UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

CONTRIBUTION TO THE DISCRIMINANT POWER OF SOME OF THE VARIABLES INVOLVED IN THE STAGING OF SEVERE EQUINE ASTHMA SYNDROME

JOANA DE SOUSA AZEVEDO SIMÕES

Orientador(es): Professora Doutora Paula Alexandra Botelho Garcia de Andrade Pimenta Tilley

Professor Doutor José Paulo Pacheco Sales Luís

Tese especialmente elaborada para a obtenção do grau de Doutor em Ciências Veterinárias na especialidade de Clínica

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Nome: Joana de Sousa Azevedo Simões

Título da Tese ou Dissertação: Contribution to the discriminant power of some of the variables involved in the staging of severe equine asthma syndrome

Ano de conclusão (indicar o da data da realização das provas públicas): 2020

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Assinatura: ____

The Road goes ever on and on
Down from the door where it began.
Now far ahead the Road has gone,
And I must follow, if I can,
Pursuing it with eager feet,
Until it joins some larger way
Where many paths and errands meet.
And whither then? I cannot say.

The Road goes ever on and on
Out from the door where it began.
Now far ahead the Road has gone,
Let others follow it who can!
Let them a journey new begin,
But I at last with weary feet
Will turn towards the lighted inn,
My evening-rest and sleep to meet.

Still round the corner there may wait
A new road or a secret gate,
And though I oft have passed them by,
A day will come at last when I
Shall take the hidden paths that run
West of the Moon, East of the Sun.

The Lord of the Rings
J.R.R. Tolkien

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The task of writing a PhD thesis is an incredible journey. At the starting point we set out full of hope and ideals, blissfully unaware of all the bumps and forks that await us further down the road. Some of our plans, pristine on paper, need to be readjusted.

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Resumo

A síndrome de asma equina (SAE) grave é uma doença respiratória crónica, recorrente e altamente prevalente em animais adultos, estando associada à domesticação dos equinos. Devido à sua natureza insidiosa, o tratamento é muitas vezes frustrante, originando perdas económicas significantes.

Quando expostos a ambientes com elevadas concentrações de partículas respiráveis, tais como esporos de fungos, ácaros, endotoxinas, pólen e outras partículas antigénicas, os animais geneticamente suscetíveis desenvolvem inflamação, hiper-reactividade e obstrução das vias aéreas. As concentrações de partículas respiráveis elevadas encontram-se normalmente presentes em sistemas de estabulação de equinos tradicionais, enquanto que em sistemas em extensivo (pastagem) estas concentrações tendem a ser menores.

Assim, no maneio da SAE grave é essencial o controlo ambiental de modo a assegurar a melhoria da função pulmonar e em alguns casos o tratamento médico com corticosteroides e broncodilatadores para reduzir os sinais clínicos de inflamação das vias aéreas e broncospasmo.

Tendo em conta a importância clínica desta síndrome, esta dissertação tem por objetivo contribuir para o conhecimento científico da SAE grave. Assim sendo, investigámos a influencia dos testes de função pulmonar no diagnóstico e estadiamento da doença e desenvolvemos um método de estadiamento, utilizando apenas equipamento portátil, o qual poderá ser utilizado na clínica em regime de ambulatório.

Ainda, investigámos a relação entre SAE grave e a resistência a parasitas gastrointestinais, a qual até à data ainda não havia sido reportada em cavalos Puro Sangue Lusitanos. Esta associação foi, contudo, descrita em equinos de outras raças diagnosticados com SAE grave ou outras hipersensibilidades múltiplas.

Por fim, considerando que a remoção de aeroalérgenos é fundamental para a remissão da SAE grave, procurámos avaliar a complacência dos donos dos animais asmáticos a um conjunto de recomendações de maneio ambiental.

Palavras-chave: Síndrome de asma equina grave; estadiamento; função pulmonar; resistência a parasitas gastrointestinal; maneio ambiental

Abstract

Severe Equine Asthma Syndrome (EAS) is a highly prevalent, chronic and recurrent respiratory disease which appears to be related to equine domestication. Due to its insidious nature, treatment may sometimes be frustrating and severe economic loses occur.

Genetically susceptible individuals develop airway inflammation, hyperresponsiveness and obstruction when exposed to environments with high concentrations of respirable dust particles, which include mould spores, mites, endotoxins, pollen and other antigenic materials.

These hazardous respirable dust concentrations are usually found in traditional equine housing systems, while lower concentrations tend to be present in outdoor systems (pasture).

The management of severe EAS essentially requires environmental control to ensure improvement of lung function and in some cases medical treatment with corticosteroids and bronchodilators to ameliorate the clinical signs of airway inflammation and bronchospasm.

Considering the clinical importance of this syndrome, the dissertation focuses on further contributing to scientific knowledge of severe EAS. As such, we investigated the influence of lung function tests on the diagnosis and staging of the disease and developed a staging method using only portable equipment, which has the potential of being used in equine ambulatory practice.

We also investigated the relation between severe EAS and resistance to gastrointestinal parasites, which had not been, to the author's knowledge, previously reported in the Lusitano breed horses. This association has been reported in other equine breeds with severe EAS or with other multiple hypersensitivities (MHS).

Lastly, because allergen avoidance is fundamental for the remission of severe EAS we examined owner compliance to a set of recommended guidelines for environmental management.

Keywords: severe equine asthma, staging, lung function, gastrointestinal parasite resistance, environmental management

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List of abbreviations:

ABG – Arterial blood gas

ABD - Abdominal

AHR – Airway hyperreactivity

BAL – Bronchoalveolar lavage

BALF – Bronchoalveolar lavage fluid

BE – Base excess

Bwt – Body weight

COPD – Chronic Obstructive Pulmonary Disease

C_{dyn} – Dynamic compliance

EAS – Equine Asthma Syndrome

EBUS – Endobronchial ultrasound

EDTA – Ethylenediamine tetraacetic acid

EPG – Egg per gram

FEM – Forced expiratory manoeuvres

FOM – Forced oscillatory mechanics

HCO_3 – Bicarbonate

HOARSI – Horse owner assessed respiratory signs index

IAD – Inflammatory Airway Disease

IBH – Insect bite hypersensitivity

IDT – Intradermal testing

IFN - Interferon

IgE – Immunoglobulin E

IM – Intramuscular

IL-4 – Interleukin 4

IL-8 – Interleukin 8

IL-17 – Interleukin 17

IL-4R – Interleukin 4 receptor

IV – Intravenous

LPG – Larvae per gram

L3 – Third stage infective larvae

MHS – Multiple hypersensitivities

miRNA – MicroRNA

MMPs – Metalloproteinases

MPO – Myeloperoxidase

NANC – Nonadrenergic-noncholinergic

NH_3 – Ammonia

P_aO_2 – Arterial oxygen pressure

P_aCO_2 – Arterial carbon dioxide pressure

PO – *Per Os*

RAO – Recurrent airway obstruction

RIB - Ribcage

RIP – Respiratory inductance plethysmography

R_L – Pulmonary resistance

R_{RS} – Resistance

sO_2 – Oxygen saturation

SPRAO – Summer Pasture-Associated Recurrent Airway Obstruction

SPT – Skin prick tests

TCO_2 – Carbon dioxide tension

Th1 – T-helper type 1

Th2 – T-helper type 2

Th17 – T-helper type 17

TW – Tracheal wash

UK – United Kingdom

W – Work of breathing

([Hist] – Histamine concentration

Δflow – Delta flow (flow differential)

ΔPpl – Transpulmonary pressure differential

Introduction

This dissertation focuses on the study of Severe Equine Asthma Syndrome (SEAS), a highly prevalent and incurable respiratory disease, which due its chronicity and recurrence induces significant losses in the equine industry.

The main on goal of the experimental work here presented was the development of a staging method for ambulatory practice, as well as the study of certain characteristics associated with SEAS, namely the parasitic resistance and owner compliance associated with management recommendations.

For this purpose, this dissertation will be divided in six chapters. The first chapter encompasses a compilation of the current scientific knowledge about SEAS, so as to provide the reader the necessary tools to better understand the experimental clinical work later presented. As such, the disease's nomenclature and economic impact, its genetic predisposition and risk factors, aetiology, pathophysiology, clinical presentation, diagnosis and management are addressed.

The objectives of experimental work and its findings are reported in the second and third chapters. The findings are presented in the form of published or submitted scientific publications. The first publication describes a staging method for SEAS using lung function tests which can be employed in ambulatory practice, the second reports helminth parasitic resistance in SEAS-affected Lusitano horses, and the third describes the owner compliance to a set of guidelines for environmental management of asthmatic horses one year after the initial diagnosis of the disease. Lastly a general discussion and conclusion integrating the three clinical studies will be addressed, as well as future areas which must be addressed in order to further improve our knowledge of this syndrome – chapters four, five and six.

CHAPTER 1

Severe Equine Asthma Syndrome

A Review

1. Severe Equine Asthma Syndrome – literature's terminology

Equine asthma syndrome is a fairly new designation for a disease that may be as old has the beginning equine domestication and stabling (Couëtil et al., 2016; Bullone & Lavoie, 2017). As such, an array of terms has so far been used to describe this clinical entity based on the state-of-the-art knowledge of the time.

With the refinement of research techniques, the syndrome's terminology evolved to encompass the new findings, like clinical signs, aetiology and pathology. As such, 'heaves', 'broken wind', 'equine emphysema', 'chronic bronchitis or bronchiolitis', 'equine chronic obstructive pulmonary disease', 'recurrent airway obstruction', among others, were once used to describe the same entity, now referred to as Equine Asthma Syndrome (EAS) (Bullone & Lavoie, 2017).

The first terms used to describe the disease centuries ago were 'broken wind' and 'heaves', due to the clinical signs exhibited by stabled horses fed hay, which tended to present severe breathing difficulties (Markham, 1656).

Later, based on *post-mortem* findings the term 'equine emphysema' was proposed (Obel & Schmiterlöw, 1948). However, since lung hyperinflation observed in severely affected horses results from air trapping and not from alveoli wall disruption, this terminology was considered inadequate (Leclere, Lavoie-Lamoureux & Lavoie, 2011b).

The discovery that neutrophils were the predominant cells found in the bronchoalveolar lavage cytology of affected-horses led to the proposal of the term chronic obstructive pulmonary disease (COPD), since human COPD is also characterised by airway neutrophilia in adult patients, as well as airway inflammation and obstruction (Lavoie, 2015). Nonetheless, this term was also dropped due to significant differences between the two clinical entities, mainly due to the reversibility of lung function changes in the equine patients (Robinson, 2001).

To encompass the variable degree of airway obstruction detected in lung function evaluation and its reversibility when the horse is removed from the offending environment, the designation 'recurrent airway obstruction' (RAO) was proposed (Derksen, Scott, Miller, Slocombe & Robinson, 1985a).

In addition, a similar condition but with different aetiology was identified, in which horses developed clinical signs of airway inflammation and obstruction while kept at pasture, and thus unlike RAO was not related to stabling and hay feeding. This syndrome was called 'summer pasture-associated recurrent airway obstruction' (SPARAO) (Seahorn & Beadle, 1993; Lavoie, 2007).

In order to simplify terminology, the use of RAO was proposed for severe cases of airway obstruction with signs of increased respiratory effort at rest and 'inflammatory airway disease' (IAD) was reserved for milder cases of respiratory impairment and airway inflammation (Robinson, 2001). However, this new nomenclature failed to encompass other similar syndromes, such as SPARAO. Thus, it was deemed necessary to further update the terminology.

Recently, researchers became aware of the similarities in phenotypical presentation, aetiology, pathology and treatment response of equine chronic non-infectious lower airway diseases and human asthma, having introduced the term 'equine asthma' (Leclere et al., 2011b; Bullone & Lavoie, 2015; Bond et al., 2018).

In 2016, an issued consensus statement by the American College of Veterinary Internal Medicine proposed the use of 'equine asthma syndrome' (EAS) when referring to the spectrum of chronic airway inflammatory diseases similar to human asthma. The panel further defined EAS presentation as varying in severity, having considered IAD as mild-moderate EAS and RAO and SPARAO as severe EAS. However, it should not be assumed that mild EAS will necessarily progress to severe EAS and these two entities should not be interpreted as a continuum (Couëtil et al., 2016; Pirie, Couëtil, Robinson & Lavoie, 2016)).

Therefore, following the panels' recommendation, severe equine asthma syndrome is currently the correct terminology to describe horses with severe airway inflammation and obstruction, cough, mucus discharge and increased respiratory effort at rest.

This broad terminology will not only allow the inclusion of different and new phenotypes and endotypes, but also simplify future information exchange (Lavoie, 2015; Pirie et al., 2016; Bond et al., 2018).

2. Epidemiology and genetics

2.1. Prevalence

Severe EAS has a significant impact on equine health and is traditionally considered a disease of the northern hemisphere, where horses are stabled throughout the year, particularly during the Winter (Hotchkiss, Reid & Christley, 2007a, b).

Although reports vary, an estimated prevalence of 14-20% has been reported in the United Kingdom (UK) (Hotchkiss et al., 2007a, b; Ireland, Christley, McGowan, Clegg & Pinchbeck, 2015), being as high as 54% when horses are housed in traditional stables (Bracher, von Fellenberg, Winder, Gruening & Hermann, 1991). This prevalence is considered representative of the northern hemisphere.

The incidence of severe EAS is also higher in countries with a cool and wet climate, such as Switzerland and the UK, whereas it becomes virtually insignificant in countries such as Australia, where a dry a warm climate is found (Bracher et al., 1991; Dixon, Railton, McGorum, 1995; Robinson, Derksen, Olszewski & Buechner-Maxwell, 1996; Ward & Couëtil, 2005). This is most likely caused by the different equine management systems (housing and feeding) practiced in such climates, and consequent exposure to disease risk factors.

2.2. Socioeconomic impact

Lower airway diseases are the most common cause of poor performance in the equine athlete (Allen, Tremaine & Franklin, 2006). Since severe EAS is a recurrent disease with no known cure up to date, it will inevitably lead to economic losses associated with environmental adjustments, therapeutic management and early retirement of affected horses (Bond et al., 2018). Some animals are refractory to treatment, which may become frustrating to some owners increasing the risk of euthanasia in severely asthmatic horses (Couëtil & Ward, 2003).

2.3. Genetics

Allergic diseases occur in most mammals, including horses, and their relevance has been increasing in western industrialized countries (Weiss, 2000; Marti et al., 2008; Hopkin, 2009; Lambrecht & Hammad, 2017).

Severe EAS is one of such diseases, where susceptible individuals develop airway inflammation and obstruction after exposure to an offending environment (aeroallergen) (Bond et al., 2018). This susceptibility is most likely due to the particular genetic makeup of the affected individuals. In fact, a strong genetic predisposition for equine asthma has been identified, although the exact genetic background for this disease still remains unclear (Ramseyer et al., 2007; Gerber, Baleri, Klukowska-Rötzler, Swinburne & Dolf, 2009).

Two modes of disease inheritance – an autosomal recessive and an autosomal dominant – have recently been described in two Warmblood families and it is believed that several major genes play a crucial role in severe EAS (Gerber et al., 2008; Gerber et al., 2009). Although inheritance mode differed between these two families, the disease clinical phenotype expressed by affected individuals was the same (Laumen, Doherr & Gerber, 2010). As such, it is believed that the overall mode of severe EAS inheritance is probably complex and that different mechanisms may be involved (Graubner, Drogemuller, Fouche & Gerber, 2012).

Several studies have also established an association with chromosome 13 and interleukin 4 receptor (IL-4R) (Jost et al., 2007; Gerber et al., 2009; Swinborne et al., 2009; Racine et al., 2011; Schnider, Rieder, Leeb, Gerber & Neuditschko, 2017). In one particular family, the expression of this receptor was increased during severe EAS exacerbation. However, this was not true in the other family, further confirming the genetic heterogeneity of the disease (Klukowska-Rotzler et al., 2012).

In humans, polymorphic differences in the interleukin 4 receptor α chain (IL4Rα) gene play an important role in the development of human asthma and other atopic diseases, since it induces isotopic switch to immunoglobulin E (IgE) and differentiation of T-helper type 2 (Th2) lymphocytes (Jost et al., 2007; Marti et al., 2008).

Likewise, *IL4Rα* plays a role in regulating susceptibility to equine asthma in some high-prevalence horse families with particular predominant Th2-type cytokine response (Lavoie et

al., 2001; Jost et al., 2007). Interestingly, this receptor has also been associated with defence against parasites in both humans and animals (Scales, Ierna & Lawrence, 2007).
The identification and differential expression analysis of MicroRNAs (miRNAs) present in the serum of severe EAS-affected horses, showed a downregulation of miR-128 and miR-744. These findings suggest that a Th2/Th17 immunological response may characterize severe EAS (Pacholewska, Kraft, Gerber & Jagannathan, 2017).
Asthmatic and non-asthmatic horses presented differences in E2F transcription and gene expression after a hay challenge, indicating that asthmatic horses may suffer from impaired epithelial regeneration associated to subepithelial remodelling (Tessier et al., 2018b).
Also, using RNA sequence it was detected a change in the PACRG and RTTN proteins of asthmatic horses, which have been related to ciliary function (Tessier, Côte & Bienzle, 2018a).

2.3.1. Parasitic resistance

In humans, as in horses, there have been several reports of a reverse relation between helminth parasitic infections and allergic diseases, such as asthma and atopy (Lynch et al., 1998; McKay, 2006; Hopkin, 2009; Neuhaus et al., 2010; Bründler et al., 2011; Medeiros et al., 2011;).
Studies conducted in two families of Warmblood horses reported a lower shedding of gastrointestinal nematode parasite eggs in asthma-affected individuals, which may suggest increased resistance to parasitic infections (Neuhaus et al., 2010; Brundler et al., 2011; Schleuniger et al., 2011; Graubner et al., 2012). In Portugal, a similar study indicated that asthmatic horses may present a lower shedding of helminth eggs (Medeiros et al., 2011).
Currently, two theories exist that may explain this relationship: the hygiene hypothesis and the genetic hypothesis.
The hygiene hypothesis states that environmental factors, such as infections and gastrointestinal parasites, determine the prevalence of allergic diseases (Strachan, 1989; Von Mutius, 2007). In essence, helminth infections would be capable of inhibiting allergic disease (Hopkin, 2009).
Nowadays, due to modernization and population hygiene, chronic infectious diseases have become less prevalent, which resulted in the loss of cellular and humoral immunoregulatory pathways thus leading to a higher prevalence of allergic diseases (Lambrecht & Hammad, 2017).
Advocating this hypothesis, studies conducted in humans found that atopic children presented a lower helminth burden and higher IgE levels in comparison with non-atopic children (Lynch et al., 1998), and that the removal of helminth infection has the potential to diminish associated

immune-regulatory mechanisms and thus promote asthma and allergy (Capron, Dombrowicz & Capron, 2004).

On the other hand, the genetic hypothesis states that the increased resistance of asthmatic-individuals to helminth infection is determined by their genetic makeup. As such, genes which confer resistance to parasites must also be responsible for an increased risk of developing allergic diseases (Neuhaus et al., 2010; Lanz et al., 2013).

This hypothesis is further supported in horses by the fact that from the two Warmblood families with a high prevalence of severe EAS, only in one was IL4Rα linked to the disease occurrence. Therefore, in one of the families one of the mechanisms that could be responsible for parasitic resistance was absent, further supporting the genetic hypothesis (Scales et al., 2007; Neuhaus et al., 2010).

On a similar note, insect bite hypersensitivity (IBH) has also been associated with the presence of airway hyperreactivity, suggesting a probable link with equine asthma, although further investigation is required (Kehrli, Jandova, Fey, Jhan & Gerber, 2015; Lanz, Brunner, Graubner, Marti & Gerber, 2017). The airways of horses with IBH showed hyperreactivity similar to the airways of horses with severe EAS, thus concluding that IBH is associated with airway hyperreactivity and decreased PaO2, even in the absence of overt respiratory clinical signs. Therefore, horses suffering from IBH appear to have a higher risk for airway hyperreactivity and might be predisposed to develop severe EAS in the future (Lanz et al., 2017).

Also, an increased risk for IBH is to be expected in severe EAS-affected horses, and multiple hypersensitivities (MHS) is significantly associated with the absence of nematode eggs in the faeces (Kehrli et al., 2015).

2.4. Risk factors

Severe EAS is usually diagnosed in adult animals (Ireland et al., 2015). In fact, some studies have reported an association between disease occurrence and the age of the horse (≥4 years) (Couëtil & Ward, 2003; Hotchkiss et al., 2007b), while another study reported an association between increased age and the risk of developing airway inflammation (Robinson, Karmaus, Holcombe, Carr & Derksen, 2006).

Breed has also been reported as a risk factor in severe EAS, with Thoroughbreds and Warmbloods presenting a higher risk than other breeds (Couëtil & Ward, 2003; Ramseyer et al., 2007). However, this may be a consequence of the type of management on which these horses were kept, or it may indicate a genetic predisposition (Marti, Gerber, Essich, Ouleha & Lazary, 1991; Ramseyer et al., 2007).

Furthermore, residence in an urbanised environment, exposure to hay and/or straw in early stages of life, as well as history of respiratory infection may also increase the risk of developing severe EAS (Hotchkiss et al., 2007b).

3. Aetiology

As previously mentioned, severe EAS-affected horses present clinical signs when exposed to offending environmental allergens (Marti et al., 1991; Marti et al., 2008).

This disease has been associated with hay feeding and stabling, mostly due to the presence of high concentrations of airborne respirable dust particles, which induce airway inflammation (Ramseyer et al., 2007; Ivester & Couëtil, 2014).

Respirable dust concentration is defined as the number of particles with an aerodynamic diameter small enough (≤ 5 µm) to reach the distal airways, and it is considered a good index of the health hazard presented by airborne dust inhalation (Clarke, 1987; Derksen & Woods, 1994; Art, McGorum & Lekeux, 2002).

In equine housing systems the amount of respirable dust concentration represents about 17 to 29% of total dust found in the environment (Woods et al., 1993). Unsurprisingly, respirable dust concentrations are significantly higher in indoor housing systems, where they are 3 to 14-fold higher than those found outdoors (McGorum, Ellison & Cullen, 1998).

