The further improvement of the indicators of innovation will be highly beneficial for the center as the number of patents and of other indicators are not as successful as it would be expected from this center. Provisions have been taken to substantially increment these indicators in the future.

The center is able to raise a substantial amount of research funding, with remarkable success in international competitions. However, the number of published research is lower than expected, but several researches are published in high impact journals for the sector. The Centre boasts a large research team and also demonstrates a considerable number of worldwide collaborations. Many of the senior scientists within the team are very experienced and well respected in their field of activities. The strength of the research is mostly in the translational stage, which is evidenced by the significant outside funding acquired from European programs. The international attractiveness in terms of funding is also growing, which is also excellent. The international outreach is very strong.

The center has a solid strategic program, which mostly builds on previous achievements and on the consolidated expertise of the center. Competence on biomedicine and animal science is rare, and the center surely has a relevant place in the regional strategies. At international level, current and programmed research may guarantee continued visibility and recognition to the center. The strategic plan could be bolder and more careful when dealing with enhancing the productivity of the center, especially in terms of published research. The center has a clear and straight focus on animal science, but it has also undertaken interesting collaborative research on agronomy and food science, thus increasing the degree of multidisciplinary research, as needed.

The proposal appears to be well organized and logical. The research team undoubtedly has sufficient critical mass to achieve the aims that it sets out. In general, the requested budget for the strategic program seems in line with the budget received by FCT in the past five years. Strangely, obsolescence of the equipment is perceived as weakness, but only a limited part of the budget is dedicated to the objective of its updating. In general, it seems that the program aims at consolidating and stabilizing existing facilities, without providing leaps toward groundbreaking and innovative studies or applications. Integration of the research center on the host University is complete and very positive, as the center is perceived like an essential part of the University itself.

Interviews to students, post-docs and integrated members of the center indicated a very good feeling toward the center activities and the capacity of the center to offer education and career opportunities.

Overall Conclusions: It is confirmed also by the site visit that the center is able to provide a very well established and comprehensive research facility in the field of animal and veterinary science, offering remarkable services and applied research for the development of animal and food science, and agriculture. The research groups are well assembled and of good scientific value and international reputation, as shown by publication number and especially by the quality of some research outputs. The capacity to raise funds, and to cooperate with industry is unquestionable. The recruitment policy is wisely built, considering a large investment in training and transfer of knowledge to young researchers. The solid program seems somehow lacking groundbreaking innovation, and strategic vision, mostly aiming at strengthening the current position of the center. This is certainly valuable, especially considering current shortage of funding, but the center might undertake a bolder turn, which would allow it to grow further at national and international level.

In 2018 the last evaluation panel visited us again and CIISA is awaiting the final report.

The exercise of reviewing the external analysis that different expert panel have made, through the years, of the center, allows to conclude:

1) A great evolution has occurred along the 25 years of CIISA's life.
2) CIISA has been increasingly perceived internationally as a major driving force for research in veterinary sciences in Portugal.
3) CIISA finally overcame the initial individualistic trend of its research teams, and is also recognized as the main and only face of research at FMV.
4) CIISA continues to implement and expand the original objectives successfully, as revealed by the number of research projects already concluded and in course, funded by several institutions and in close collaboration with national and international partners.

Final Remarks … and the Future Looks Bright

Following the 2013 tremendous efforts of the coordination and executive board to mount the Self-evaluation report and to plan re-structuring, CIISA went to elections in May 2014 and I decided that I should no longer be a candidate. After 22 years of dedication to development of our research center and many consecutive elections won as the coordinator, I decided it was time for new teams and new ideas to take place. After all, the child born in 1992 has reached adulthood and no longer needs the former guidance.

The modern societies are increasingly confronted with animal health related issues that, in the last few years, were shown to have considerable impact in the global economy and in public health. In addition, claims for the development of novel environmental sustainable systems for animal production and agriculture are justifiably increasing. CIISA is in a privileged situation to study the main aspects related to animal health and production, considering the multidisciplinarity of its research teams. The main contributions of

this center to significantly improve animals' and consumers' quality of life, are noticeable in all main fields of veterinary sciences. Major impacts of CIISA's research, have been noticed in the past and are expected in the future, in the development of novel diagnostic strategies and therapies, innovative biotechnological products, new sustainable production systems and to significantly improve food safety and nutritional quality of animal products.

Now, that we are celebrating his 25th birthday and Jubilee, I want to wish that this child, young adult, never grows old. I know he won't because it keeps in a great company. All of you, young researchers, full of energy, scientific curiosity and irreverence. The main qualities that, together with freedom to choose your one ways, are the most important characteristics needed to accomplish great Research!

Long life to CIISA.
Luis Tavares

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