As such, studies have classified equine indoor stable environments as hazardous for human respiratory health, due to a high concentration of respirable dust particles, such as endotoxins and moulds, which were considerably above recommended levels (McGorum et al., 1998; Mazan et al., 2009). Thus, it is only logical that the same environment would have a negative impact on equine respiratory health, particularly in individuals genetically predisposed for developing airway inflammation.

No single aeroallergen has been, up to date, pinpointed as the sole major risk factor for severe EAS development, and researchers hypothesise a synergistic effect of several dust components, with horses often exhibiting hypersensitivity to multiple allergens (McPherson et al., 1979; Pirie, Collie, Dixon & McGorum, 2003a; Pirie, Dixon & McGorum, 2003b).

Because aeroallergens have a wide range of size, with the smallest being fungal spores (0.3 to 30 µm in diameter) and the largest pollen grains (10 to 100 µm in diameter), a greater significance has been attributed to the smaller ones which can penetrate the airway of horses (Robinson, 2001; Pirie & McGorum, 2017).

However, pollen grains although larger in size are still present in the respirable dust found indoors and outdoors, and they are thought to play a role in the disease pathogenesis, as is the case in human respiratory allergies (Burge, 2002; Ward and Couëtil, 2005).

Some of the most hazardous (pro-inflammatory) agents found in the stables include fungal spores (>50 species identified), bacterial endotoxins, forage and storage mites, microbial toxins, peptidoglycan, proteases, pollen and plant debris, as well as inorganic dust (McGorum & Pirie, 2008). Although spores such as *Aspergillus fumigatus*, *Faenia rectivirgula* and *Thermoactinomyces vulgaris* have been widely recognised as significant risk factors, a considerable number of horses have shown an increased sensitivity to mite allergens (Clarke, 1987; Seguin et al., 2012; Niedzwiedz, Jaworski & Kubiak, 2015; Klier et al., 2018).

Other pollutants, especially high levels of ammonia, may further aggravate airway inflammation, which is why a good stable and bed hygiene and ventilation are fundamental for the wellbeing of asthmatic horses (Lawrence, Bump & McLaren, 1988; Pirie, 2014).

Urban pollution has been identified as a significant risk factor for asthmatic humans and it may also have a detrimental impact on equine respiratory health (Ward & Couëtil, 2005).

While in the most common phenotypical presentation of severe EAS disease exacerbation occurs with exposure to indoor aeroallergens, mainly mites and fungi spores, in pasture-associated severe EAS exacerbation occurs paradoxically by exposure to pollen and other outdoor allergens (Seahorn & Beadle, 1993; Seahorn, Groves, Harrington & Beadle, 1996; Swiderski et al., 2017; Ferrari et al., 2018)

Environmental conditions also appear to impact disease manifestation, mainly by conditioning the concentration of environmental aeroallergens. Minimum temperature and humidity will likely affect pollen and fungal concentrations, two of the major pro-inflammatory agents of severe EAS (Ward & Couëtil, 2005; Costa, Johnson, Baur & Beadle, 2006; Bullone, Murcia & Lavoie, 2016).

It is believed that horses require a prolonged exposure to high concentrations of respirable dust in order to develop clinical signs of severe EAS (Hotchkiss et al., 2007b).

Nonetheless, equine traditional management, which consist of stall housing, hay feeding and straw bedding, leads to an increased exposure to high concentrations of respirable dust particles and is associated with an increased incidence of severe EAS (Clarke, 1987; Derksen, 1993; Kirschvink et al., 2002). In this environment, respirable dust concentrations around the horse's breathing zone can reach alarming values of 17.51 mg/m^3, due to the dust content of hay and straw. Thus, the use of alternative feeds and bedding materials suffice to reduce dust concentrations to 0.52 mg/m^3, with feed having a greater impact (Woods et al., 1993; Clements & Pirie, 2007a).

The dust concentration found in hay can be affected by the season (heat and humidity) and by storage conditions (type of bale and storage facilities) (Woods et al., 1993, McGorum et al., 1998).

A study by Robinson and colleagues reported that horses fed hay in round bales had an increased risk of >20% neutrophil counts in the tracheal wash (Robinson et al., 2006).

Also, hay bailed while containing a high moisture content presents an increased number of fungi and actinomycetes spores (*Aspergillus fumigatus, Faenia rectivirgula* and *Thermoactinomyces vulgaris* (Clarke, 1987; Seguin et al., 2010; Seguin et al., 2012). The inhalation of *A. fumigatus and F. rectivirgula* has been shown to result in neutrophilic airway inflammation in severe EAS-affected horses (McGorum, Dixon & Halliwell, 1993; Derksen et

al., 1988). Fungal infection can also further contribute to airway inflammation, one of the hallmark features of equine asthma (Dauvillier, ter Woort & van Erck-Westergren, 2018).
Additionally, due to airway hyperreactivity, which is one of the clinical features found in severe EAS-affected horses, inhalation of dry or cold air and of particulate matter, such as plant materials, can further aggravate airway inflammation and obstruction (Davis et al., 2006; Pirie, 2014).

4. Pathophysiology

The pathological changes which characterise severe EAS have been vastly described in the literature. Airway inflammation along with neutrophil recruitment, mucus plugging, bronchospasm and airway remodelling, which includes airway wall thickening and smooth muscle hyperplasia, are some of the most common features of the disease (Couëtil et al., 2016).
However, a complete understanding of the immunological mechanisms involved in this disease has still not fully been achieved, with conflicting results having been reported in the literature. A significant number of cytokines, chemokines, genes and, inflammatory and resident airway cells appear to play a part in severe asthma exacerbation (Art, Bureau & Robinson, 2008).
There are studies supporting the involvement of a hypersensitive response during disease exacerbation, but unlike human asthma, an early phase response to aeroallergens has yet to be identified in horses (McGorum et al., 1993; Robinson et al., 1996; Deaton et al., 2007). Nonetheless, the delayed phase response has been thoroughly described in asthmatic horses, ultimately resulting in neutrophil recruitment into the airways and in an increase of $CD4^+$ T cells in the bronchoalveolar lavage fluid (BALF) (McGorum et al., 1993; Kleiber et al., 1999).
The role of mast cells remains somewhat controversial, since severe EAS-affected horses present a higher number of chymase positive mast cells in their bronchial walls in comparison with healthy controls, although no differences were found pertaining to the amount of immunoglobulin E (IgE) bound cells in their lungs (van der Haegen et al., 2005; Künzle et al., 2007).
Genetics may be able to explain the conflicting reports on the role of IgE, since it seems to play an important role in determining the allergen specific IgE response (Schmallenbach et al., 1998; Eder et al., 2000; Künzle et al., 2007; Scharrenberg et al., 2010).
The precise cytokine profile characteristic of severe EAS is still under investigation and conflicting reports on the roles of interleukin (IL) 4, 5, 13 and interferon (IFN) γ can be found in the literature. In fact, some studies support a Th2 associated response, others indicate a mixed cellular response, and others still found no suggestion of a specific Th1 nor Th2 response (Giguère et al., 2002; Ainsworth et al., 2003; Cordeau, Joubert, Dewachi, Hamid & Lavoie, 2004; Horohov, Beadle, Mouch & Pourciau, 2005; Kleiber et al., 2005).

Such differences may also be the result of genetic variations and of the complex mode of inheritance identified in severe EAS, which may in turn be linked to a diversity of immunological responses, converging into the same disease phenotype (Art et al., 2008; Graubner et al., 2012; Pirie, 2014).

Another likely hypothesis is that basic methodological differences in these studies, such as time of sample collection, resulted in a lack of consistent results (Horohov et al., 2005; Art et al., 2008; Pietra, Cinotti, Ducci, Guinti & Peli, 2011).

Recently, new evidence of the involvement of Th17 or regulatory cells in the pathogenesis of the disease has been described and the authors have hypothesised a mixed Th17 and Th2 immunological response in affected horses (Pacholewska et al., 2017; Frodella, et al., 2019).

Neutrophil recruitment, and consequent airway infiltration, is a hallmark of severe equine asthma, and, T cell activation and epithelial cells play an important role in this process. The expression of IL-8 and IL-17 interleukins was found to be increased in asthmatic horses after their exposure to organic dust (Debrue, Hamilton, Joubert, Lajoie-Kadoch & Lavoie, 2005; Ainsworth et al., 2006). In addition, IL-4 induces the expression of chemotactic factors in pulmonary endothelial cells, further contributing to neutrophil recruitment (Lavoie-Lamoureux et al., 2010).

The neutrophilic activation and infiltration, besides engaging the inflammatory cascade of equine asthma also plays and important role on the pathology of severe EAS. Although not a requirement for pulmonary dysfunction, neutrophils are associated with the presence of pulmonary lesions due to epithelial lesion and impaired repair (Tessier et al., 2017; Bullone, Joubert, Gagn, Lavoie & Elie, 2018; Uberti & Morán, 2018).

Clara cell secretory protein may also play a part in airway inflammation by inhibiting the oxidative burst of luminal neutrophils, further improving phagocytosis (Katavolos et al., 2009; Katavolos, Ackerley, Clark & Bienzle, 2011).

Both the role of alveolar macrophages and epithelial cells remains dubious, with some authors reporting differences in cytokine and chemokine expression between asthmatic and control horses, whilst others have failed to do so (Laan, Bull, Pirie & Fink-Gremmels, 2005; Ainsworth, Wagner, Erb, Young & Retallick, 2007; Rilhimaki et al., 2008; Reyner, Wagner, Young & Ainsworth, 2009; Ainsworth, Matychak, Reyner, Erb & Young, 2009; Joubert, Cordeau & Lavoie, 2011).

Several inflammatory markers have been identified in asthmatic horses during disease exacerbation, such as the neutrophil derived enzyme myeloperoxidase (MPO) and several metalloproteinases (MMPs) derived from neutrophils, lymphocites, epithelial cells and macrophages (Raulo, Sorsa & Maisi, 2000; Nevalainem et al., 2002; Art et al., 2006; Barton, Shety, Bondzio, Einspanier & Gehlen, 2016).

Likewise, markers of oxidative stress, such as oxidised glutathione, reduction in ascorbic acid and increased hydrogen peroxide, are present in the airways and breath of asthmatic horses in exacerbation (Art, Kirschvink, Smith & Lekeux, 1999; Deaton et al., 2005; Deaton et al., 2006; Tan et al., 2010).

Another persistent finding in severe EAS-affected horses is the presence of mucus accumulation within the airway lumen (Gerber et al., 2003; Gerber et al., 2004a, b).

This phenomenon occurs due to changes in the rheological properties of mucus, namely an increase in viscoelasticity, which then leads to a decrease in its clearance by the mucociliary and cough mechanisms (Gerber, King, Schneider & Robinson, 2000; Jefcoat et al., 2001; Gerber et al., 2004b).

Also, increased mucus production, may be related to an over expression of the mucin gene EqMUC5AC and an increased number of mucus cells (Gerber et al., 2003; Lugo et al., 2006; Gerber et al., 2009).

Due to the persistence of low-grade airway inflammation, asthmatic horses maintain a certain degree of mucus accumulation even whilst in disease remission, which is associated with changes in the mucus glycoproteins (Jefcoat et al., 2001; Gerber et al., 2004a, b).

In horses, although cholinergic and adrenergic innervation occurs, the inhibitory innervation of the distal airways results mainly from nonadrenergic-noncholinergic (NANC) pathways (Olszewski, Robinson & Derksen, 1997; Matera, Amorena & Lucisano, 2002).

In asthmatic horses there appears to be a flaw in the innervation of the lower airways, including a defective inhibitory NANC response, a decreased response to cholinergic activation and to the inhibitory function of prostanoids, an increased production of epithelial-derived relaxing factor, as well as a dysfunction of α_2 and β_2 receptors and of muscarinic-autoreceptors, which further contribute to bronchospasm and airway obstruction (Yu, Wang, Robinson & Derksen, 1994; Olszewski et al., 1997; Zhang, Robinson & Zhu, 1999; Abraham, Kottke, Dhein & Ungemach, 2006; Abraham, Kottke, Ammer, Dhein & Ungemach, 2007; Venugopal et al., 2009).

The peptide endothelin appears to also play a significant role in the occurrence of bronchoconstriction in equine asthmatic patients (Fluvio, Emanuele, Francesca, Matteo & Beniamino, 2012a; Fluvio et al., 2012b).

Airway patency is further compromised by the inflammatory-associated changes that take place in bronchi and bronchioles. Increased thickness of the epithelium, submucosa and smooth muscle along with the loss of ciliated cells and their replacement by a hyperplastic epithelium in larger airways, result in a decreased airway lumen and contribute to obstruction (Kaup, Drommer, Damsch & Deegen, 1990).

Asthmatic horses may present emphysema, not because of alveoli destruction but due to its incapacity of collapsing (‘air entrapment’) in cases of severe airway obstruction (Marinkovic, Aleksic-Kovacevic & Plamenac, 2007a, b).

5. Clinical signs

Exercise intolerance, cough and increased expiratory effort characterise severe EAS-affected animals, although clinical signs vary with disease severity, although cough is usually the first sign reported. It begins as a soft persistent cough, but with disease progression paroxysmal bouts of cough occur (Allen & Franklin, 2007; Tilley, Sales Luís & Branco Ferreira, 2012b).

Exercise intolerance is also one of the earliest clinical signs, becoming more evident in severely asthmatic horses. It should be noted that depending on the expected performance for a particular horse, decrease in exercise ability may be missed (Robinson, Derksen, Jackson, Peroni & Gerber, 2001).

Overall, the observed clinical signs result from airway inflammation, obstruction and hyperresponsiveness and from mucus hypersecretion when susceptible horses are exposed to offending aeroallergens (Pirie, 2014).

Nasal discharge may also be observed in some animals due to mucus accumulation in the tracheobronchial tree (Tilley, Sales Luís & Branco Ferreira, 2012c). Nonetheless, the regular swallowing of these secretions renders this clinical sign occasional (Pirie, 2014).

Severe disease-induced airway obstruction leads to overt respiratory distress in the resting horse with an abnormal breathing pattern (Robinson et al., 1996). A rapid inspiration and a longer exhalation, with thoracic collapse followed by abdominal lift, characterise the adopted breathing pattern (Léguillette, 2003). The increased abdominal effort observed corresponds to a rapid abdominal movement, which does not impart a significant contribution to ventilation when compared to rib cage movement (Hoffman, Oura, Riedelberger & Mazan, 2007). Eventually, hypertrophy of the external abdominal oblique muscle occurs, and a ‘heave’ line can be observed in chronic cases. Likewise, in an attempt to minimise the resistance provided by the upper airways, nasal flaring can be observed. Increased respiratory effort also results in exaggerated anal movement during breathing (Robinson et al., 2000; Tilley et al., 2012b).

Often, severely affected horses present significant body mass loss or even cachexia. These animals present increased oxygen consumption and energy expenditure due to the breathing effort required for trying to overcome the expiratory obstruction (Mazan, Deveney, DeWitt, Benedice & Hoffman, 2004). In addition, the respiratory distress and tachypnoea lead to a decrease in food intake, which results in caloric imbalance and this energetic deficit contributes to weight loss (Robinson et al. 2001; Couëtil & Ward, 2003).

Upon clinical examination horses are afebrile and, depending on the degree of airway obstruction, abnormal findings in thoracic auscultation will vary. An increase in bronchovesicular sounds, end-expiratory wheezes and inspiratory crackles may be heard (Léguillette, 2003). Wheezes result from airway narrowing during expiration, while the sudden opening of collapsed airways produces crackles (Pirie, 2014). Mucus hypersecretion and pooling along the trachea produces a 'fluttering' sound. The sounds of mucus flutter and wheezes can sometimes be heard at the nostrils without the use of a stethoscope.

Paradoxically, in some animals presenting with significant breathing effort, lung auscultation will be atypically quiet, as a consequence of little airflow occurring due to the severe airway obstruction. Due to lung hyperinflation, because of air trapping in the alveoli, auscultation may reveal an increased lung field (Robinson et al., 2001; Christmann, Buechner-Maxwell, Witonsky & Hite, 2009).

With disease progression gas exchange may be affected, resulting in increased respiratory and heart rates, and in extreme cases the horse may become cyanotic (Robinson et al., 2001). Nonetheless, it is important to remember that one of the key characteristics of severe EAS is the reversibility of clinical signs when the asthmatic horse is removed from the offending environment. The improvement of clinical signs is related to disease severity, but in general progress should be expected in a week span (Thompson & McPherson, 1984).

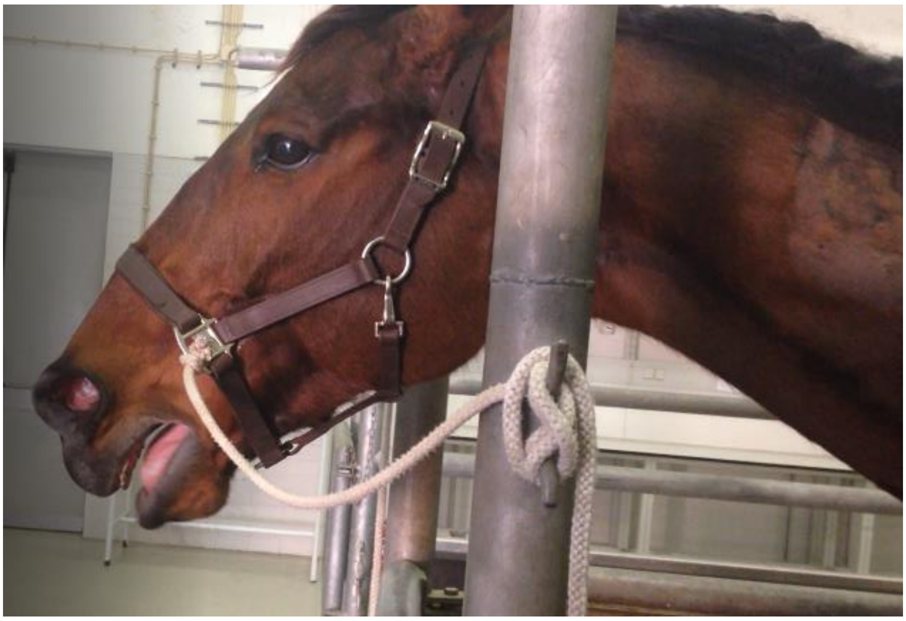

Fig. 1 – Marked nasal flare and cough in a severe EAS-affected horse.

6. Diagnosis

6.1. 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 (Robinson et al., 2001). The vast majority of horses present with a history of chronic and persistent cough, which can be seasonal or associated with stabling and hay feeding (Lavoie, 2007; Bosshard & Gerber, 2014). These animals are also at risk of contracting opportunistic respiratory infections (Theegarten et al., 2008; Juhn, 2014).

Several studies have proposed the use of clinical scores to evaluate and monitor disease severity. The scores are usually based on the most frequent clinical signs observed during severe EAS exacerbation, namely cough, nasal flaring, nasal discharge, abdominal lift and even exercise intolerance (Miskovic, Couëtil & Thompson, 2007; Tilley et al., 2012c; Rettmer, Hoffman, Lanz, Oertly & Gerber, 2015).

However, although clinical history and signs can provide a correct diagnosis of moderately and severely asthmatic horses, clinical scores alone have proved unsuccessful in identifying low-grade obstruction (mild and subclinical cases) (Coëtil, Rosenthal, DeNicola & Chilcoat, 2001; Laumen et al., 2010; Bosshard & Gerber, 2014).

Clinical scores based on nasal flaring and abdominal effort were found to correlate with standard lung mechanics, though exercise intolerance showed no connection with cough nor with airway dysfunction (Rush et al., 1998; Robinson et al., 2000; Benedice, Mazan & Hoffman, 2008). Although cough has been reported as an indicator of airway inflammation, it only manifests when a certain degree of inflammation is present in the horses' airways. Horses in remission and kept in a controlled environment maintain some degree of airway inflammation and hyperresponsiveness, although unaccompanied by the usual clinical signs (Robinson et al., 2003).

Since clinical signs scores proved insufficient to correctly stage severe EAS and because other diseases, such as dynamic upper airway obstruction and mild equine asthma, can mimic its low-grade presentations, ancillary diagnostic tests play an important role in disease characterisation and differential diagnosis (Robinson et al., 2000; Couëtil et al., 2001; Pirie, 2014; Rettmer et al., 2015).

6.2. Venous blood and serum analysis

Routine haematology and biochemistry are usually within normal values, but they can be helpful to rule out other differential diagnosis, such as pneumonia (Robinson et al., 2001; Allen & Franklin, 2007).

6.3. Thoracic radiography

In the adult equine, x-ray imaging of the lungs can be challenging, particularly due to the depth of the equine chest and the superimposition of the forelimb on the cranial thoracic field. On average the x-ray beam must penetrate almost one meter of bone, muscle and lung tissue. As such, a large amount of body fat may further compromise this task (Dunkel, Gibbs & Weller, 2013). This can be particular true in the Lusitano breed which tends to present a higher body condition.

In order to completely visualise the equine thorax, four overlapping imaging fields should be considered – craniodorsal, caudodorsal, cranioventral and caudoventral (Farrow, 1981). Each view will provide the clinician with important information for evaluating respiratory disease (Barton, Schulze, Doherr & Gehlen, 2018). However, in smaller horses a lesser number of projections may suffice.

In 2016, the ACVIM suggested that thoracic radiographs alone were insufficient to diagnose severe EAS. However, they allowed the exclusion of alternative diagnoses, thus providing the confirmation of asthma (Couëtil et al., 2016). This recommendation corresponds to the reality in everyday clinical practice, where economic factors play an important role in the choice of the most suitable diagnostic test.

Nonetheless, several studies have reported radiographic findings characteristic of severe EAS. Radiographic imaging of the equine asthmatic lung showed an increased bronchovascular and interstitial pattern (Robinson, 2001) and thickening of smaller bronchi bifurcations related to disease severity (Koch et al., 2007). The presence of an interstitial and a bronchial interstitial pattern in the lungs has also been associated with severe clinical signs of asthma (Bakos, 2008).

In chronic cases, the x-rays may reflect irreversible changes in the lung parenchyma, such as generalised increase of lung opacity, interstitial infiltration, caudally displaced lung borders, increased radiolucency with concave diaphragm and even bronchiectasis (Lavoie, Dalle, Breton & Hélie, 2004; Allen & Franklin, 2007; Barakzai & McAllister, 2007; Lavoie, 2007).

Tilley et al. (2012c) found significant differences in thoracic radiographic changes in pulmonary pattern and tracheal and bronchial wall thickness which correlated with clinical signs and other indicators of airway inflammation and therefore could be used in the staging of severe EAS.

Nonetheless, the radiographic changes that can be seen in severe EAS cases are not pathognomonic of this disease (Kutasi, Balogh, Lajos, Nagy & Szenci, 2011; Couëtil et al., 2016).

Also, in mild cases of severe EAS with discreet airway inflammation, little or no changes will be found in the thoracic x-ray, which is why this technique alone does not allow the identification of mild and subclinical cases (Barton et al., 2018).

Regardless, radiographic observations can be particularly helpful in ruling out alternative diagnoses and evaluating pulmonary sequelae of severe EAS.

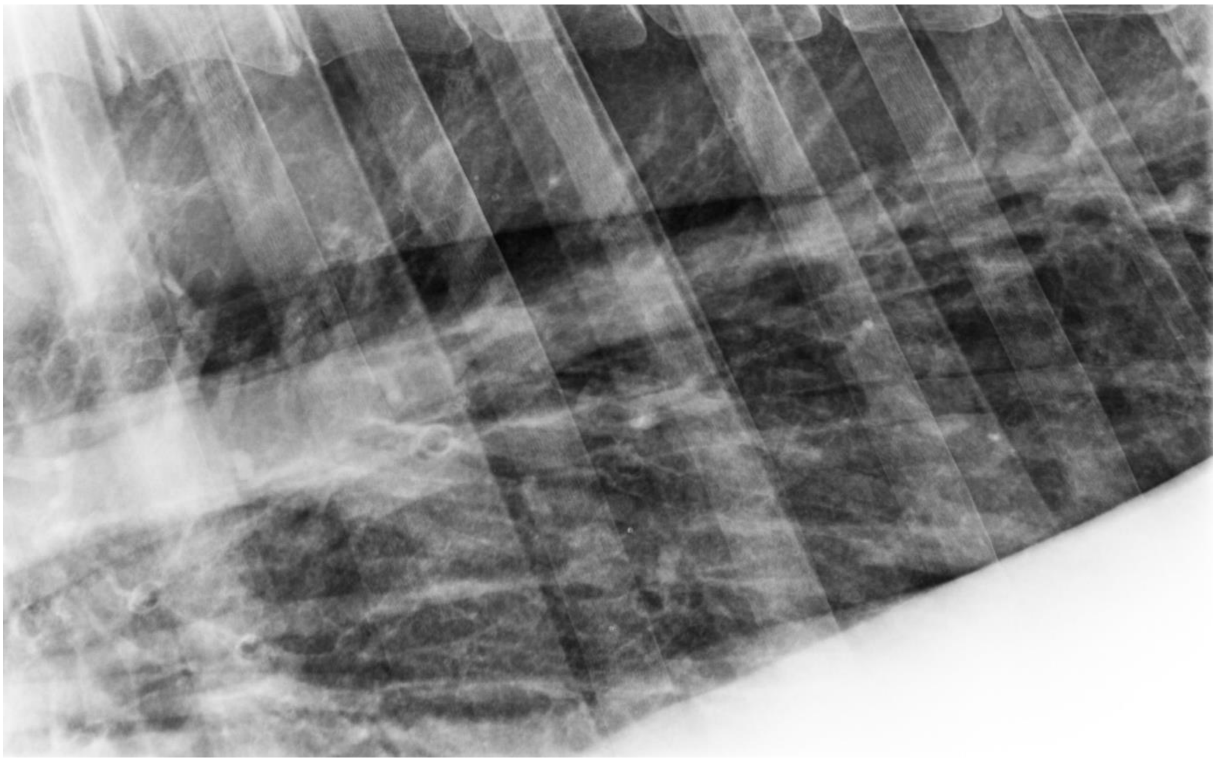

Fig. 2 – Lung x-ray imaging of an asthmatic horse during disease exacerbation. Note the bronchial interstitial pattern accompanied by thickening of bronchial walls.

6.4. Respiratory endoscopy

Respiratory endoscopy allows the assessment of mucosal oedema and/or hyperaemia, mucopurulent secretions, bronchospasm and foreign bodies, making its use commonplace in equine practice in cases presenting with respiratory disease (Hare and Viel, 1998; Kutasi et al., 2011).

The use of endoscopy, by itself or in association with other techniques (bronchoalveolar lavage and tracheal lavage), in severe EAS allows the evaluation of airway inflammation, and also contributes to the exclusion of other differentials (Couëtil et al., 2016).

An association between upper and lower airway inflammation has been extensively documented in human medicine, giving rise to the expression “one airway, one disease” and asthmatic humans will often present with rhinitis as well as lower airway inflammation (Jeffery & Haahtela, 2006). However, no association was found in the horse and the results from upper and lower airway examination should be interpreted independently (Koblinger et al., 2011).

Endoscopic evaluation of severe EAS horses is characterised by the presence of mucus or mucopurulent secretions in the tracheal and bronchial lumen, due to hypersecretion, decreased clearance and changes in mucus rheological properties (Gerber et al., 2000; Gerber et al., 2003; Robinson et al., 2003). Depending on its quantity, the mucus exudate can form a small pool at the lowest point of the trachea, or if it becomes more profuse form rafts or streams along the tracheal wall (Gerber et al., 2004 a, b).

As a result, several endoscopic scoring systems have been reported in the literature for assessing mucus quantity and quality and airway oedema/thickness, having been used in the evaluation of the asthmatic horse (Dixon et al., 1995; Hare and Viel, 1998; Gerber et al., 2004b; Koch et al., 2007; Tilley et al., 2012c).

Mucus accumulation was found to correlate well with clinical scores for severe EAS (Costa, Seahorn & Moore, 2000), as well as with cough frequency (Robinson et al., 2003). A correlation between tracheal mucus and lower airway inflammation has also been reported (Koblinger et al., 2011) and airway obstruction due to mucus presence can persist in severe asthmatic horses in remission (Gerber et al., 2004a).

In 2004, Gerber and co-workers developed a mucus scoring system for quantitative and qualitative classification of secretions observed in the tracheal lumen which has been extensively used in clinical practice and which presented a correlation with cytological indicators of airway inflammation (Gerber et al., 2004b).

An increase in the thickness of the tracheal septum (carina), a consequence of airway wall inflammation, is commonly observed in severe EAS (Feutz, Riley, Adamec & Couëtil, 2011). However, Koch et al (2007) reported no correlation between septum thickness and severe EAS clinical, endoscopic or cytological features.

In 2012, building on the works by Gerber et al (2004b) and Koch et al (2007), the team of investigators used an endoscopy scoring system, which evaluated airway mucosal hyperaemia, septum thickness and tracheal mucus quantity and its characteristics. Only the mucus scores had a high correlation with clinical and inflammatory features of severe EAS and were included in a proposed model of severe EAS staging (Tilley et al., 2012c).

However, mucus accumulation cannot be considered a sensitive indicator of severe EAS, since it is not an exclusive feature of this disease and has been observed in cases of mild and moderate EAS (formerly IAD) (Couëtil et al., 2016). Also, in severe asthmatic horses in remission little or no differences were found in endoscopic mucus scores compared to healthy horses (Gerber et al., 1998; 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).

Fig. 3 – Respiratory endoscopic examination of an asthmatic horse.

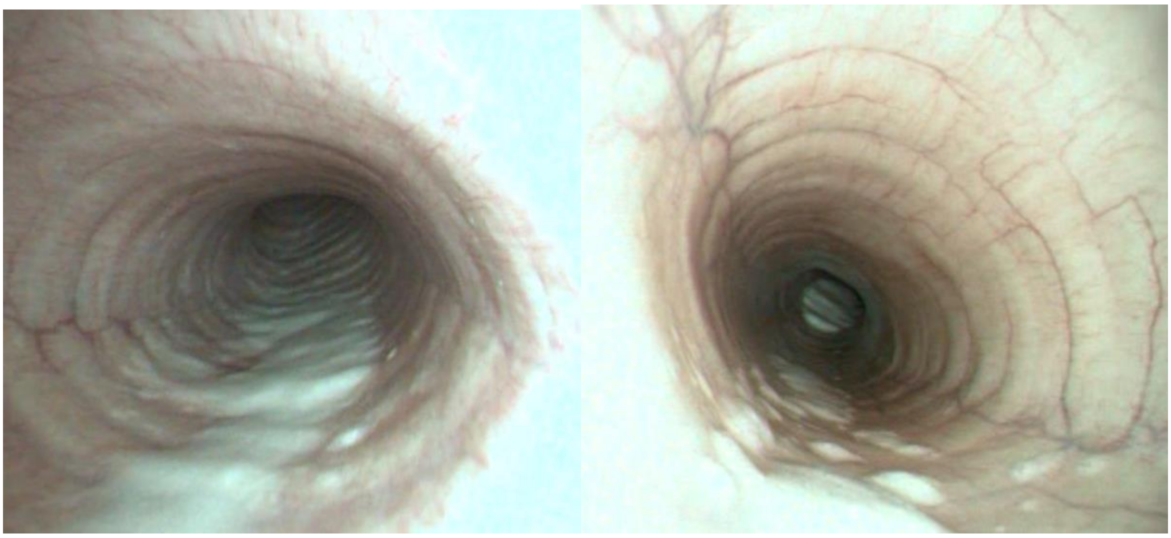

Fig. 4 – Tracheal endoscopy of asthmatic horses. Note the increased accumulation of mucus on the ventral aspect of the trachea.

6.5. Tracheal wash (TW) cytology

The tracheal wash (TW) allows sampling of secretions found in the asthmatic horses' trachea, thus providing information on airway inflammation.

The technique may be performed either percutaneously or transendoscopicaly, the latter being a less invasive option (Allen & Franklin, 2007; Couëtil & Hawkins, 2013). Saline is infused into the tracheal lumen and the loosen secretions are aspirated for cytological analysis (Robinson et al., 2001).

Despite being relatively easy to perform, TW has not been included in the guidelines for severe EAS diagnosis (Couëtil et al., 2016). This is mainly due to reports of poor correlation between neutrophil counts in the TW and in the BALF, which may indicate that the cellular population found in the tracheal lumen is not representative of the lower airways (Derksen, Brown, Sonea, Darien & Robinson, 1989; Malikides, Hughes, Hodgson & Hodgson, 2003; Fraipont et al 2011). Also, a large variability of inflammatory cells can be observed in the TW cytology of healthy horses, making results' interpretation challenging (Robinson, 2001; Allen & Franklin, 2007).

However, Rossi and co-workers reported a good correlation between neutrophil percentage in TW and BALF cytology in healthy horses and in horses with respiratory disease (Rossi et al., 2018).

Nonetheless, TW neutrophilia has been associated with cough and mucus (Derksen et al., 1989; Christley et al., 2001; Robinson et al., 2006), but not with poor performance (Holcombe et al., 2006).

6.6. Bronchoalveolar lavage (BAL)

Bronchoalveolar lavage fluid (BALF) cytology is considered the reference technique for the diagnosis and monitoring of generalised lung disease, enabling the assessment of lower airway inflammation characteristic of severe EAS (Hewson & Viel, 2002; Jean, Vrins, Beauchamp & Lavoie, 2011; Pirie, 2014).

Unlike TW, BAL provides direct insight of the small airways and alveoli, being a better representative of potential gas exchange impairment and thus allowing the diagnostic of severe EAS (Hoffman, 1999; Hoffman & Mazan, 1999; Couëtil et al., 2016).

BAL can be easily performed in the standing lightly sedated horse and in field conditions (Hoffman, 1999; Mazan & Hoffman, 2003). The technique can be executed using a flexible endoscope or 'blindly' with an equine BAL catheter equipped with an inflatable cuff (Couëtil & Hopkins, 2013). The catheter or endoscope is advanced through the nasopharynx and into the trachea, until it is firmly wedged in a small calibre bronchus, which depends on the diameter of the catheter/endoscope (Hoffman, 2008; Tilley et al., 2012c).

In asthmatic horses it is to be expected that the volume of fluid retrieved is significantly smaller than the volume originally instilled into the horses' airways, due to airway collapse and obstruction when the fluid is aspirated (Hoffman, 2008; Couëtil & Hopkins, 2013).

The collected BALF sample is representative of the entire lung and studies have shown no significant differences in cell populations between both lungs, with the exception of mast cells (Jean et al., 2011).

In healthy animals, the collected sample will have a superficial layer of foam (pulmonary surfactant) and contain <400 cells/μl. The most abundant cell populations are alveolar macrophages (40-70%), followed by lymphocytes (30-60%), and in smaller numbers neutrophils (<5%), mast cells (<2%) and eosinophils (<1%) (Robinson, 2001; Allen & Franklin, 2007; Couëtil & Hopkins, 2013).

The cytological BALF profile of the asthmatic horse is usually characterised by neutrophilia (>20% neutrophils) and a reduction in macrophage and lymphocyte percentages (Couëtil et al., 2007; Tilley et al., 2012b, c). However, a wide range of neutrophil percentage (10-98%) has been identified in this syndrome and in severely affected chronic cases an increased amount of mucus can be observed in the form of Curschmann's spirals (Hoffman, 2008; Couëtil & Hopkins, 2013).

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, horses may also exhibit a paucigranulocytic phenotype with moderate (5-20%) or no increase in neutrophil percentage (Leclere et al., 2011b; Bullone & 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, Uddin & Koenderman, 2015).

Nonetheless, BALF 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 (Léguillette, 2003; Morán, Araya, Ortloff & Folch, 2009). As such asthmatic horses in clinical remission can present only a slightly elevated neutrophil count (Miskovic et al., 2007).

Increased neutrophil percentages in BALF have been correlated with the occurrence of cough and with higher mucus scores in severe EAS affected horses (Robinson et al., 2003; Benedice et al., 2008).

However, neutrophilia does not appear to be a requirement for the lung function deterioration observed in severe EAS (Fairbairn, Page, Lees & Cunningham, 1993b; Couëtil et al., 2006b). On the other hand, BALF neutrophilia does not normalise if the asthmatic horse is kept in the offending environment, despite treatment with corticosteroids (Couëtil et al., 2006a; Leclere et al., 2012). In fact, stabling, hay feeding and exposure to mould have also been associated with BALF neutrophilia (Fairbairn, Page, Lees & Cunningham, 1993a, b; Holcombe et al., 2001; Rush & Mair, 2004).

It was also reported that neutrophil percentage in BALF could be used to stage severe EAS as it correlated well with the clinical signs exhibited by asthmatic horses, with the changes observed in the thoracic x-ray and with mucus score determined using endoscopy (Tilley et al., 2012c).

It is relevant to mention that the BAL procedure should always be performed after lung function assessment, since it has been shown to result in a transient improvement of pulmonary resistance (R_L), probably from mucus clearance (Léguillette & Lavoie, 2006).

Fig. 5 – Bronchoalveolar lavage proceeding in a severe EAS-affected horse.

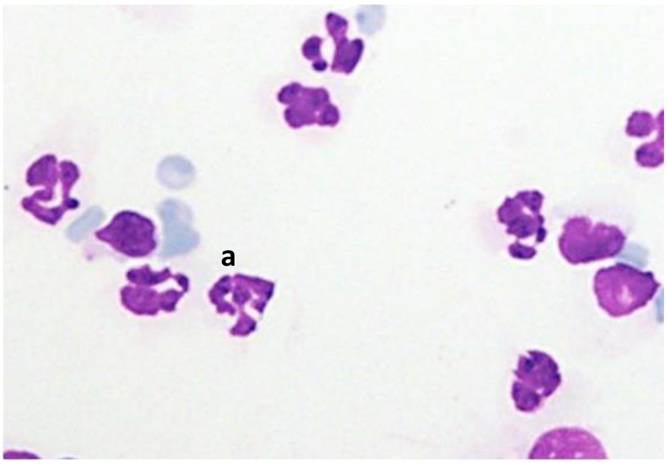

Fig. 6 – Bronchoalveolar lavage cytology of a severe EAS-affected horse, showing an increased number of neutrophils (a) – magnification 2000x.

6.7. Pulmonary function tests

Pulmonary function testing has become an important tool in the evaluation of the respiratory system. With technologic evolution, equipment has become more user-friendly; as such, lung function testing has become available in equine practice. Nonetheless, depending on the invasiveness of the test, owners may be reticent, particularly if serial testing is necessary.

The evaluation of pulmonary function mainly assesses ventilation based on lung mechanics, and gas exchange through arterial blood gas analysis (Couëtil & Hawkins, 2013).

In the horse, pulmonary function is assessed using dynamic measurements, since static testing requires general anaesthesia (Mazan & Hoffman, 2003).

These tests can be particularly useful in mild clinical cases of severe EAS, which present airway obstruction despite the clinical signs not being evident (Robinson et al., 2000).

In general, pulmonary function tests are used to determine the presence, degree and location of respiratory dysfunction. However, in severe EAS they allow assessment of disease severity, response to treatment (immediate and long-term response) and also provide information on breathing strategy (Robinson, 2001; Hoffman, 2002; Mazan & Hoffman, 2003).

Also, because pulmonary function tests provide a measurement of airway obstruction, they can be helpful in imparting the need to perform environmental changes crucial for severe EAS management (Hoffman, 2002).

Currently, there are an array of techniques available for the assessment of respiratory function, although no single test can be considered perfect, as they all have their strengths and weaknesses, and their selection will be determined by the clinician’s needs.

Some tests are more suitable for research purposes, while others can be used in clinical practice, and although the gold standard remains conventional lung mechanics, techniques like forced oscillatory mechanics (FOM) (van Erck, Votion, Art & Lekeux, 2006), flowmetric plethysmography (Hoffman, Kuehn, Riedelberger, Kupcinkas & Miskovic, 2001), forced expiratory manoeuvres (FEM) (Couëtil, Rosenthal & Simpson, 2000), tidal breathing flow-volume loops (Herholz et al., 2003), volumetric capnography (Herholz et al., 2002) and arterial gases analysis (Gerber, Schott II & Robinson, 2011; Rettmer et al., 2015) have been applied in the diagnosis of severe EAS.

6.7.1. Arterial blood gas (ABG) analysis

Arterial blood gas (ABG) analysis provides information on the efficiency of lung gas exchange (Chevalier & Divers, 2003).

It allows the assessment of alveolar ventilation, oxygenation and acid-base balance, thus providing an estimation of the respiratory compromise observed in severe EAS (Irizarry & Reiss, 2009).

In the adult horse this procedure can be easily performed in non-sedated animals, with blood being collected from the transverse facial or the carotid arteries.

In order to ensure an accurate measurement of ABG parameters, the sample must be collected anaerobically and processed as quickly as possible (Picandet, Jeanneret & Lavoie 2007). Also, sedation should be avoided, especially with α2-agonists, since they decrease minute ventilation which results in hypoxemia and hypercapnia (Nyman et al., 2009).

Horses affected with severe EAS will usually present with lower values of P_aO_2, sO_2 and pH and increased values of P_aCO_2, however hypoxemia is the most common finding (Sánchez, Couëtil, Ward & Clark, 2005; Stopyra, Sobiech & Waclawska-Matyjasik, 2012).

In healthy adult horses, P_aO_2 ranges between 90 and 100 mmHg (Couëtil & Hawkins, 2013). But in asthmatic horses, as disease progresses, hypoxemia becomes more severe (Nuytten Deprez, Picavet, van den Hende & Muylle, 1988; Couëtil & DeNicola, 1999, Sanchez et al., 2005).

Due to airway inflammation, remodelling and mucus accumulation gas exchange becomes impaired (ventilation perfusion mismatch and dead space ventilation) and animals in exacerbation may present values of P_aO_2<80 mmHg (Nyman et al., 1991; Stopyra et al., 2012; Couëtil & Hawkins, 2013). However, asthmatic horses may present normal P_aO_2 values in early stages of inflammation and while in disease remission (Ferro, Ferrucci, Zucca, Di Fabio & Castoldi, 2002).

While providing important information on disease severity, ABG analysis lacks sensitivity in identifying mild cases of severe EAS (Stopyra et al., 2012).

Fig. 7 – Arterial blood collection from the transverse facial artery.

6.7.2. Conventional lung mechanics

Conventional lung mechanics is considered the gold standard for pulmonary function testing, having been used in research for more than fifty years (Gillespie, Tyler & Eberly, 1966; Derksen, Slocombe, Brown, Rook & Robinson, 1982; Stadler & Deegen, 1986).

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 ΔPpl>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).

One of the inconveniences of this technique is the oesophageal catheter, which inevitably induces the horse to swallow against it, interfering with pleural pressure readings (Mazan & Hoffman, 2003).

Severely affected-asthmatic horses usually present increased R_L, W and ΔPpl, and a decreased C_{dyn}. This pattern results from airway obstruction (first small airways and then the larger ones), which leads to the need of a higher pressure to move the air column, and eventually to changes in breathing strategy (Robinson et al., 1999; Robinson, 2000).

The pneumotachographic assessment of flow reflects the horse's adopted breathing strategy, with high values of flow in the beginning of expiration. This is usually accompanied by end-expiratory pressures indicative of bronchoconstriction (higher than zero) (Derksen, Robinson, Armstrong & Slocombe, 1985b; Petsche, Derksen & Robinson, 1994; Hoffman, 2002).

However, this technique has a very low sensitivity, especially in cases of small airway obstruction, where baseline readings are within normal range (Robinson, 2001). The small airways are responsible for <20% of airway resistance, which explains why alterations in this parameter are only registered when obstruction becomes more severe (Macklem & Mead, 1967; Hoffman, 2002; Herholz et al., 2003; van Erck, Votion, Art & Lekeux, 2004). Therefore, this method's sensitivity is quite similar to that of clinical examination, since it does not identify low-grade airway obstruction (Derksen et al., 1982, Robinson et al., 2000, Couëtil et al., 2001; Miskovic et al., 2007).

Nonetheless, when combined with histamine bronchoprovocation its sensitivity increases, and despite its limitations it is still a very useful tool in the assessment respiratory function (Doucet, Vrins & Ford-Hutchinson, 1991).

6.7.3. Flowmetric plethysmography

Horse's size determined that traditional plethysmographic testing could not be employed in the same way that is used in humans, where patients sit in a whole-body chamber, the plethysmograph. Konno and Mead (1967) introduced the "boxless" plethysmographic technique that allowed assessment of thoracic and abdominal compartments, and which eventually could be used in large animals.

Flowmetrics plethysmography was developed for equines and derives from the boxless plethysmography (Hoffman et al., 2001). Essentially, flowmetrics combines the use of respiratory inductance plethysmography (RIP) and pneumotachography (Hoffman, 2002).

RIP allows evaluation of thoracic and abdominal contributions to ventilation through the placement of two elastic transducer bands, one in the thorax and the other in the abdomen, which reflect changes in each compartment cross-sectional area (Konno & Mead, 1967; Miller, Hoffman & Hunter, 2000). The signal of the bands is then converted into signals of volume and flow (Hoffman, 2002). On the other hand, the pneumotachograph measures airflow at the level of the horse's nostrils (Mazan & Hoffman, 2003).

Flowmetrics is based on the knowledge that due to gas compression during expiration and its expansion at inspiration, the flow measured by the bands and by the pneumotachograph will be different, and that gas compression increases as airway obstruction worsens (Hoffman et al., 2001).

The software calculates airway obstruction by subtracting the flow registered with the bands from that measured at peak expiration by the pneumotachograph. This measurement is referred to as delta flow (Δflow) (Hoffman, 2002).

In cases of bronchoconstriction, such as 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).

The information gathered using flowmetrics can also be used independently to assess other respiratory parameters.

The inductance plethysmography also gives information on the thoracic and abdominal contributions to ventilation, on thoracoabdominal synchrony and on end-expiratory lung volume (Miller et al., 2000; Mazan & Hoffman, 2003).

Thoracoabdominal asynchrony is usually present in severe EAS-affected horses, but it was not considered as reliable in the assessment of airway obstruction as flowmetrics (Mazan & Hoffman, 2003).

Flowmetrics is a non-invasive technique and thus does not require the use of sedation, unless it is associated with histamine bronchoprovocation (Hoffman, 2002).

The sensitivity of this test is similar to that of conventional lung mechanics and flowmetric variables correlate well with conventional measurements of R_L, C_{dyn} and ΔPpl (Hoffman et al., 2001).

However, the association of histamine bronchoprovocation to assess airway responsiveness increases the sensitivity of flowmetrics, allowing detection of severe EAS-affected horses in remission (Rettmer et al., 2015; Wichtel, Gomez, Burton, Wichtel & Hoffman, 2016).

In addition, this is one of the few pulmonary function tests available for field assessment of asthmatic horses (Nole-Walston et al., 2009). The commercially available Open Pleth™, comprises a facemask, a pneumotachograph, a thoracic elastic band (to be placed between the 9 and the 11th intercostals spaces), an abdominal elastic band (placed behind the last rib) and a RIP interface, and it is easy to employ and well tolerated by the horse.

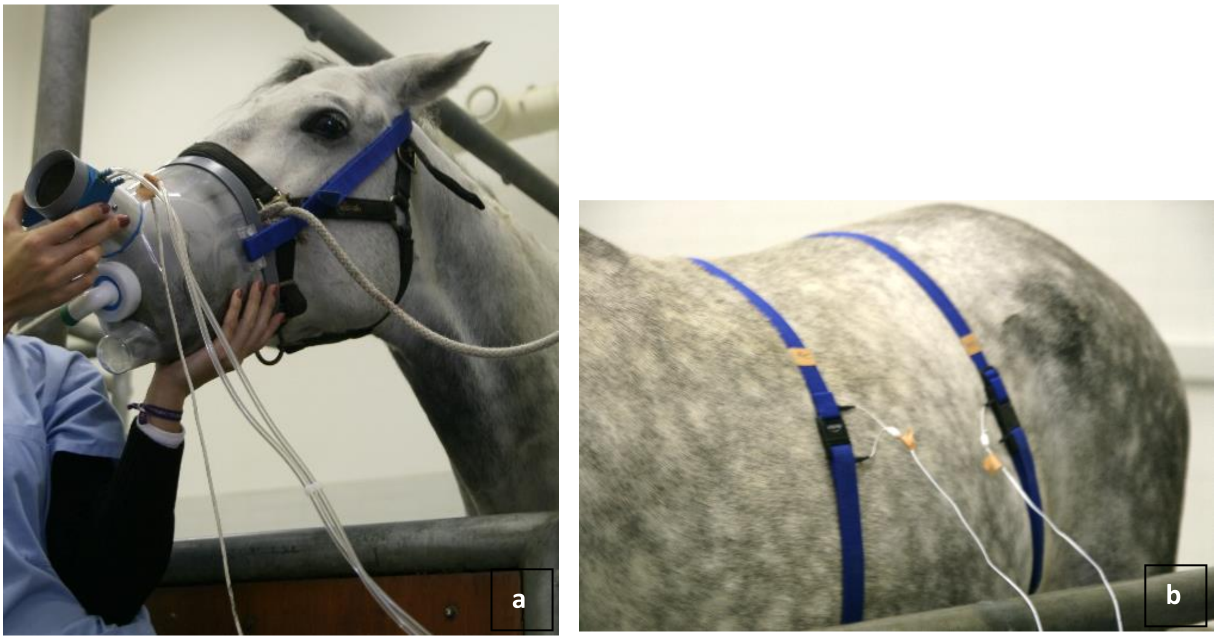

Fig. 8 – Open Pleth System™. a- Aeromask™ and pneumotacograph; b- thoracic and abdominal RIP bands

6.7.4. Forced oscillatory mechanics (FOM)

FOM is the most sensitive test available for detecting small changes in respiratory resistance (Mazan & Hoffman, 2003). It is used in equine practice for assessing lung function, but the equipment it requires is not suited for field testing.

This technique is based on pressure/flow relationship and uses an oscillating signal, which can be pressure from an airflow or from a loudspeaker, which is superimposed on the horse's tidal breathing via a facemask (Hoffman & Mazan, 1999; Couëtil & Hawkins 2013). The input signal is superior to that which is generated by the horse, and the horse's pressure-flow signal from spontaneous breathing is filtered out (Young, Tesarowski & Viel, 1997). The recorded measurements are used to determine respiratory impedance (Z_{rs}) to airflow (Hoffman & Mazan, 1999).

With FOM, it is possible to assess the spatial patterns of lung constriction, since depending on the frequency used it will indicate whether upper or lower airways are involved and if the constriction is homogenous or not (Hoffman, 2002; van Erck, Votion, Art & Lekeux, 2006).

In horses with severe EAS, a frequency dependence of respiratory resistance is observed, due to the presence of airway obstruction (Young et al., 1997). This frequency dependency is identified even in cases of low-grade obstruction (Mazan & Hoffman, 2003).

However, in horses in disease exacerbation, FOM testing poses a problem since these animals have a noisier breathing which interferes in signal coherence. Likewise, histamine bronchoprovocation is better not associated with this procedure (Hoffman, 2002).

Nonetheless, FOM has been widely used in equine pulmonary function assessment, with impulse oscillometry showing very promising results in the identification of airway obstruction and in the diagnosis of severe EAS (van Erck, Votion, Kirschvink, Art & Lekeux, 2003; van Erck et al., 2004; van Erck 2006; Richard et al., 2009). However, it is important to note that daily variations of resistance were registered, so approximately the same time of day should be used for repeated measurements (Onmaz, Stoklas-Schmidt & van den Hoven, 2013).

6.7.5. Forced expiratory maneuvers (FEM)

This pulmonary function test is fairly new in the equine practice, although in human medicine it is a key test in the diagnosis of asthma. The FEV_1 is the test commonly used to detect airflow obstruction in humans and it is based on the patient's forced expiratory volume during one second Evidently, this test requires the patient's cooperation, thus being unpractical in horses. This method is based on the notion that above a certain intrathoracic pressure, flow reaches its maximum, ceasing to increase (Niewoehner, Collins & Erbland, 2000; Hoffman, 2002).

In horses, FEM is an invasive technique, requiring nasotracheal intubation in order to perform mechanical ventilation. Thus, the horse needs to be sedated, usually with an α-2 agonist and butorphanol (Couëtil et al., 2000). The lungs are ventilated until the pressure-flow curve reaches a plateau (hyperventilated) and then a vacuum reservoir suctions the air from the inflated lungs, as a result forced expiratory measurements of volume and flow are computed (Couëtil & Hawkins, 2013).

This technique has been shown to detect low-grade peripheral airway obstruction in severe EAS-affected horses in remission (Couëtil et al., 2001; Miskovic et al., 2007).

6.7.6. Evaluation of airway reactivity

This test assesses the reversible narrowing of airways (sensitivity) in response to a bronchoconstrictor agonist stimulus of a certain magnitude. This is a fundamental characteristic of the respiratory system (Mazan & Hoffman, 2003). However, some individuals will exhibit airway hyperreactivity (AHR), meaning a high sensitivity to bronchoconstrictive stimuli, resulting in an exaggerated airway narrowing response (Swiderski et al., 2017).

AHR is due to smooth muscle activation, which in the presence of inflammation, mucus accumulation and epithelial hyperplasia, results in a significant reduction of airway calibre and an exponential increase in airway resistance (Broadstone, LeBlanc, Derksen & Robinson, 1991; LeBlanc, Broadstone, Derksen & Robinson, 1991; Wiggs, Bosken, Paré, James & Hogg, 1992; Couëtil & Hawkins, 2013).

As such, severe asthmatic horses in exacerbation will invariably present AHR (Vandenput et al., 1998; Couëtil et al., 2016). This feature, although in lesser magnitude, is also observed in horses in disease remission, thus proving useful in assessing cases of low-grade obstruction without clinical signs (Derkson et al., 1985a; Mazan, Hoffman & Manjerovic, 1999; Nolen-Walston et al., 2009; Rettmer et al., 2015; Wichtel et al., 2016).

For the assessment of airway reactivity, the stimuli used can be specific – an allergen (e.g. fungal spores) – or not – histamine or methacoline (Pirie, Collie, Dixon & McGorum, 2002; Couëtil & Hawkin; 2013). However, histamine and methacoline are most common choices in research and clinical practice (Derksen et al., 1985a; Derksen et al., 1985b; Doucet et al., 1991; van Erck et al., 2003). Also, it has been reported that humans with mild AHR have a n increased responsiveness to histamine in comparison with methacoline (Choi et al., 2007).

Almost every pulmonary function test can be used to assess airway reactivity. First, a baseline reading of respiratory parameters is performed after which saline (negative control), followed by increased concentrations of histamine (2, 4, 8, 16, 32 mg/ml) or methacoline (0.001, 0.01, 0.1, 1.0 and 3 mg/ml) are nebulised. Respiratory function is measured after each nebulisation and the test is terminated when the desired endpoint is reached or if the horse shows any severe clinical signs of respiratory discomfort (Klein & Deegen, 1986; Hoffman, 2002; Rettmer et al., 2015).

The endpoint will depend on the test used for monitoring the pulmonary function. In the case of conventional testing a 35% decrease in C_{dyn} is considered the end point; in flowmetric plethysmography a 35% increase of Δflow; and in FOM a 100% increase in resistance (R_{RS}) at 1Hz frequency (Hoffman & Mazan, 1999; Wichtel et al., 2016).

As previously mentioned, histamine/methacoline bronchoprovocation has the advantage of increasing the sensitivity of pulmonary function tests for detection of low-grade airway obstruction (Hoffman, 2002; Mazan & Hoffman, 2003; Couëtil & Hawkins, 2013). The technique is fairly simple and the use of increasing doses of the bronchoconstrictor agonist makes testing safer. Nonetheless, if the patient develops severe bronchoconstriction the administration of a fast-acting intravenous bronchodilator and of a corticosteroid will reverse the undesirable effects.

6.7.7. Bronchodilator challenge

In clinical cases, where the asthmatic horse presents a severe compromise of baseline pulmonary function it is recommended to perform a bronchodilator challenge (Hoffman, 2002). This will allow the assessment of the degree of bronchospam and its contribution to the registered respiratory parameters. It is also a good indicator of therapeutic response (Hoffman & Mazan, 1999; Couëtil & Hawkins, 2013).

Like in bronchoprovocation testing, after a baseline reading, a bronchodilator aerosol is administered, usually albuterol (450-900 µg), and pulmonary function is measured again after 15 minutes (Mazan & Hoffman, 2003).

An improvement of 50% in airway resistance is considered a positive response to this test in severe EAS-affected horses in exacerbation (Hoffman & Mazan, 1999).

6.8. Immunological testing

There is still some controversy surrounding immunological testing in severe EAS diagnosis, mainly due to contradictory reports.

In one study, it was 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 (Tahon et al., 2009).

In contrast, Niedzwiedz and co-workers found that severe EAS horses had significantly higher concentrations of specific IgE against mites (Niedzwiedz et al., 2015).

However, Tilley and coworkers reported that serum IgE did not reliably detect allergen hypersensitivity in severe EAS horses. In the same study the first use of skin prick tests (SPT) in horses was also reported (Tilley et al., 2012a).

In human medicine, SPT are used in the diagnosis of asthma and other allergic diseases, since they are minimally invasive and provide confirmation of sensitization in IgE-mediated diseases. In horses, SPT proved to be more reliable than *in vitro* IgE tests in identifying allergen hypersensitivity (Tilley et al., 2012a).

6.9. New imaging techniques – EBUS

Although BALF cytology and lung function testing remain the cornerstone of severe EAS diagnosis and research, novel methods to assess airway remodelling have been developed.

One such technique is the endobronchial ultrasound (EBUS), which only recently has been employed in the equine practice, mainly for research purposes.

The EBUS is an imaging technique that enables assessment of remodelling in the distal small airway. It is minimally invasive, allowing imaging of the bronchial wall using a miniature radial ultrasound probe inserted through the working channel of a videoendoscope (Bullone & Lavoie, 2017a).

In horses, it has been shown to allow differentiation of healthy and asthmatic horses based on airway remodelling (Bullone & Lavoie, 2015). Nonetheless, the technique is still experimental in horses and further studies are required.

6.10. Staging methods for severe EAS

Because severe EAS is a chronic disease and has been proven to have a significant impact on the equine population the development of staging methods became essential to optimise equine medical care. As such, several staging methods for severe EAS have been published in recent years, following different methodologies.

One such method, the Horse Owner Assessed Respiratory Signs Index (HOARSI) relies solemnly on information reported by horse owners to identify the presence of respiratory disease. The questionnaire scoring system is based on clinical history and signs of cough frequency, nasal discharge, breathing at rest and the horse's performance (Ramseyer et al., 2007). It has been primarily used to identify diseased asthmatic horses (with mild, moderate or severe EAS) and has shown a good validation and repeatability (Gerber et al., 2009; Laumen et al., 2010; Rettmer et al., 2015). However, it does not provide information on the degree of severity of each clinical entity and may misdiagnose horses in disease remission or in early inflammation stages of severe EAS.

Alternatively, the severe EAS clinical staging method encompasses clinical history reports 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 (Tilley et al., 2012c). Unfortunately, this method is not suited to be used in field conditions, which limits its applicability to diagnostic and research centres.

7. Management of severe equine asthma syndrome

To date, there is no cure for severe EAS, making it a chronic disease. In fact, the name recurrent airway obstruction referred specifically to the recurring nature of the syndrome, since susceptible horses will develop airway inflammation and obstruction when exposed to environmental triggers. Thus, we speak of disease management, in which our goal is to maintain susceptible horses in clinical remission (without clinical signs).

Because severe EAS is an allergic disease, allergen avoidance is essential to ensure airway health, making environmental management the cornerstone for the successful management of this syndrome.

In addition, medical therapy can help improve pulmonary function and airway inflammation, alleviating respiratory distress. In general, therapeutic goals are to control airway inflammation and to revert bronchospasm, and the choice of drugs will depend on disease severity and whether environmental control can be optimised (Leclere et al., 2012; Barton et al., 2016).

When environmental management with effective allergen voidance is not possible or when inflammation and bronchospasm are very severe, rescue therapy with corticosteroids and bronchodilators is highly effective for improving lung function (Calzetta et al., 2017). Thus, this two drug families will be addressed in detail in this chapter.

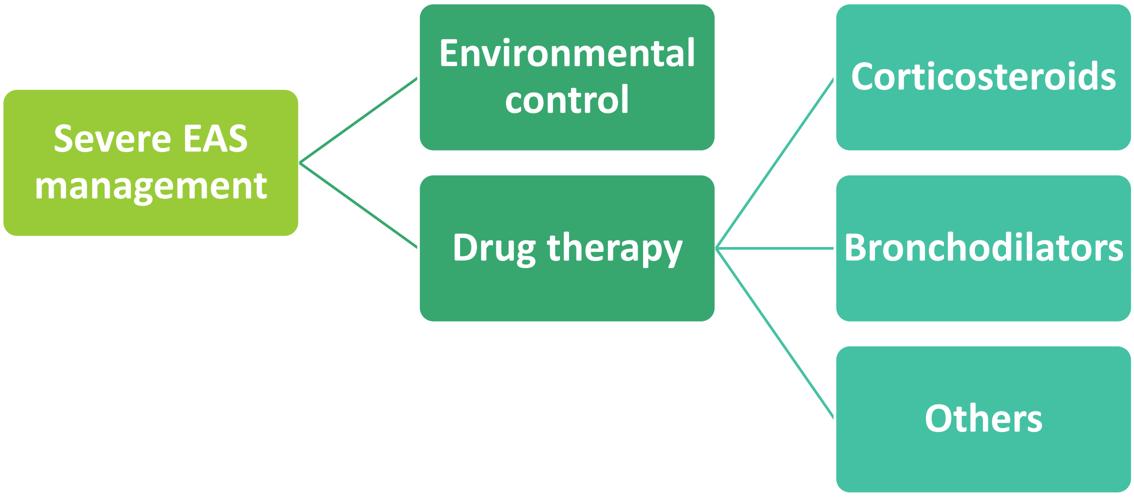

Fig. 9 – Basic principles of severe EAS management.

7.1. Environmental management

When adopting measures of environmental control, it is important to consider the two major phenotypes that characterise severe EAS – the RAO and SPARAO. Although the former is the most common, perhaps because of the Mediterranean climate, SPARAO-affected horses are relatively frequent in Portugal (Costa et al., 2006; Bullone et al., 2016).

RAO-affected horses tend to exhibit increased sensitisation to aeroallergens found indoors (i.e. moulds, mites), while in SPARAO clinical exacerbation occurs when animals come in contact with outdoors aeroallergens (i.e. pollen) (Ferrari et al., 2018). Basically, animals with these phenotypes will present susceptibility to different allergens, which will determine the implementation of environmental control catered to the animal's specific needs. However, some horses present a mix of both phenotypes, making environmental management more challenging (Couëtil et al., 2016).

Nonetheless, environmental management is essential in improving the quality of respirable air by controlling the sources of respirable dust particles in the horse's environment, such as bedding, feed, housing, ventilation, ridding arena pavement material and adjustment of periods of increased stable activity (Couëtil et al., 2005; Ivester & Couëtil, 2014). Still, bedding and feed, particularly hay, represent the major sources of respirable dust and their adjustment is fundamental to ensure asthmatic horse's respiratory health (Woods et al., 1993; Vandenput, Istasse, Nicks & Lekeux, 1997; McGorum et al., 1998).

Depending on the affected horse's age and the disease severity, clinical signs may still be observed weeks after the implementation of environmental changes (Thompson & McPherson, 1984), but generally pulmonary function improves after 3 days (Jackson, Berney, Jefcoat & Robinson, 2000).

Albeit environmental changes being essential in severe EAS management, this is a challenging process, involving profound modifications in traditional management practices and in some cases an additional economic investment. Thus, owner cooperation and commitment is essential to ensure the success of environmental management (Robinson et al., 2001; Couëtil et al., 2003; Boivin, Pilon, Lavoie & Leclere, 2018).

7.1.1. Bedding

In the traditional equine management system, straw is the conventionally adopted bedding material, which may in part be related to the fact that it is a by-product of farming and was once a cheap and easily available material. However, straw is no longer the most affordable bedding material, since its cost has significantly increased with modern day agricultural practices.

Good quality straw though, meets the asthmatic horse's low-dust requirements. But since, the quality of this material tends to fluctuate depending on season and may even vary between bales from the same batch (Vandenput et al., 1998). As such, it is safer to opt for an alternative material.

Furthermore, the use of straw bedding was implicated as a significant contributor to a high concentration of respirable dust particles, in particular around the horse's breathing zone, making it an unsuitable bedding material for severe EAS-affected horses (Auger & Moore-Colyer, 2017).

Saw dust is also a hazardous material for asthmatic's respiratory health due to its extremely high level of dust, making it a far worse choice than straw (Tanner et al., 1998).

Non-conventional bedding alternatives, like cardboard, shredded paper and stable mats, are sometimes met with some resistance from more conservative owners or caretakers, despite being better suited for the asthmatic horse (Tanner et al., 1998; Vandenput, Duvivier, Votion, Art & Lekeux, 1998b; Kirschvink et al., 2002; Clements & Pirie, 2007b).

Wood shavings are far better accepted, and there are even commercial brands of aspirated wood shavings making this material easily available. This material has a low concentration of airborne respirable dust particles and has been found to be a better alternative to straw bedding in two different housing systems (Auger & Moore-Colyer, 2017).

A study by Seedorf and co-workers, evaluated the use of a bio compost as a bedding alternative for horses, but found it unsuitable due to its high concentration of bacteria, whose antigens could trigger respiratory disease, like severe EAS, thus concluding that wood shavings were a better alternative (Seedorf, Schröder, Köhler & Hartung, 2007).

Crushed wood pellets is another low-dust alternative, providing a better air quality in equine stables than beddings with straw or peat with wood shavings (Kwiatkowska-Stenzel, Witkowska, Sowińska & Stopyra, 2017).

Nonetheless, it remains essential to ensure proper bedding hygiene by removing wet bedding material to prevent increased concentrations of ammonia (NH_3), bacterial endotoxins and fungal spores, which can significantly impair the efficiency of a low-dust bedding and endanger the horse's respiratory health (Seedorf et al., 2007).

7.1.2. Feed

Perhaps, more important than bedding is the adjustment of the horse's diet, to suit its low dust requirements.

Equine asthma has since long been associated with the practice of hay feeding, and hay is one of the most significant risk factors in disease exacerbation (Markham, 1656; Fairbairn, 1993a). Therefore, it should be removed from the asthmatic horse's diet and an alternative forage source should be provided (Vandenput et al., 1998b; Clements & Pirie, 2007b).

The best option is putting the horse at pasture and feeding it green grass, which not only accommodates the asthmatic horse's need for a low-dust feed but also provides an environment with lower respirable dust concentrations (Jackson et al., 2000). At pasture and without exposure to hay, lung function and airway inflammation improve significantly within 3 weeks (Robinson et al., 2001).

When green grass is unavailable, the asthmatic horse can be kept at pasture and fed an alternative source of forage. Likewise, when it is not possible to keep the horse at pasture, dietary adjustments should be performed and exposure to hay should be removed or at the very least limited (Robinson et al., 2001).

Rolled oats also have a very high dust content, and although it can be reduced by adding molasses, they should be avoided in severe EAS horses due to their high number of allergens (Vandenput et al., 1997; Robinson et al., 2001).

More suitable alternatives include haylage, silage, a complete pelleted or cubed diet or chopped dry forage. These have been shown to improve or maintain normal levels of lung function, due to their low-dust and aeroallergens concentrations (Thompson & McPherson, 1984; Vandenput et al., 1998a, b; Jackson et al., 2000; Seguin et al., 2010).

As a last resource, hay can be soaked prior to feeding, which has been shown to reduce its concentration of respirable dust by 90%. Nonetheless, in order for this procedure to be effective, the hay needs to be completely immersed in water for 20-30 min and fed to the horse in small quantities, to prevent it from dying out which would then result in the release of higher levels of airborne dust particles (Blackman & Moore-Colyer, 1998; Robinson et al., 2001;

Swain 2004; Clements & Pirie, 2007b; Hotchkiss et al., 2007b; Pirie 2014; Moore-Colyer, Taylor & James, 2016).

Sprinkling water onto the hay's surface is insufficient, since the centre of the hay will remain dry, failing to reduce the concentration of airborne allergens (Clarke, 1987).

Studies comparing feeding and bedding regimens found that steamed hay produced the lowest concentration of airborne respirable dust, mould and bacteria (Moore-Colyer et al., 2016; Auger & Moore-Colyer, 2017). Nevertheless, BALF neutrophilia persists with steamed hay feeding, despite the reduction in tracheal mucus score (Orard et al., 2018).

In cases, where the asthmatic horse is stabled with other horses which are being fed hay, it is still important to modify the affected animal's diet (Jackson et al., 2000).

Although a suitable diet greatly improves lung function, there may still be some residual airway obstruction, which is why environmental modifications should be addressed as a whole (Jackson et al., 2000).

7.1.3. Housing and ventilation

The type of housing and ventilation determine the concentration and nature of the aeroallergens the horse is exposed to.

While asthmatic horses with the RAO phenotype benefit from being kept at pasture, horses with pasture asthma will gain from being housed indoors (Robinson et al., 2001; Ivester & Couëtil, 2014).

The air quality associated with equine stables has thoroughly been described in the literature, and it was deemed hazardous not only for stable employees but also to the horse's respiratory health (Elfman, Riihimäki, Pringle & Wålinder, 2009; Mazan et al., 2009; Saastamoinen et al., 2015). This is greatly associated not only with the type of feed and bedding materials used, but also with the stable's design and consequently its ventilation.

Good ventilation ensures the removal of airborne dust and toxic gases, such as ammonia, thus ensuring cleaner air and, consequently, a reduction of airway inflammation in asthmatic horses (Woods et al., 1993; Riihimäki, Raine, Elfman, Bohlin & Pringle, 2009; Pirie, 2014; Saastamoinen, Särkkihärvi & Hyyppä, 2015).

Thus, in cases where the asthmatic horse must remain indoors and pasture is not a feasible alternative, it is fundamental to ensure the stables possess at least two entries above the level of the horse's body to allow an efficient air circulation. Likewise, close proximity to hay should be avoided at all times, and as such hay and straw must be stored outside the stables to prevent increasing the concentration of respirable dust particles (Whittaker, Hughes, Parkin & Love, 2009).

In addition, in order to further optimise air quality, the management practices of neighbouring stalls should also be addressed, which includes adapting the bedding and feed materials of other horses that reside in the same stable as the severe EAS-affected animal. This may not be well accepted by the horses' owners or caretakers, because of economic reasons, but the fact remains that the effect of a careful environmental management can be lost if proximity to potentially hazardous bedding and feeding materials is not addressed (Vandenput et al., 1997; Clements & Pirie 2007b). However, if this proves to be an unachievable goal, the asthmatic horse will still greatly benefit from environmental changes implemented in its stall alone (Jackson et al., 2000).

Nonetheless, even in a low-dust environment subclinical inflammation and airway obstruction may persist in asthmatic horses kept indoors (Vandenput et al., 1998a; Votion et al., 1999; Miskovic et al., 2007).

For this reason, in severe EAS management it is highly recommended that asthmatic horses be kept at pasture or at the very least turned out for the largest amount of time possible (Dixon et al., 1995; Holcombe et al., 2001; DeLuca, Erb, Young, Perkins & Ainsworth, 2008; Leclere, Lefebvre-Lavoie, Beauchamp & Lavoie, 2010; Leclere, et al., 2011a, b).

Still, many caretakers are reluctant to keep their horses outdoors in winter or overnight mainly out of fear for the horse's health in the cold weather. As such it is important to remind them that horses fair well in cold weather and that being stabled will have a far worse impact on their health than enduring the low temperatures of our Mediterranean climate (Robinson et al., 2001).

Temperature also plays an important role in environmental control. A study by Bullone and co-workers, suggested that severe asthmatic horses in disease exacerbation may benefit from a controlled stable temperature, since environmental conditions of high temperature and humidity resulted in a decline in pulmonary function (Bullone et al., 2016).

7.1.4. Stable activity

During times of increased stable activity, such as mucking out, dusting and grooming, concentrations of respirable dust tend to increase significantly, which usually induces cough bouts in affected horses (Clarke et al., 1987). The asthmatic horses should be kept outdoors during those periods, even if the stables are very well ventilated, to prevent exposure to the higher concentration of airborne dust particles (Whittaker et al., 2009).

7.1.5. Ridding arena

The surface used in the ridding arena may also be an important source of aeroallergens, especially in horses that spend a considerable amount of time training in poorly ventilated arenas. A very dry and dusty surface will inevitably produce a high concentration of airborne dust particles, which can be prevented by watering the pavement material (Rapp et al., 1992; Wasko et al., 2011; Lühe, Mielenz, Schulz, Dreyer-Rendelsmann & Kemper, 2017).

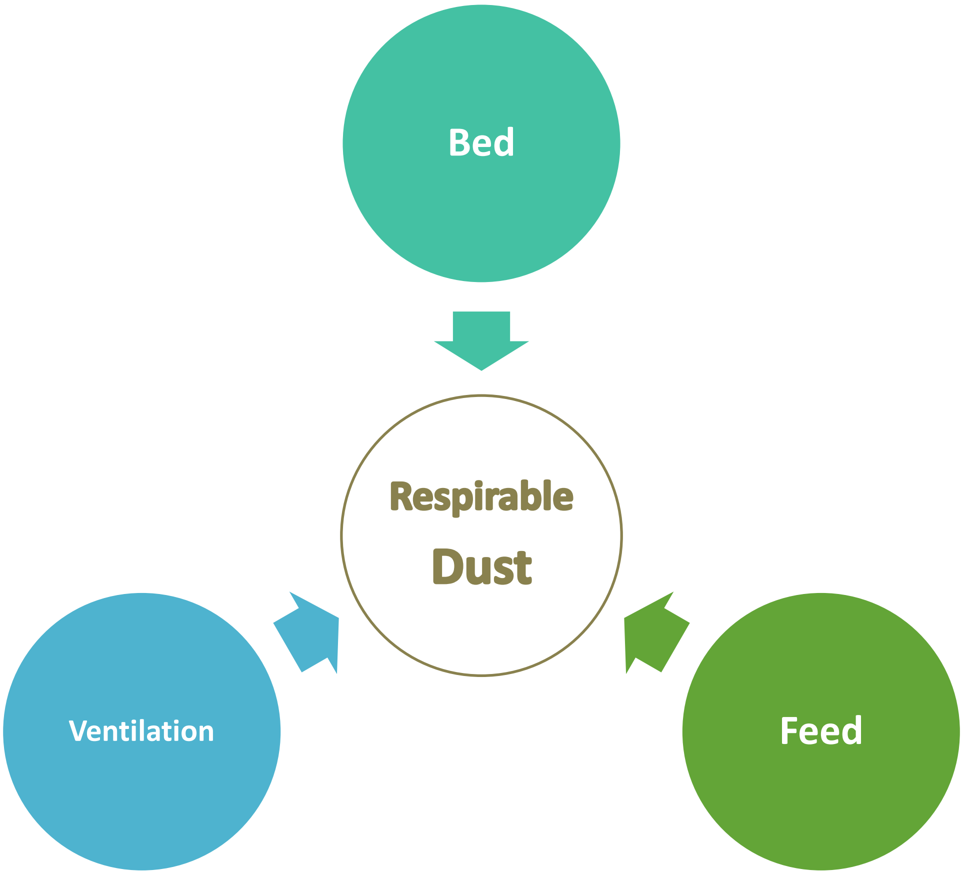

Fig. 10 – Three major aspects of environmental management.

Overall, it has been demonstrated that the sole exposure of a susceptible horse to the stable environment for only 7 hours is enough to trigger airway hyperreactivity, which persists for more than 3 days (Fairbairn et al., 1993a).

7.2. Therapeutic Management

7.2.1. Corticosteroids

The development of lower airway inflammation in response to aeroallergen exposure is the main pathological feature of severe EAS. As such, in parallel with allergen avoidance, corticosteroids are highly effective in controlling the inflammatory response and returning lung function to normal (Williamson & Davis, 2007; Gerber et al., 2011; Mora Pereira, Groover, Wooldridge & Calwell, 2018).

Although highly effective in controlling airway inflammation, systemic corticosteroid therapy is not without potential severe side effects (Dauvillier et al., 2011; Mora Pereira et al., 2018). In the horse, amongst the adverse effects usually associated with this class of drugs, such as adrenal suppression, the increased risk of developing laminitis is particularly worrisome (Slack et al., 2000; Couëtil et al., 2005; Bailey & Elliot, 2007; Cornelisse & Robinson, 2013; Leclere, 2017). The systemic effects can be significantly reduced when corticosteroids are administered by inhalation (Dauvillier et al., 2011; Mora Pereira et al., 2018).

However, it is important to note that corticosteroid administration is not a substitute for environmental management, but a complementary approach that will result in a faster clinical improvement of airway inflammation and especially obstruction (Giguère et al., 2002; Lavoie et al., 2002; Leclere et al., 2010). When used alone in the management of severe EAS, drug effects are short lived and inflammatory control is inefficient (Rush et al., 1998; Couëtil et al., 2006a).

In horses with severe airway obstruction it is best to initiate treatment with systemic corticosteroid, since this will allow a faster relief of respiratory distress. Also, severe bronchoconstriction will prevent adequate deposition of an aerosolised steroid, rendering this route more suited for long-term treatment and for horses with moderate airway obstruction (Rush et al., 1998; Robinson, Berney, Behan & Derksen, 2009; Mora Pereira et al., 2018).

Currently, there are several options of corticosteroids available for equine patients; nonetheless dexamethasone, prednisolone, beclomethasone and fluticasone are the most popular choices, having presented very good results in cases of severe EAS (Couëtil & Hawkins, 2013; Calzetta et al., 2017).

7.2.1.1. Systemic corticosteroids

Despite having the disadvantage of higher probability of occurrence of adverse effects, the enteral and parenteral routes do not require the use of special equipment, thus being more economic than the inhalation therapy (Pirie & McGorum, 2017; Mora Pereira et al., 2018).

Dexamethasone administered systemically, significantly reduces airway inflammation, with a reduction in neutrophil percentage observed in BALF, and improves pulmonary function (Robinson et al., 2010).

This drug can be administered IV, IM or PO at a dosage of 0.04 to 0.1mg/kg body weight (bwt) q. 24h for up to 2 weeks (Williamson & Davis, 2007; Leclere et al., 2010).

Oral dexamethasone has been used in the treatment of severe EAS with significant success. It has a good oral bioavailability and, in association with environmental changes, has been associated with less treatment failures (Cornelisse et al., 2004; DeLuca et al., 2008; Grady, Davis, Kukanich & Sherck, 2010). Also, in a study by Leclere and co-workers, asthmatic horses treated with oral dexamethasone presented pulmonary function values similar to those of asthmatic horses kept at pasture (Leclere et al., 2010).

Dexamethasone administered IV has a faster onset of action, making it more suitable for horses in severe respiratory distress, since oral administration has a peak effect at 24h.

However, this route has a longer duration of action (Cornelisse et al., 2004; Couëtil et al., 2006a; Lavoie et al., 2019). Thus, dexamethasone IV should be used in severe affected horses, after which treatment may then be continued orally (Robinson et al., 2010).

Doses of dexamethasone PO of 0.164 up to 0.05mg/kg bwt q. 24h are described in the treatment of severe EAS (Cornelisse et al., 2004; DeLuca et al., 2008; Leclere et al., 2010).

Prednisolone at a dose of 2mg/kg bwt PO given to severe asthmatic horses during exacerbation has been shown to improve pulmonary function, with maximum improvement registered 7 days after treatment. However, the same study found that oral dexamethasone (0.05mg/kg bwt) was more effective compared to prednisolone (Leclere et al., 2010).

Another study found that oral prednisolone (1mg/kg) combined with environmental management had a similar effect to dexamethasone administered IM (Courouće-Malblanc, Fortier, Pronost, Siliart & Brachet, 2008). Prednisolone formulations are usually available in the form of tablets, which are grinded and mixed with water and molasses to be administered PO with a syringe.

Other corticosteroids have also been described in the treatment of severe EAS. Prednisone does not seem to rapidly improve airway function and clinical signs and thus its use is not recommended in asthmatic horses (Jackson et al., 2000; Couëtil et al., 2005). This may be related to a lower bioavailability of this drug (Peroni, Stanley, Kolias-Baker & Robinson, 2002). On the other hand, triamcinolone acetonide (0.09mg/kg btw IM, single injection) and isoflupredone acetate (0.03mg/kg bwt q.24h IV/PO/IM) significantly improve lung function (Lapointe, Lavoie & Vrins, 1993; Picandet, Leguillette & Lavoie, 2003). Unfortunately, treatment with isoflupredone acetate is associated with the development of hypokalaemia and treatment with triamcinolone acetonide is associated with an increased risk of laminitis (Picandet et al., 2003; French, Pollitt & Pass, 2000).

Fig. 11 – Systemic corticosteroids for severe EAS treatment ordered according to their potency.

7.2.1.2. Inhaled corticosteroids

Unlike systemically administered corticosteroids, inhalation therapy with these drugs has significantly less adverse effects, allowing delivery of high concentrations directly to inflamed airways (Votion et al., 1997; Durham, 2001). It does, however, require the use of specific equipment, such as the Aeromask™, AeroHippus™, Equine Haler™ or other option specially developed for equine and is therefore more expensive (Pirie & McGorum, 2017). Because the equipment determines the drug concentration that is deposited in airways, its choice will determine the dosage of aerosolised corticosteroid administered to the horses (Mora Pereira et al., 2018).

Treatment with fluticasone propionate was found to improve pulmonary function in severe EAS-affected horses (Couëtil et al., 2005). Long-term treatment with fluticasone was not associated with adverse effects, making this drug suitable for maintenance therapy (Dauvillier et al., 2011; Bullone, Vargas, Elce, Martin & Lavoie, 2017b).

Early treatment with fluticasone in association with environmental management was found to improve the recovery speed of asthmatic horses (Couëtil et al., 2005).

However, fluticasone propionate (6000μg q.12h aerosol) is not as efficient as dexamethasone (0.1mg/kg bwt q.24h IV) in improving pulmonary function, particularly during disease exacerbation. Nonetheless, when used prophylactically it prevents the occurrence of airway obstruction, since it contributes to reversing peripheral airway smooth muscle remodelling, but it does not decrease intraluminal neutrophilia (Robinson et al., 2009; Bullone et al., 2017b). Treatment doses of aerosolised fluticasone range from 2000μg to 6000μg q.12h, for up to 21 days (Ivester & Couëtil, 2014).

In severe EAS-affected horses, treatment with aerosolised beclomethasone improves pulmonary function and PaO_2, reduces neutrophilic pulmonary inflammation and prevents alterations in lymphocyte subpopulation (Rush et al.,1998; Ammann, Vrins & Lavoie, 1998; Rush et al., 2000; Couëtil et al., 2006a).

Conflicting results can be found when comparing this drug's efficacy to dexamethasone. Rush and co-workers (1998) reported that improvement in pulmonary function following treatment with beclomethasone (1320μg q.12h aerosol) was less marked than with dexamethasone (0.1mg/kg bwt IV); while Couëtil and colleagues reported significant improvement in pulmonary function following treatment with beclomethasone (0.5mg q.12h aerosol) compared with dexamethasone administered IM (Rush et al., 1998; Couëtil et al., 2006a). The benefits of beclomethasone treatment become more evident and effective when combined with environmental management (Rush et al., 1998). For the treatment of severe EAS of dose of 500μg to 3750μg q. 12h, is recommended for up to 7 days (Ivester & Couëtil, 2014). Beclomethasone may cause adrenal depression, but this effect is dose-dependent (Rush et al., 2000).

Other inhaled lipophilic corticosteroids that have demonstrated good efficacy include budenoside (1400µg q. 12h) (Kampmann, Ohnesorge, Venner & Deegen, 2001). In severe asthmatic horses, treatment with inhaled budenoside (1800µg q. 12h) resulted in lung function improvement, although cortisol suppression was also observed (Lavoie et al., 2019).

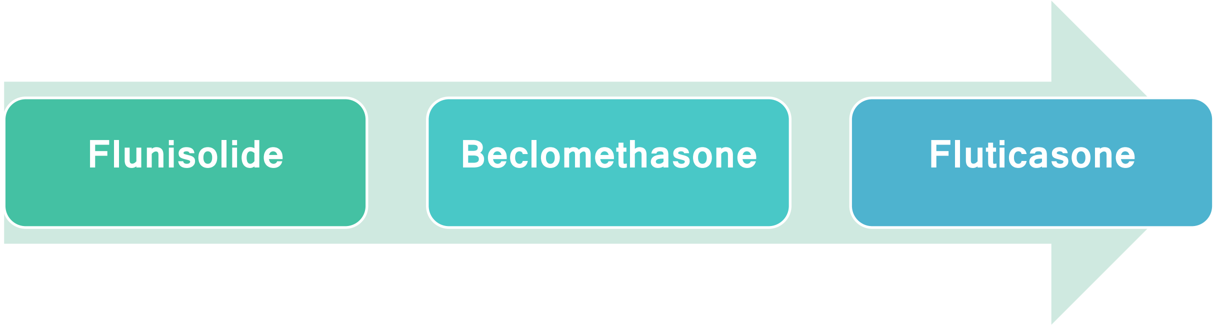

Fig. 12 – Inhaled corticosteroids used in the treatment of severe EAS ordered by increasing potency.

7.2.2. Bronchodilators

Although corticosteroids are highly effective in relieving the clinical signs of severe equine asthma, some horses also require treatment with bronchodilators. These drugs will reduce airway obstruction and improve deposition of other inhaled drugs (Rush, Hoskinson, Davis, Matson & Hakala, 1999; Couëtil & Hawkins, 2013).

In horses, bronchodilator therapy can be systemic or inhaled and usually consists of anticholinergic and β_2-adrenoreceptor agonists, although other substances may be used (Laan, Bull, van Nieuwsstadt & Fink-Gremmels, 2006; Cunningham & Dunkel, 2008; Couëtil & Hawkins, 2013).

7.2.2.1. β_2-adrenoreceptor agonists

In asthmatic horses, specific β_2-adrenoreceptor agonists such as clenbuterol, albuterol, pirbuterol, formoterol or salmeterol may be used to revert airway obstruction caused by bronchospasm (Camargo et al., 2007; Calzetta et al., 2019a).

Non-selective agonists are generally avoided due to their cardiovascular side effects. This is the case of tretoquinol (also known as trimetoquinol), which despite its fast-acting nature and bronchodilator effect, results in significant side effects when administered IV or intra-tracheally, acting as a cardiac stimulant. Nevertheless, this drug appears to be fairly safe when administered by aerosolization in horses (Camargo et al., 2006; Camargo et al., 2007).

The β_2-adrenoreceptor agonists duration of action is usually short and these drugs may be administered by aerosol, orally or intravenously (Couëtil & Hawkins, 2013; Pirie & McGorum, 2017).
The administration by aerosol inhalation requires the use of masks or aerosol chambers specially developed for horses, but this route leads to fewer side effects (Robinson, 2001). Nonetheless, in cases of severe bronchospasm and airway inflammation, asthmatic animals will benefit of an initial IV treatment, for faster and more efficient onset of action.
Independent of route of administration, treatment with β_2-agonist significantly reduces pulmonary resistance (R_L) and ΔPpl_{max} (Bertin, Ivester & Couëtil, 2011; Calzetta et al., 2017).
In prolonged treatments, such as with clenbuterol, a drug-induced downregulation of the β_2 receptors may occur, and incremental doses of β_2-agonist will be necessary to maintain the desired effect, which would increase the risk of side effects. However, this downregulation can be easily prevented by the association of corticosteroids (Abraham, Brodde & Ungemach, 2001; Abraham, Brodde & Ungemach, 2002; Couëtil & Hawkins, 2013).
Clenbuterol is the most commonly used systemic β_2-agonist in the treatment of severe EAS. It greatly improves clinical signs due to its bronchodilator effect and increases mucociliary clearance (Kearns & McKeever, 2009; Pirie, 2014). Some studies have also reported an anti-inflammatory effect of clenbuterol by reducing airway neutrophilic inflammation and airway cell cytokine expression, *in vivo* and *ex vivo*, respectively (Laan et al., 2006; van den Hoven, Duvigneau, Hartl & Gemeiner, 2006).
Clenbuterol can be administered orally or intravenously with dosages of 0.8µg/kg every 12h to 3.2µg/kg q 12h (Erichsen, Aviad, Schultz & Kennedy, 1994; Cunnigham & Dunkel, 2008). Of the two, the oral route is generally preferred due to its convenience however, caution should be used when high dosages are employed. Side effects associated with high doses of this drug may include tremors, sudoresis, tachycardia and excitement (Abraham et al., 2001; Abraham et al., 2002; Kearns & McKeever, 2009).
Studies have indicated that therapy with aerosolised albuterol, levalbuterol and pirbuterol, despite these drugs' short duration of action (1-2h), has minimal side effects and significantly improves bronchospasm (Derksen et al., 1992; Derksen et al., 1999; Bertin et al., 2011; Arroyo, Couëtil, Nogradi, Kamarudin & Ivester, 2016).
Because of its longer duration of action, approximately 8h after administration of 210µg by aerosol, salmeterol is more suited for the treatment of severe EAS (Robinson, 2001). Furthermore, when combined with aerosolised fluticasone it reversed smooth muscle remodelling and decreased airway neutrophilia (Bullone et al., 2017b).

7.2.2.2. Anticholinergics

Anticholinergic drugs induce bronchodilation through their action on the parasympathetic nervous system. Although these substances are highly effective their use has been discouraged in asthmatic horses due to their severe side effects when administered systemically (de Lagarde et al., 2014). However, because bronchospasm in severe EAS-affected animals is primarily mediated by the cholinergic system, anticholinergic drugs are very effective in the treatment of this disease (Derksen & Robinson, 2002).

Atropine is a potent and fast-acting bronchodilator, resulting in improvement of ΔPpl_{max} and clinical signs (Couëtil & Hawkins, 2013; Pirie, 2014). Because this drug is non-selective and is easily absorption, deleterious side effects, such as ileus and abdominal pain, may occur after its administration As such, atropine is reserved for 'rescue treatment' as a single dose IV and is not used routinely (Gosens, Zaagsma, Meurs & Halayko, 2006; de Lagarde et al., 2014). N-butylscopolammonium bromide, which also has anticholinergic properties appears to be a safer option than atropine to reduce bronchospasm (de Lagarde et al., 2014; Pirie et al., 2014). Glycopyrrolate has a similar effect to atropine, but fewer side effects reducing the risk of 'colic' in the horse. This drug can be administered IV or by inhalation (Art, de Moffarts, van Erck, Becker & Lekux, 2003; Couëtil & Hawkins, 2013).

Ipratropium bromide is a dose-dependent anticholinergic bronchodilator which has been reported as a fairly safe option in horses. When inhaled it produces bronchodilation for a longer duration than a β adrenergic agonist (Robinson, Derksen, Berney & Goossens, 1993; Duvivier et al., 1999; Robinson, 2001). However, it should be administered every 8 hours in order to be effective.

Tiotropium bromide is a long acting drug which is selective for M3 receptors and is currently being investigated for long term use in the treatment of severe EAS (Calzetta et al., 2017). It has shown promising results and may be combined with a β2- agonist in order to reduce the dosage of both drugs and their side effects, while potentiating the bronchodilator effect (Calzetta et al., 2019b).

7.2.2.3. Others

Furosemide, in addition to its diuretic effect, also produces bronchorelaxation in horses. The exact mechanism of action by which this drug improves airway function is still not fully understood (Broadstone et al., 1991). In the treatment of severe EAS furosemide is administered by inhalation (Pirie & McGorum, 2017).

7.2.3. Other therapeutic approaches

Since mucus clearance depends on its viscosity, treatment with saline may help its elimination either through systemic administration or through inhalation (Robinson, 2001; Pirie & McGorum, 2017). However, conflicting results have been reported on the benefits of this practice (Jean, Vrins & Lavoie, 2004; Fey, 2010).

When environmental and therapeutic management prove insufficient, either because complete antigenic void was not possible or due to persistence of clinical signs, immunotherapy may prove to be a suitable alternative (Tilley, Sale Luís & Branco Ferreira, 2012a). This therapy is fairly novel in equine medicine, but it has been successfully used in human and feline asthmatic patients (Holgate & Polosa, 2008; Mueller et al., 2018).

New therapeutic options are currently being developed. For instance, a nanoparticullate CpG immunotherapy is currently being developed with promising results (Klier et al., 2018).

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CHAPTER 2

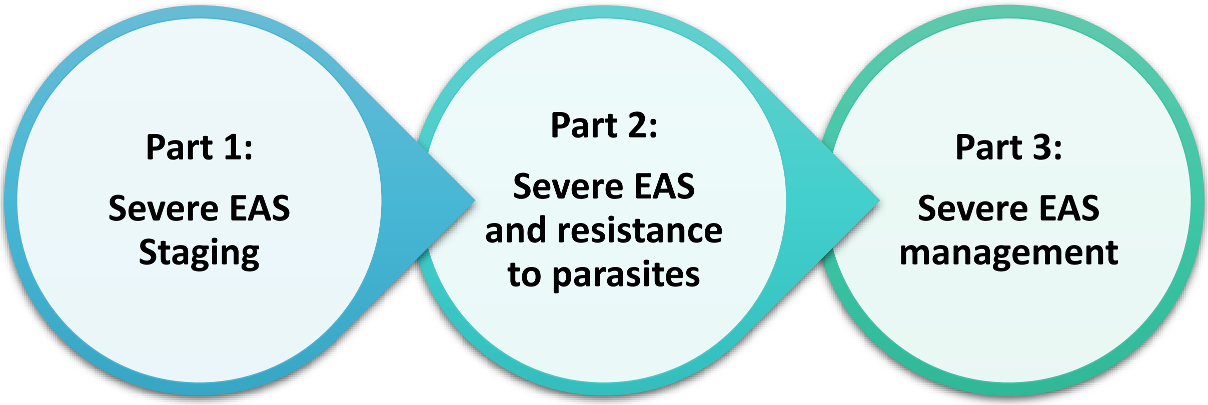
Objectives

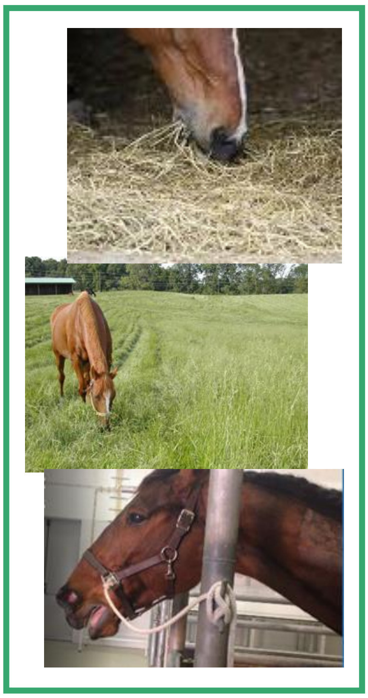
Severe Equine Asthma Syndrome is a chronic incurable respiratory disease affecting adult horses. It has been associated with stabling, where horses are persistently exposed to a high concentration of respirable dust particles, resulting in airway inflammation, hyperreactivity and obstruction. The affected horses require life-long specific management to avoid exposure to offending aeroallergens and thus prevent the development of irreversible pathological changes to their airway physiology. Seldom, some animals prove to be refractory to treatment which often complicates disease management and may result in the precocious death.

Because a large number of stabled horses are athletes, severe EAS affects performance and compromises the horses' winnings, which in association with the investment necessary to ensure disease remission through environmental management and pharmacological treatment, results in significant economic losses.

As such, the main objectives and approaches of this study were:

1. Evaluate the contribution of several variables to the staging of severe Equine Asthma Syndrome;
    - 1.1. Determine the contribution of lung function testing to the diagnosis and staging of severe Equine Asthma Syndrome;
    - 1.2. Develop discriminant functions for the diagnosis and staging of severe Equine Asthma Syndrome;
    - 1.3. Contribute to disease staging and diagnosis in ambulatory practice;
2. Contribute to the knowledge of the relationship between severe Equine Asthma Syndrome and gastrointestinal helminth infection;
    - 2.1. Determine if Lusitano asthmatic horses have lower gastrointestinal parasite counts than healthy animals;
    - 2.2. Compare the helminth species identified in asthmatic horses' coprology with those found in healthy animals;
    - 2.3. Characterize the helminth population found in the faeces of Lusitano horses in a Mediterranean climate.
3. Assess owner compliance to an environmental management protocol;
    - 3.1. Determine and characterise the asthmatic horses' clinical status based on owner assessment and need for pharmacological treatment;
    - 3.2. Assess the influence of environmental parameters on clinical signs;
    - 3.3. Determine compliance to environmental management guidelines.


Part 1:
Severe EAS Staging
Part 2:
Severe EAS and resistance to parasites
Part 3:
Severe EAS management



CHAPTER 3

Experimental work

1. Contribution of lung function tests to the staging of severe equine asthma syndrome in the field

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Abstract

Staging methods are useful tools for monitoring disease and response to treatment, and because severe Equine Asthma Syndrome (EAS) has a high prevalence in the equine population, a clinical staging method can provide important information to optimize equine care.

Our team has previously developed and published a clinical staging method for severe EAS and in the present study we further evaluated information provided by lung function tests, in order to determine their contribution to disease staging. Using discriminant analysis we set out to produce a new staging method with applicability in the field. Differences between group means ($P<0.05$) were observed for clinical score, bronchoalveolar lavage fluid neutrophil percentage, pleural pressure (ΔPpl), P_aO_2 and histamine concentration and the linear functions obtained explained 99.3% of the data variability, with 94.7% of cases grouped correctly and a cross-validation of 86.8%. Thus, this staging model showed very good results and the discriminant linear functions may be used to identify and stage severe EAS. This method can be used in the field and also in diagnostic and research centres.

Keywords: severe equine asthma syndrome; disease staging; discriminant power; lung function tests; diagnosis

1. Introduction

Severe equine asthma syndrome (EAS), previously named Recurrent Airway Obstruction (RAO), is the most common pulmonary disease in adult horses, with a reported prevalence of 14% (Hotchkiss, Reid & Christley, 2007).

It is believed, that as its' human counterpart, equine asthma spurns from the interaction of genetic and environmental factors. When genetically susceptible individuals are exposed to offending aeroallergens usually found in the horses' environment, such as moulds, mites, bacterial endotoxins and even pollen, airway inflammation, obstruction and hyperresponsiveness occur (Costa et al., 2000; Robinson, Derksen, Jackson, Peroni & Gerber, 2001; Marti et al., 2008; Tilley, Sales Luís & Branco Ferreira 2012a,b). Affected animals exhibit increased respiratory effort at rest, coughing and even poor performance (Pirie, 2014). Because the disease has a chronic and insidious development it leads to significant economic losses, with many equine athletes retiring before their time.

Although severe EAS phenotype can be easily identified when disease exacerbation occurs, mild clinical cases prove harder to diagnose (Robinson et al., 2000). For this purpose, ancillary diagnostic tests provide useful data, particularly lung function evaluation which is capable of detecting small changes due to airway obstruction and hyperresponsiveness (Hoffman, Kuehn, Riedelberger, Kupcinkas & Miskovic, 2001; Miskovic, Couëtil & Thompson, 2007; Pirie, 2014; Rettmer, Hoffman, Lanz, Oertly & Gerber, 2015).

In order to evaluate disease progression and establish a management protocol, Tilley, Sales Luís and Branco Ferreira (2012c) developed a clinical staging method for severe EAS based on clinical examination, mucus characteristics observed during respiratory endoscopy, neutrophil percentage present in the bronchoalveolar lavage fluid (BALF) and changes observed in the thoracic x-ray. Because this clinical staging method did not take into account information provided by lung function evaluation, the present study aimed to evaluate the contribution of data obtained from arterial blood gas analysis, pleural pressure measurement, flowmetric plesthysmography and histamine bronchoprovocation for severe EAS staging.

2. Materials and Methods

2.1. Population

Twenty one horses with severe EAS phenotype (Couëtil et al., 2016) and 21 healthy controls from the same stables without signs of respiratory disease, aged between 8 and 23 years were examined. Age average and standard deviation was 13±4.3 years in the severe EAS group and 14.9±4.8 years in the Control group. Age was not significantly different between the 2 groups ($P= 0.15$; t-test) and presented a normal distribution (Shapiro-Wilk test).

All animals had a history of being fed hay for at least two months (Ramseyer et al., 2007) and severe EAS-affected horses were examined when clinical signs were more severe, according to their owners, that is to say they were all examined in exacerbation. None of the horses had been medicated with corticosteroids, bronchodilators or anti-histaminics in the two weeks previous to their examination, nor had history of having been subjected to immunotherapy.

2.2. Study design

The horses were examined at the equine hospital, of the Faculty of Veterinary Medicine, by two independent clinicians for disease staging and lung function evaluation. Upon the horse's arrival at the hospital, testing was performed in the following order: clinical examination, arterial blood gas (ABG) analysis, thoracic x-ray, pleural pressure measurement, flowmetric plethysmography and bronchoprovocation with histamine. Finally, respiratory endoscopy and bronchoalveolar lavage (BAL) were executed 2 hours after bronchoprovocation testing, since it has been reported that bronchoprovocation does not alter BALF cytology (Perkins et al., 2008; Wichtel, Gomez, Burton, Wichtel & Hoffman, 2015). Once clinical examination, ABG analysis and thoracic x-ray had been performed, all horses were sedated with detomidine (0.01mg/kg bwt; Domosedan®[a]), in order to carry out the other tests. For the BALF procedure, butorphanol (0.01 mg/kg bwt; Dolorex®[b]) was administered to reduce the cough reflex and provide mild analgesia. Because butorphanol increases sedation depth and produces ataxia, which would influence lung function assessment, namely plethysmography, it was only administered before the BAL, this being the reason why this test was performed last (Taylor, Browning & Harris, 1988; Sellon, Monroe, Roberts & Papich, 2001). If the horse showed signs of severe bronchoconstriction, a bronchodilator was also administered (clenbuterol) previous to this procedure. After sedation, care was taken to prevent nasal oedema by supporting the horse's head with a headstand.
All procedures were previously approved by the horses' owners and an informed consent form was signed either by the owner or its' representative before examining the animals.

2.3. Clinical staging

For Severe EAS (or RAO) staging, the clinical staging sheet developed by Tilley et al. (2012c) and previously used in other studies (Haltmayer, Reiser, Scharmel & van den Hoven, 2013; Niedzwiedz &Jaworski, 2014 a,b; Niedzwiedz, Jaworski, Tykalowski & Smialek, 2014; Niedzwiedz et al., 2016; Decloedt et al., 2017; Barton, Schulze, Doherr & Gehlen, 2018), was used. This involved clinical examination, thoracic x-ray, respiratory endoscopy and bronchoalveolar lavage fluid (BALF) cytology. All observed variables were classified by two independent clinicians. Horses were then allocated to one of five stages, according to the severity of clinical signs and pathophysiological alterations encountered: stage 0 (horses without respiratory disease), stage 1 (horses with the least severe form of RAO), stage 2 (mild presentation of RAO), stage 3 (moderate presentation of RAO) and stage 4 (horses with the most severe form of RAO).

2.3.1. Clinical exam

Horses were examined for intensity of nasal flare, cough and abdominal lift so that clinical score could be determined (Tilley et al., 2012c).

2.3.2. Thoracic x-ray

Three to four x-rays, depending on the horse's size, were taken at end inspiration to visualise the thoracic trachea and the lung (Mair & Gibbs, 1990). An x-ray generator (850 mAs, 125 kV; Philips) Optimus[c] with a vertical bucky (Philips Bucky Diagnostic[c]) and a Regius cassette RP45110 10x14 (Konica Minolta Inc.[d]) were used. The exposure factors were in the range of 90-100 kV and 80-100 mAs and images were developed with the Regius Model 110s (Konica Minolta Inc. [d]).

Images were evaluated for interstitial pattern, bronchial radiopacity, tracheal thickening and bronchial thickening, and thoracic x-ray score was determined (Tilley et al., 2012c).

2.3.3. Respiratory endoscopy

Respiratory endoscopy was carried out with a flexible video bronchoscope (10.4 x 3000 mm Karl Storz[e], PV-G 28-300) and mucus characteristics (accumulation, colour, viscosity, localization and stickiness) were used to determine endoscopy score (Tilley et al., 2012c).

2.3.4. Bronchoalveolar lavage fluid (BALF) cytology

For the BAL technique, a silicone catheter (240cm, 2.5 I.D, COOK[f]) was passed through the nostril into the trachea and advanced until it reached the smallest calibre bronchi possible. Lidocaine (20 ml of 20mg/ml Anestesin®[g]) was instilled to reduce the intensity of the cough reflex before reaching the carina region. Once the catheter was wedged, the cuff was inflated with air and the catheter was held securely at the nostrils. Up to 500 ml of warm sterile saline were instilled through the catheter using 100 ml syringes and a stop-cock. Fluid was aspirated with the same syringes and the samples were pooled together in a sterile cup (Viel, 1980; Viel, 1997; Hoffman & Viel, 1997; Hoffman, 2008). A 10 ml aliquot was placed in an EDTA tube and processed immediately to avoid cell deterioration. Using a hemacytometer, cellularity was verified to be ≤ 265 cells/µl in the healthy controls and higher than this value in severe EAS horses. The aliquots were then centrifuged (300 g for 10 min) and the supernatant was decanted. The pellet was then placed in a clean slide, using a plastic transfer pipette, to make the smear. The smears were air-dried, fixed with methanol for 5 min and stained with a Giemsa's (Merk[h]) 20% solution.

Ten smears were made for each horse and 400 nucleated cells (x1000 magnification) were counted by an experienced clinical pathologist, blinded to the horse's medical history. Neutrophil percentage was registered for BALF score (Tilley et al., 2012c).

2.4. Arterial blood gas (ABG) analysis

A sample of arterial blood was collected from the transverse facial artery using a heparinized 2ml syringe and a 23G 1'' needle and was immediately processed. Care was taken to ensure that the sample was not exposed to the atmospheric air. ABG analysis was conducted with a portable analyser (VetScan i-STAT[i]) and with the i-STAT CG4+ cartridges[i]. Measurements of pH, P_aCO_2, P_aO_2, TCO_2, HCO_3, BE, sO_2 and lactate were recorded for each animal. The i–STAT analyser allows for direct measurement of pH, P_aO2, P_aCO2 and lactate, while the remaining parameters are calculated by the software.

2.5. Pleural pressure measurement

Pleural pressure (ΔPpl) was estimated by means of an oesophageal balloon catheter, placed in the distal third of the oesophagus (intrathoracic oesophagus), connected to a hand-held pressure transducer (Dppl device N V1.01[j]). The pressure transducer was calibrated according to manufacturer's specifications before each use, and distance from the horse's nostril to the distal third of the oesophagus was also marked on the oesophageal catheter beforehand. The oesophageal catheter, a semi-rigid Teflon probe (220 cm long, OD: 6 mm diameter) with holes in one extremity and sealed with a condom, was introduced through the nostril with the help of a nasogastric tube (150 cm long, OD: 19 mm), which served as a guide. Once the catheter was in position it was connected to the pressure transducer and the balloon was inflated with 5ml of air. ΔPpl, the difference between pressure during inspiration and expiration, was recorded for each horse. Artefacts caused by swallowing where excluded from ΔPpl calculations.

2.6. Plethysmography and bronchoprovocation

In order to further assess airway function, flowmetric plethysmography (Open Pleth™ System[k]) was performed in association with histamine bronchoprovocation.

Horses were fitted with an airtight mask and a pneumotachograph was attached to it. Thoracic and abdominal inductance bands were placed in the 11th intercostal space (RIB band) and caudal to the last rib (ABD band), respectively. The system was calibrated prior to its use, according to the manufacturer's instructions, and the software calculated the Δflow, which can be defined as the difference between the flow signal from the pneumotachograph and the flow signal from the bands.

Baseline measurements were recorded for 2 min before beginning bronchoprovocation, and if a maximal ∆flow >3.5 l/s was recorded during baseline measurements the histamine protocol was aborted.

Using a nebuliser compressor and a kit provided by the system, a 0.9% sterile saline solution (negative control) followed by increasing concentrations of a histamine (histamine diphosphate[l]) solution of 2, 4, 8, 16 and 32 mg/ml were nebulised. After each nebulisation, measurements were recorded for 3 min. Histamine protocol was terminated if the Δflow value increased >50% of that recorded during saline measurement, if the horses coughed repeatedly and/or an increase in abdominal breathing pattern was observed or if the highest dose of histamine was reached. Measurements recorded included ∆flow and histamine concentration which resulted in a positive response to bronchoprovocation testing.

2.7. Statistical analysis

Results were analysed using IBM SPSS Statistics V21.0.0 software[m] and an α-level of $P<0.05$ was considered significant for all statistical tests.

Assumptions for discriminant analysis were evaluated using Kolmogorov-Smirnov test to confirm that data had a normal distribution. Data symmetry and kurtosis were also assessed.

A discriminant analysis model based on severe EAS stage groups was conducted on the independent variables deemed significant, after group means comparison with an ANOVA test. Descriptive statistics, such as mean and standard deviation, as well as group correlation matrices, method cross-validation and discriminant analysis canonical functions are presented.

The clinical staging for severe EAS was considered the dependent variable, and the independent variables, clinical score, endoscopy score, x-ray score, BALF neutrophil percentage, ABG analysis parameters (pH, PCO_2, PO_2, HCO3, BE, TCO_2, sO_2 and Lactate), ΔPpl, Δflow and maximum histamine concentration ([Hist]), were evaluated for inclusion in the discriminant model.

3. Results

Using the clinical staging method described by Tilley et al. (2012c), our population of 42 horses was characterized as stage 0 (n=21), stage 1 (n=7), stage 2 (n=10) and stage 3 (n=4). In order to perform the discriminant analysis, all collected data was tested for basic assumptions, namely normal distribution, data symmetry and kurtosis. According to Seber (1984), in the presence of moderate deviations, and taking into account that the majority of tests used in discriminant analysis are relatively robust, it is generally preferable to use the original variables instead of the transformed ones. In this analysis, although the normal distribution hypothesis is frequently violated, an analysis of the symmetry coefficients (which vary between -2.602 and 2.183) and of flattening (between -0.673 and 6.174) revealed that our data, with the exception of the variable pH, did not present heavy-tails that to our knowledge would justify using corrective measures.

Because the independent variables P_aCO_2, HCO_3, endoscopy and x-ray score did not present a normal distribution they were excluded from the discriminant analysis model. As previously mentioned, although it displayed a normal distribution, the variable pH was also not considered in the analysis due to its high kurtosis value.

When comparing group means, no differences were found using the variables BE, Lactate, TCO_2, sO_2, Δflow and P_aO_2 (P>0.05). Also, the variables sO_2 and Δflow were related with the third non-significant discriminant function, and therefore were excluded from the model.

Although the variable P_aO_2 was not considered statistically significant (P=0.062), the α-value tended towards significance and because it possessed some discriminant power it was still included in our analysis. Descriptive statistics for significant independent variables are presented in table 1.

Table 1 – Descriptive statistics (mean and standard deviation) for severe EAS-affected and control horses and significance level of group mean differences.

	Stage 0 (n=21)	Stage 1 (n=7)	Stage 2 (n=10)	Stage 3 (n=4)	Total (n=42)	Sig.
Clinical score	0.48 ± 0.22	1.00 ± 0.00	1.89 ± 0.60	3.00 ± 0.00	0.84 ± 1.05	0.000
Neutrophil %	10.71 ± 4.86	30.6 ± 6.6.9	49.89 ± 9.57	53.00 ± 11.14	25.95 ± 19.43	0.000
ΔPpl	9.56 ± 2.83	13.20 ± 1.10	14.33 ± 1.73	13.00 ± 6.25	11.61 ± 3.36	0.002
P_aO2	101.48 ± 11.32	95.40 ± 2.41	93.33 ± 3.00	91.00 ± 2.00	97.92 ± 9.44	0.062
Histamine concentration	32.00 ± 0.00	32.00 ± 0.00	13.78 ± 11.33	0.90 ± 3.06	25.53 ± 11.25	0.000

The correlation matrices between groups were also analysed (table 2). Normally, when variables possess a very high correlation, one of them is automatically excluded from the model because both variables would account for an equal amount of data variability. All variables that displayed significant differences between group means, namely clinical score, BALF neutrophil percentage, ΔPpl and histamine concentration ($P<0.05$) and also P_aO_2 ($P=0.062$), did not present high values of correlation, and thus were all included in the discriminant analysis model (table 1).

Table 2 – Discriminant analysis correlation matrices between groups.

	Clinical Score	Neutrophil %	ΔPpl	P_aO_2	Histamine concentration
Clinical Score	1.000	-0.225	0.140	-0.061	-0.098
Neutrophil %	-0.225	1.000	0.144	-0.177	0.041
ΔPpl	0.140	0.144	1.000	-0.412	-0.139
P_aO_2	-0.061	-0.177	-0.412	1.000	0.023
Histamine concentration	-0.098	0.041	-0.139	0.023	1.000

The first two canonical linear functions obtained were statistically significant and together explained most of the data variability found in our studied population. The first function accounted for 97.3% of the variability and the second function for 2%. Although the second function describes only a small percentage of variability it is still significant, and both functions together explain 99.3% of data variability.

The discriminant analysis of our staging method was validated resulting in a classification matrix where 94.7% of the original cases were grouped correctly and where 86.8% of cross-validated cases were correctly classified, having thus confirmed a major contribution of the independent variables used in this staging model (figure 13). These results validate the discriminant analysis performed and make it possible to discriminate between groups using the following functions:

Stage 0

X0= -134.357 + (2.84x CS) + (0.409x Neut %) + (3.787 x ΔPpl) + (1.835 x P_aO_2) + (1.233 x [Hist])

Stage 1

X1= -158.722 + (13.743x CS) + (0.943x Neut %) + (3.899 x ΔPpl) + (1.871 x P_aO_2) + (1.277 x [Hist])

Stage 2

X2= -180.730 + (23.698x CS) + (1.501x Neut %) + (3.569 x ΔPpl) + (1.910 x P_aO_2) + (0.691 x [Hist])

Stage 3

X3= -205.453 + (34.672x CS) + (1.717x Neut %) + (3.005 x ΔPpl) + (1.866 x P_aO_2) + (0.411 x [Hist])

CS – clinical score; Neut % - neutrophil percentage in BALF cytology; ΔPpl – Differential pleural pressure; P_aO_2 – arterial blood oxygen partial pressure; [Hist] – maximum histamine concentration tolerated in bronchoprovocation.

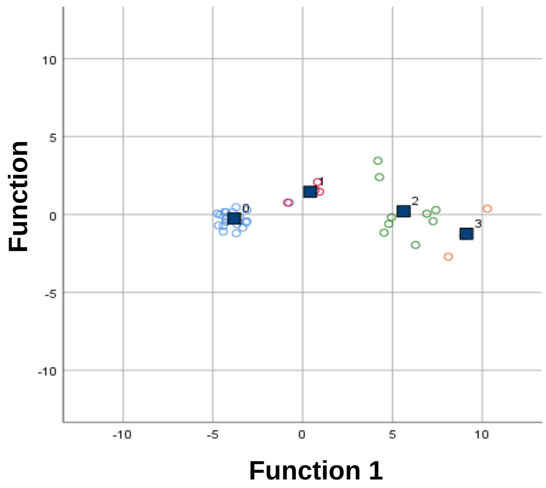

Fig. 13 – Discriminant analysis model adjusted on severe EAS staging suggested (canonical discriminant functions). Black square – Mean discriminant score (group centroid).

4. Discussion

Staging methods are important tools to assess quality of care, monitor disease progression and determine effective treatment strategies, while providing prognostic information. They also enable information exchange in an easy and universal way.

Because severe EAS is a chronic recurrent disease, treatment can sometimes be challenging (Robinson et al., 2001; Pirie, 2014). In some cases, owners may be reluctant to keep asthmatic horses, particularly if they are refractory to treatment.

Disease staging gives asthmatic horse owners an objective way of evaluating their horse's respiratory health and can also help to increase awareness of the relevance and effectiveness of environmental management in maintaining these animals in disease remission.

With this in mind, our group has previously developed a clinical staging method which enables disease characterization, according to its severity, through clinical parameters, such as physical examination, thoracic x-ray, respiratory endoscopy and bronchoalveolar lavage fluid cytology (Tilley et al., 2012c). However, this method is more suited for a hospital setting, since it requires the use of an x-ray generator with sufficient power to allow for the correct visualisation of the pulmonary parenchyma. Also, it does not take into account lung function evaluation, which provides important information on airway mechanics and function (Robinson et al., 2000; Hoffman et al., 2001).

As such, we set out to evaluate other parameters which could further contribute to severe EAS staging and ideally also allow staging in a field setting. Thus, we developed a discriminant model based on the aforementioned variables.

Because the discriminant analysis requires data with a normal distribution, some of the variables were removed from the model. As such, the variables thoracic x-ray and endoscopy score, although part of the original clinical staging method (Tilley et al., 2012c) were not included. The thoracic x-ray score was based on the evaluation of pulmonary pattern and of tracheal and bronchial thickness, radiologic findings which are common in this disease. However, although thoracic x-ray may contribute to severe EAS diagnosis, this information alone is non-specific for this disease (Kutasi, Balogh, Lajos, Nagy & Szenci, 2011; Couëtil et al., 2016). The endoscopy score was based on tracheal mucus distribution and its observed characteristics. In severe asthma-affected horses changes in the viscoelastic properties of mucus lead to a decreased clearance, which associated with an increased mucus production results in pooling along the trachea and bronchi (Gerber, King, Schneider & Robinson, 2000; Gerber, Lindberg, Berney & Robinson, 2004; Tilley et al., 2012a, c). However, in cases with mild clinical signs or in horses in remission little or no differences from healthy horses are observed (Gerber et al., 1998; Rettmer et al., 2015).

Clinical scores alone have also been considered insufficient to correctly differentiate mild stages of severe EAS from healthy animals (Robinson et al., 2000; Miskovic et al., 2007). In this study, grading of cough, nasal flare and abdominal lift were evaluated in order to attribute a clinical score to each animal. Nasal flare and abdominal lift observed in affected horses result from the recruitment of accessory muscles in an attempt to decrease airway resistance. We found that although some asthmatic horses in remission did not present obvious clinical signs, group means were significantly different. Furthermore, asthmatic horses in remission maintain some degree of airway inflammation and hyperresponsiveness, in spite of presenting discreet clinical signs (Rettmer et al., 2015).

Airway inflammation is one of the key features of equine asthma and the BAL technique has been used to identify changes in lung cytology. Differential cytologic counts are used to diagnose equine asthma cases, with neutrophil count (>20%) being the most significant parameter. Since severe EAS is a diffuse lung disease, a single aliquot of the lavage fluid collected is considered representative of the entire lung (Hoffman & Viel, 1997; Hoffman, 2008; Jean, Vrins, Beauchamp & Lavoie, 2011). In previously published work, neutrophil percentage was considered the most significant parameter in BALF cytology for staging severe EAS (Tilley et al., 2012c), and in the present study significant differences were also observed between group means.

Until recently the majority of lung function tests used in equine clinic were complicated and invasive procedures, and thus reserved for use in clinical research and in laboratory settings. Nowadays, several equipments have made lung function evaluation available in the field due to their portability. Because one of our goals was the possibility of implementing this staging model in a field scenario, all the equipment chosen can be easily used outside a diagnostic centre.

In this study P_aO_2 was found to be the most significant parameter in the ABG analysis, with differences between group means tending towards statistical significance (P=0.62). This is in agreement with other published data, since, as disease progresses, hypoxaemia tends to become more severe (Nuytten, Deprez, Picavet, Van Den Hende & Muylle, 1988; Couëtil & DeNicola, 1999; Sánchez, Couëtil, Ward & Clark, 2005). For this reason, we chose to include the value of P_aO_2 in our staging model, as it accounted for some of the variability found in this population.

Indirect pleural pressure (transpulmonary pressure) measurement has long since been used to evaluate conventional lung mechanics. The cut off value adopted for considering a positive diagnosis of Severe EAS varies between $\Delta Ppl \geq 12$ cm H_2O (Pirie et al., 1990) and $\Delta Ppl > 15$ cm H_2O (Couëtil et al., 2016). Group means obtained in the present study were in agreement with Pirie, Pirie, Cranston and Wright (1990), however, they were lower than the reference cut off value proposed by Couëtil et al. (2016). This could be explained by the administration of detomidine, an α2-agonist, which induces airway smooth muscle relaxation and a subsequent decrease in distal airway obstruction (Broadstone, Gray, Robinson & Derksen, 1992). As such, there may have still been an underestimation of airway obstruction (ΔPpl) in our asthmatic population. Nonetheless, the results obtained showed significant differences between groups, with ΔPpl increasing as disease severity progressed.

Furthermore, until recently the equipment available for ΔPpl measurement was more suited for diagnostic centres, but the Dppl device N V1.01, a hand held device with a battery included, can easily be used in a field setting due to its portability, ensuring that severe EAS staging is not limited to laboratories and clinical diagnostic centres.

The Open Pleth™ also allows field testing of airway obstruction and of airway hyperresponsiveness, when associated with histamine bronchoprovocation. This equipment can be easily used in the field, as was done by Rettmer et al (2015), although an assistant may be necessary to ensure the horse doesn't lower its head while sedated, which could result in the development of nasal oedema, thus interfering with the pneumotagraphic assessment of flow. In this study, we found no significant differences of ∆flow between groups (P=0.913). This may have resulted from a lack of power of this variable due to the size of our sample. However, Rettmer et al (2015) reported similar findings in their work. Nevertheless, maximum histamine concentration ([Hist]) tolerated by the horses during bronchoprovocation provided significant differences between groups (P=0.00), but an overlap of values was observed in healthy and stage 1 horses, where mean concentration and standard deviation were the same. So, our group of stage 1 asthmatic horses showed no signs of airway hyperresponsiveness, having tolerated maximum histamine concentration (32 mg/ml), which could imply that this parameter alone would prove insufficient to correctly differentiate between healthy horses and those with the mildest form of the disease (stage 1), which while presenting some airway inflammation (BALF neutrophil >20%), had no airway hyperresponsiveness.

Therefore, discriminant analysis resulted in two significant canonical functions, which included the parameters clinical score, BALF neutrophil percentage, ΔPpl, arterial blood PO_2 and maximum histamine concentration ([Hist]). The first function explained the majority of data observed in this study, while the second explained a further 2% of the observed variability.

Consequently, there is enough data to support the use of the new staging method presented here. This staging method shows very promising results with a high percentage of cases correctly identified. As previously mentioned, some overlap of values was seen between stages and this could be related to the small number of horses that were included in each stage, which implies a lack of power of some parameters. Nonetheless, the discriminant model proposed here showed very good results in this population of horses.

Also, it confirmed a major impact of some variables which can easily be evaluated in the field for identifying and staging cases of severe EAS, and the removal of endoscopy and thoracic x-ray from the staging model simplified the methodology and allowed field use. Nonetheless, some precaution should be taken to ensure equipment safety and we recommend light sedation with an α2-agonist for this purpose.

As such, it is the author's conviction that the discriminant linear functions presented here may be used to correctly identify and stage severe EAS, although further method validation should still be performed in a larger number of asthmatic animals with different degrees of disease severity and also in healthy horses.

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Study Limitations

Because of the limited size of our sample and the fact that no horses characterised as stage 4 were included in the examined population, this staging model should be tested in a larger number of animals and validated for stage 4 asthmatic horses.

Declarations of interest

None.

Ethical animal research

Horses described in this work where clinical cases presented to the hospital for respiratory evaluation. All owners where previously informed of the test protocol and signed an informed consent form specific for this work. All procedures performed were in accordance with the ethical standards of the Portuguese and European Union legislation for animal experiments and this research was approved by the Ethics and Welfare Comity (CEBEA) of the Faculty of Veterinary Medicine of the University of Lisbon (FMV-ULisboa) under the reference 007/2014.

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Authorship

All authors contributed to the study design, study execution, interpretation of findings and approved the final manuscript.

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2. A study on intestinal parasitic infection in Lusitano horses with severe Equine Asthma Syndrome

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Abstract

The relation between parasite infection and allergic diseases has long intrigued the scientific community. Although this interaction has been recognized and studied in the human population, only eight years ago was it identified in horses.

In the present study, faecal samples were collected from 20 horses diagnosed with severe Equine Asthma Syndrome (EAS) and from 20 healthy mates belonging to the same Lusitano farms in Portugal. Strongyle egg count per gram (EPG), as well as infective larval stages (L3) count (LPG) and identification were assessed for all animals, and deworming protocols were registered. Significant differences were found between the two groups ($P<0.05$), with mean EPG and LPG being significantly lower in horses with severe EAS. This corroborates the tendency for horses with EAS to have an increased resistance to parasite infection. No differences between the two groups were found in what concerns egg and L3 larvae identification, and strongyle type eggs and *Cyathostomum sensu latum* larvae had the highest representation in both groups of the studied population.

Keywords: Severe Equine Asthma Syndrome; parasitology; horse strongyles; cyathostomins; natural resistance; Lusitano breed.

1. Introduction

The prevalence of allergic diseases has been increasing at an astounding rate in Western industrialized countries (Weiss, 2000; Tilley, Sales Luís & Branco Ferreira, 2012a,b,c; Lambrecht & Hammad, 2017). These types of diseases occur in most mammals, and horses are no exception (Marti et al., 2008). In fact, severe Equine Asthma Syndrome (EAS) was first described more than 2000 years ago and it is now the most prevalent lower respiratory tract disease of horses in the Northern Hemisphere (Tilley et al., 2012b; Moran & Folch, 2011). The disease is triggered when susceptible animals are exposed to hay and dust particles which are usually present in barns (Robinson, 2001; Marti et al., 2008; Pirie, 2014; Couëtil et al., 2016). Consequently, affected horses develop lower airway inflammation and obstruction associated with coughing, increased respiratory effort at rest, decreased clearance and increased mucus production, as well as exercise intolerance (Robinson, 2001; Gerber, Lindberg, Berney & Robinson, 2004; Marti et al., 2008; Tilley et al., 2012b,c).

There have been several reports of a reverse relationship between resistance to certain parasitic infections and allergic diseases, such as asthma and atopy in humans and horses (Lynch et al., 1998; McKay, 2006; Hopkin, 2009; Bründler et al., 2011; Medeiros et al., 2011). An explanation for this occurrence was first proposed by the hygiene hypothesis, which states that environmental factors, such as infections and intestinal parasites, play an important role on the prevalence of allergic disease (Strachan, 1989; Von Mutius, 2007; Tilley, Sales Luís, Branco Ferreira, 2012a). It is believed that Westernization and the disappearance of chronic infectious diseases resulted in the loss of cellular and humoral immunoregulatory pathways and consequently in the development of allergic diseases (Lambrecht & Hammad, 2017). On the other hand, some authors alternatively claim that the resistance to helminth infection observed in asthmatic individuals is due to genetic background, in which the genes conferring resistance to parasites are also responsible for an increased risk of developing allergic diseases (Neuhaus et al., 2010; Lanz et al., 2013).

In this research, we studied the helminth composition and burden, namely regarding horse strongyles, through faecal counts of eggs per gram (EPG) and larvae per gram (LPG), of healthy and severe EAS affected horses from the same Lusitano farms in Portugal. Our purpose was to determine if the parasitic load was indeed significantly lower in severe EAS horses managed under a Mediterranean climate, as suggested by previous authors in other geographic areas, considering that the characteristics of this climate contribute to heavy parasitic burdens. We also aimed to assess if there were any differences on egg and larvae identification between the two groups.

2. Material and methods

2.1. Horses and faecal sample collection

Samples were collected from 40 adult vaccinated Lusitanos, 20 clinically healthy horses and 20 horses diagnosed with severe Equine Asthma Syndrome (EAS), according to the Clinical Staging of severe EAS method (Tilley et al., 2012c). Horse's ages were between 9 and 16 years old (mean age 13 ± 2 years). The horses were divided in two groups – severe EAS (EAS) group and Control (C) group. Inclusion in the severe EAS group was decided by a clinical staging score ≥ 1, while control horses presented clinical staging score = 0.

Severe EAS diagnosis and Clinical Staging were performed at the Equine Hospital of the Faculty of Veterinary Medicine of the University of Lisbon. The horses were examined by two independent clinicians for clinical exam score, endoscopy score, thoracic x-ray and bronchoalveolar lavage cytology score, according to the methodology described by Tilley et al. (2012c).

Each asthmatic animal was paired with a healthy control from the same farm. Deworming protocols (date of treatment and drug used) and environmental management (housing and diet) were the same for each pair. All horses were dewormed with ivermectin, either twice a year (every six months) or once a year.

All faecal samples were obtained directly from the rectum or from freshly void faeces and were examined on the same day. Sample collection was performed simultaneously in each severe EAS-Control pair.

2.2. Faecal analysis

The faecal analysis involved quantitative and qualitative methods for egg and larvae count and their identification, respectively. The coproscopic and faecal culture techniques described below are based on Thienpont, Rochette and Vanparjis (1986), Madeira de Carvalho (2001) and Madeira de Carvalho et al. (2007; 2008a, b).

2.2.1. Egg Count – McMaster Technique

The McMaster technique was used to assess the number of strongyle eggs per gram of faeces (EPG). The faecal samples were homogenized and two grams were diluted in 28 ml of a 25% sugar solution with 1.2-1.25 density. After filtration the sample was placed in a McMaster chamber and the number of eggs was counted using an optical microscope with a 10x objective. The number of eggs was multiplied by 50 to obtain total EPG (Thienpont et al., 1986).

2.2.2. Egg Identification

2.2.2.1. Flotation method

With the remaining faecal suspension, a test tube was filled and a coverslip was placed on top. After 15 minutes, the eggs attached to the coverslip were identified through microscopic observation (Thienpont et al., 1986; Madeira de Carvalho et al., 2007).

2.2.2.2. Sedimentation method

After 15 minutes, the excess supernatant liquid of the previous technique was discarded and the sediment was stained with methylene blue, homogenized and observed under the microscope for egg identification, according to Thienpont et al. (1986) and Madeira de Carvalho et al. (2007).

2.2.3. Larvae count and identification – Faecal culture

In order to count and identify horse strongyle infective larvae stages (L3), the homogenized faecal samples were placed in previously identified plastic cups, weighed and covered with aluminium foil. An average of 47g (±10g) of faeces was placed in each cup. To provide oxygen and moisture, a hole was made in the middle of the faecal sample, the aluminium foil was pierced and a source of water, with approximately 500ml, was placed along with the sample cups inside the incubator set at 26-28ºC. After 14 days, the samples were removed from the incubator and, after discarding the aluminium foil, the cups were filled with water and covered with a Petri dish. The cups were then inverted, in order to completely fill the Petri dish with water, and they were left undisturbed for 24 hours. Afterwards, the liquid was collected into 10 ml centrifuge tubes. The samples were then centrifuged for 5 minutes at 1500 rpm and an aliquot of 100 µl was observed under the microscope in order to count and identify the L3 (Madeira de Carvalho, 2001; Madeira de Carvalho et al., 2007). The identification of horse strongyle L3 to the genus/species level was performed according to Madeira de Carvalho et al. (2008a, b).

To calculate the number of infective larvae per gram (LPG) of faeces the following formula was employed:

$LPG = (N \times F) / W$

LPG- Larvae per gram of faeces; N- Number of larvae per 100 µl; F- Dilution factor to reach total volume of water (100); W- Weight of sample in grams.

2.5. Statistical analysis

All data was stored in a Microsoft Excel 2007 worksheet. Descriptive statistics such as arithmetic mean, standard deviation, and absolute and relative frequencies are presented. The results were analysed using IBM SPSS Statistics V21.0.0 software. Because data did not present a normal distribution, the differences between the severe EAS and control groups were analysed using the Mann-Whitney U test and were considered significant if p≤0.05.

3. Results

3.1. Deworming and management

All horses were dewormed with ivermectin (n= 40), and treatment frequency was either yearly (n=18) or twice a year (n=22) but was the same for each severe EAS-Control pair. Environmental management, mainly housing and diet, was also the same in all EAS-control pairs (n=40), and each pair had access to the same spaces.

3.2. Quantitative methods

3.2.1. Egg Count (EPG)

A total of 24 animals (60%) presented a positive egg count. Mean EPG was nine times higher in the control group (992.5±1535.3) than in the severe EAS group (107.5± 196.7) and mean EPG was significantly different between the two groups (p=0.043) (Figure 14).

3.2.2. Larvae Count (LPG)

As previously mentioned, 24 animals (60%) showed a positive L3 count. When comparing the severe EAS and control group, significant statistical differences were found (p=0.040) and control group LPG mean was ten times higher (1056.7±1598.5) than the severe EAS group (156.00±289.1) (Figure 15).

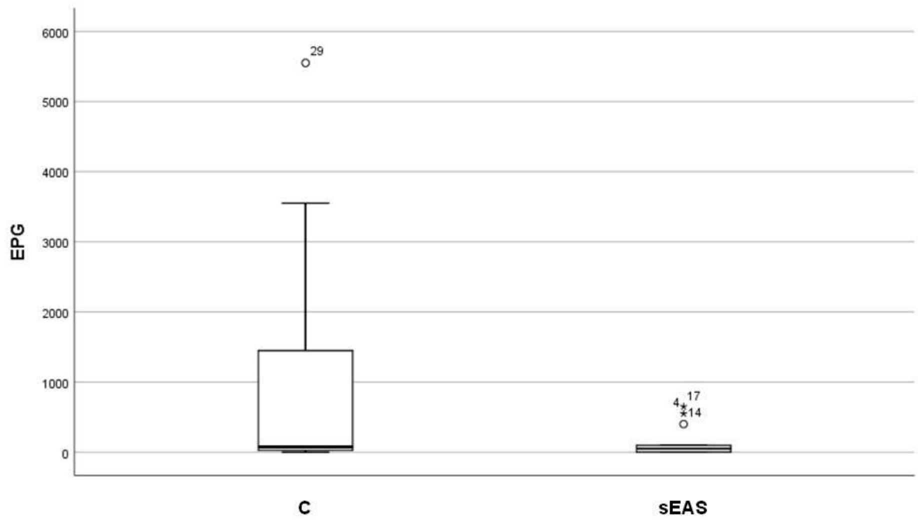

Fig. 14 – Strongyle egg count per gram (EPG) in the Control group (C) and in the severe Equine Asthma Syndrome (sEAS) group.

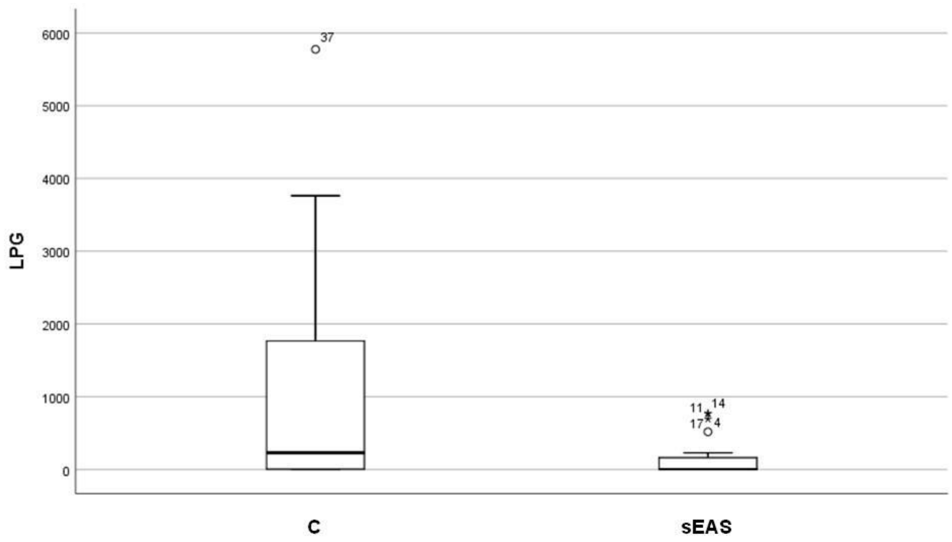

Fig. 15 – Larvae count per gram (LPG) in the Control group (C) and in the severe Equine Asthma Syndrome (sEAS) group.

3.3. Qualitative methods

3.3.1. Egg Identification

Nematode eggs were observed in 24 animals (60%) with the flotation method and in 11 animals (27.5%) with the sedimentation method. The observed eggs were identified as strongyle type (95%) and as *Strongyloides westeri* eggs (5%). *Strongyloides westeri* eggs were found in one horse from the control group, while strongyle type eggs were present in all the animals positive for nematode eggs.

3.3.2. Larvae Identification

Infective larval stages (L3) were observed in 24 animals (60%) and regarding their abundance 0.11% of all L3 observed were identified as *Triodontophorus* spp., 0.23% as *Poteriostomum* spp., 27.93% as *Strongyloides westeri* and 71.73% as *Cyathostomum sensu latum*. *Cyathostomum sensu latum* larvae were highly prevalent in both groups of horses. However, in the control horses *Triodontophorus* spp. and *Poteriostomum* spp. larvae were identified in one of the faecal samples and *Strongyloides westeri* in another. These species were not observed in the corresponding severe EAS-mate faeces (Figure 16).

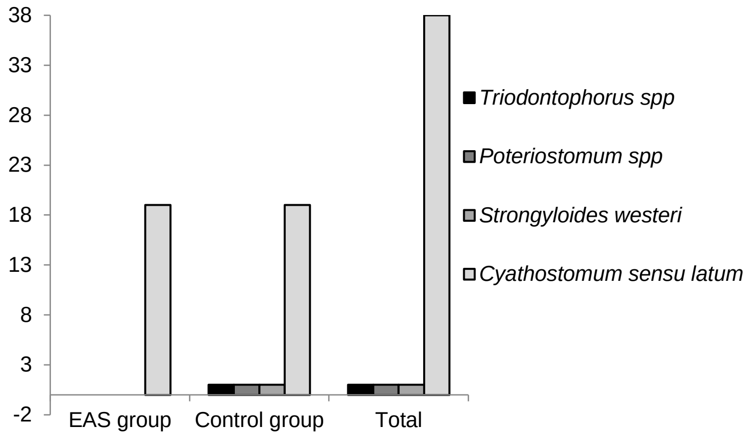

Fig. 16 – Number of horses positive for the larvae species identified per group of animals and in the total population.

4. Discussion

In horses, intestinal parasites have been recognized as a cause of clinical disease since the Roman Empire (Klei & Chapman, 1999). However, only in the last two decades an association between allergic diseases and intestinal parasite infection has been extensively reported. Moreover, although explanatory theories differ in proposed causality, allergic individuals seem to present lower parasitic counts than their healthy counterparts (Lynch et al., 1998; Capron, Dombrowicz & Capron, 2004; Hopkin, 2009; Neuhaus et al., 2010). It has been proposed that protection against intestinal nematodes may have been an evolutionary advantage, which due to a common genetic basis increased the risk for allergic diseases, such as asthma and atopy. In this study, we proposed to investigate the intestinal parasitic burden of horses diagnosed with severe EAS and compare it to healthy control mates in Portugal. In addition, we identified the eggs and the larvae in all animals to assess if any differences in intestinal helminth population were present between the groups, and if these parasites prevalence was similar to that described in the literature for Portugal. For this purpose, the McMaster egg counting technique and also flotation and sedimentation for egg identification were performed. Depending on their size, the eggs may either float, being identified with the flotation technique, or become deposited in the sediment, requiring identification through the sedimentation technique.

To minimise confounding variables every asthmatic horse was paired with a healthy mate from the same farm and both horses had an identical deworming program, diet, housing and had access to the same pastures. As such, all animals were treated with ivermectin, either yearly or twice yearly. Because severe EAS is a disease which is characteristic of adult horses, all animals had ages between 9 and 16 years, and each pair was approximately the same age (mean age difference 2 years ± 2.6 years).

This study shows a pattern of lower shedding of parasitic eggs in horses affected by severe EAS, since EPG and LPG group means were found to be significantly different. Non-asthmatic animals (control group) were found to shed on average nine times more eggs and ten times more infective larvae than the asthmatic horses. Therefore, this data appears to be in agreement with other studies which describe an increased resistance to intestinal strongylid nematodes in severe EAS-affected horses, when compared to pasture control mates (Neuhaus et al., 2010; Bründler et al., 2011; Medeiros et al., 2011).

Some animals (9 asthmatic horses and 5 control horses) had null parasitic counts independent of the group they were allocated to, which could be related to the selected deworming program. Most of these horses were competing in either show jumping or dressage, and as such, were dewormed twice a year. This greatly limits the risk of intestinal parasitic infection and most likely contributed to the EPG and LPG counts observed.

In this study, no differences were found in the egg and the larvae species identified in this population of horses, and the majority of the observed eggs were identified as strongyle type, which is in accordance with other reports of gastrointestinal nematode prevalence (Lopes et al., 2017; Stancampiano, Usai, Marigo & Rinnovati, 2017). The exception was one animal, allocated to the control group, which presented a high percentage of *Strongyloides westeri* eggs and larvae. This is a fairly uncommon finding in asymptomatic adult horses, since most adult horses develop a natural resistance to this nematode, and its presence is usually seen in young foals (Miller, Bellaw, Lyons & Nielsen, 2017).

Larvae identification results also showed a high abundance of cyathostomins infection in our study population, particularly of *Cyathostomum sensu latum* (71.73%). These findings attest to the high prevalence of these nematodes in Portugal and also agree with other published reports [23; 24; 29]. In the modern era, cyathostomin nematodes have become highly prevalent in parasitic infections of mature horses and concerning reports of their increased resistance to anthelmintic treatment are abundant in the scientific literature (Klei & Chapman, 1999; Kaplan & Nielsen, 2010; Kaplan & Vidyashankar, 2012).

Although two control horses, one with *Strongyloides westeri* and the other with *Triodontophorus* spp. and *Poteriostomum* spp., presented nematode species not identified in the severe EAS population, conclusions cannot be extrapolated from this small sized population and further investigation needs to be conducted.

Some of the studied horses, especially the healthy mates, presented an alarmingly high EPG and LPG count even though the owners reported anthelmintic treatment in the last year. This could alert to a possible helminth resistance to the treatment, or the use of unsuitable/off label anthelmintics, sub dosages, or even a lack of hygiene that promotes reinfection of the horse by the larvae it previously shed as faecal eggs, or still to the heavy parasite burden associated with the Mediterranean climate. Furthermore, all the animals in this study were dewormed at a specific time and always with the same drug, and previous diagnostic surveillance was not performed to determine the need for treatment, nor control its efficacy. Diagnostic surveillance is an essential tool for the assessment of an adequate prophylactic treatment protocol for helminth infection and for the prevention of indiscriminate use of anthelmintics (Kaplan & Nielsen, 2010). Thus, owner education is essential to promote the most suitable parasite control program and to prevent the development of anthelminthic resistance.

In conclusion, asthmatic horses presented a significantly lower mean EPG and LPG, indicating a relationship between the presence of allergic diseases, namely Equine Asthma Syndrome, and intestinal nematode infection. It appears that this group of Lusitano asthmatic horses in a Mediterranean climate also have an increased resistance to intestinal nematodes and as such these animals may require fewer treatments with anthelmintics to control parasitic infections. However, the presence of allergic diseases seemed to have no impact on infective nematode species, since no differences were observed between the two groups, with strongyle type eggs and cyathostomin larvae being highly abundant and prevalent, reflecting trends previously reported in Portugal.

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Ethical statement

All procedures performed were in accordance with the ethical standards of the Ethics and Welfare Comity of the Faculty of Veterinary Medicine of the University of Lisbon.

Declarations of interest

None

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Abbreviations

EAS – Equine Asthma Syndrome

EPG – Egg Per Gram

LPG – Larvae Per Gram

L3 – Third stage infective larvae

3. Owner compliance to environmental management protocol for severe Equine Asthma Syndrome

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Abstract

Severe equine asthma (SEA) syndrome is a chronic recurrent respiratory disease, common among adult horses. The disease occurs in genetically susceptible individuals after their exposure to organic dust. Thus, environmental management has proved essential in controlling airway challenge and disease exacerbation. This is a demanding process that can only be achieved through the horse owners' cooperation. One year after initial diagnosis of SEA in a group of 39 horses, owner compliance to an environmental management protocol was evaluated. The overall compliance to the protocol was poor and the horses' clinical health and need for pharmacological management was related to the successful implementation of the environmental recommendations provided upon disease diagnosis.

Keywords

Severe equine asthma syndrome; owner compliance; environmental management; clinical signs

1. Introduction

Severe Equine Asthma (SEA) syndrome is a chronic recurrent respiratory disease which appears to be related to domestication (Couëtil et al., 2016; Bullone & Lavoie, 2017). The disease is fairly common in the Northern hemisphere where horses are stabled during part of the year and in the UK there is an estimated prevalence of 14% (Hotchkiss, Reid & Christley, 2007).

This naturally occurring illness occurs in genetically predisposed individuals after exposure to environmental risk factors (organic dust) (Marti, Gerber, Essich, Oulehla & Lazary, 1991; Leclere, Lavoie-Lamoureux, Gélinas-Lymburner, David & Martin, 2011). Feed and bedding materials are the main sources of respirable dust particles, which include mould spores, mites, endotoxins, antigenic materials, and pollen (Robinson, Karmaus, Holcombe, Carr & Derksen, 2006; Tilley, Sales Luís & Branco Ferreira, 2012). Also, traditional housing systems have significantly higher respirable dust concentrations than outdoor systems (McGorum, Ellison & Cullen, 1998).

When exposed to organic dust, susceptible horses present with airway inflammation and obstruction as well as bronchial hyper-responsiveness. The most common clinical signs are cough, nasal discharge, increased respiratory efforts at rest and exercise intolerance (Leclere, Lavoie-Lamoureux & Lavoie, 2011).

Management of SEA requires environmental control and medical treatment. Medical treatment focuses on controlling airway inflammation through corticosteroid therapy and reducing bronchospasm with bronchodilators (Robinson, Derksen, Jackson, Peroni & Gerber, 2001). However, environmental management should be the primary goal since clinical signs and lung function quickly improve in a low dust environment, even without medication (Holcombe et al., 2001; Couëtil & Ward, 2003). Ideally, asthmatic horses should be kept at pasture all day and fed grass or a completely cubed or pelleted diet. Alternatively housing facilities, bedding materials, stable management and feeding, should be adapted to minimise airway challenge and accommodate the requirements of the asthmatic horse (Robinson et al., 2001; Clements & Pirie, 2007).

2. Material and Methods

2.1. Horses

Severe equine asthma was diagnosed in 39 adult horses from independent locations, aged between 8 and 18 years (mean age 12.6 ± 3.7). The horses were referred to the Equine Teaching Hospital of Lisbon's Veterinary College (University of Lisbon) and were examined during disease exacerbation.

All animals were vaccinated for tetanus and influenza and had a history of being fed hay in the last two months prior to examination. Pharmacological treatment, with either corticosteroids or bronchodilators, was not administered in the 2 weeks prior to the horse's clinical evaluation at the Equine Teaching Hospital.

2.2. Severe equine asthma syndrome staging

SEA diagnosis was based on clinical examination, thoracic x-ray, bronchial endoscopy and bronchoalveolar lavage fluid cytology, according to Clinical staging method published by Tilley et al. (Tilley, Sales Luís & Branco Ferreira, 2012b).

A score was attributed to each parameter, based on specific clinical variables, and the sum of the scores indicated the stage of the horse. The clinical score involved the evaluation of cough, nasal flare and abdominal lift. The thoracic x-ray score required assessment of interstitial pattern, bronchial radiopacity, tracheal thickening and bronchial thickening. The endoscopy score was determined based on mucus characteristics, namely accumulation, colour, localization and thickness and apparent viscosity. The bronchoalveolar lavage fluid cytology score was based on neutrophil percentage. Horses were considered to be SEA-affected if staged ≥ 1 (Tilley, Sales Luís & Branco Ferreira, 2012b).

2.3. Environmental management – initial evaluation

Upon disease diagnosis, a questionnaire was given to the owners in order to obtain information concerning the housing and feeding conditions of each horse as well as the clinical signs perceived by the owners.

The questionnaire was comprised of 12 closed questions and each interview was conducted by two veterinary practitioners trained for consistency (table 3). For each animal, the housing ventilation was classified as poor (<2 openings to facilitate air circulation) or good (≥2 sources for air circulation). Furthermore, it was ascertained if the horse was stabled or kept at pasture, the amount of time each horse spent outdoors (≤ or > than 6 hours), if the horse was turned out or kept inside during cleaning of the stables, the type of bedding material (high or low dust content) and if the horse was fed dry hay or another forage alternative. Owners were questioned whether the affected horses exhibited signs of nasal discharge, increased respiratory effort at rest and cough. Similarly, the need of pharmacological management, with corticosteroids and bronchodilators was registered as well as the frequency of the clinical signs observed by the owner (rare, occasional or frequent). Pharmacological treatment was administered systemically (intravenous or intramuscular) for a short period of time as a way of controlling overt clinical signs and was administered under the supervision of the referring veterinarian practitioners. The use of topical aerosol therapy was not reported for any of the horses included in the study. If the horse owner was unaware of the type of medication that had been administered to horse, this information was provided by the referring veterinarian.

Table 3 – Horse owner questionnaire used in both interviews to evaluate environmental management

Environmental management questionnaire

1. Is your horse stabled?
   □ Stabled □ Pasture
2. How would you describe the housing ventilation?
   □ Poor (< 2 distinct openings to facilitate ventilation)
   □ Good (≥ 2 distinct openings to facilitate ventilation)
3. On average how much time does the horse spend outdoors daily?
   □ <6h □ >6h
4. During cleaning and mucking out does the horse stay inside the stables?
   □ Yes □ No
5. How is the dust content of your horses' bedding material?
   □ Low dust content (wood shavings, grass, rubber mattress)
   □ High dust content (sawdust, straw)
6. What type of forage do you feed your horse?
   □ Unsoaked hay □ Soaked hay > 20min □ Other: ____________
7. Does your horse have nasal discharge?
   □ Yes □ No
8. Does your horse have an increased breathing effort at rest?
   □ Yes □ No
9. Does your horse have episodes of cough?
   □ Yes □ No
10. Are the clinical signs associated with:
    □ Hay feeding □ Season □ Exercise □ No trigger factor identified
11. How often does the horse exhibit clinical signs?
    □ Rarely □ Occasionally □ Frequently
12. Was treatment with corticosteroids and bronchodilators required to manage clinical signs?
    □ Yes □ No

Final owner assessment:

Do you feel the horse has improved since the initial diagnosis?

□ Yes □ No

Additionally, we asked the owners to identify the main trigger of their horses' clinical sign (feed, season, exercise).

After the initial evaluation, a set of guidelines comprising six management recommendations was provided to each interviewee to promote asthma remission (table 4). The six parameters of the environmental protocol were quality of ventilation, type of housing, amount of time spent outdoors, removal of the horse during cleaning of the stables, type and dust content of bedding material used and type of forage fed (dry hay or soaked hay).

Table 4 – Environmental management guidelines given to each SEA-affected horse owner after diagnosis

Key goal: Improve air quality and reduce horse's exposure to respirable dust

Parameter	
	Housing
1	Housing must have **good ventilation** (at least two openings for fresh air circulation);
2	Avoid stabling and opt for keeping your horse at **pasture**;
3	**Turn out** your horse for the largest amount of time possible, at least 12 hours a day;
4	During grooming and cleaning of the stables horses should stay **outside**;
	Bedding material
5	Preferential use of commercial **dust-free wood shavings**, cardboard or other low dust option and avoid materials with a potentially high dust content, such as saw dust and straw;
	Forage and feed
6	**Do not** feed dry hay, because of its' high dust concentration; Opt for **cubed or pelleted diet** or **completely immerse the hay in water for 20-30 min** before feeding it to your horse.

2.4. Environmental management – follow up

One year after initial environmental management evaluation, a telephone follow up interview was conducted for all the participants to ascertain owner compliance to the environmental management recommendations provided a year prior. All interviews were conducted by the same veterinarian using the same 12 questions from before. Additional enquiries were made to ascertain whether the clinical status of the horse had improved (table 3).

2.5. Evaluation of owner compliance and influence of environmental parameters on clinical signs

In order to ascertain whether the owners had followed the provided guidelines (table 5) and whether these changes had had a positive effect on the horses' respiratory health, the information obtained via telephone interview was compared with the data collected a year earlier.

The statistical analysis was performed using IBM SPSS Statistics V21.0.0 software[a] and cross tabulation analysis in association with the z test was used to compare proportions. An α-level of 0.05 was considered significant.

Owner compliance to environmental management was classified as poor (0 to 2 parameters), acceptable (3 to 4 parameters) or good (5 to 6 parameters), depending on the number of environmental parameters adopted (table 5).

3. Results

3.1. Clinical signs

In the initial evaluation, all horses presented with cough and increased respiratory effort at rest (n=39) and a large number also exhibited nasal discharge (n=22). The occurrence of these clinical signs was described as either frequent (n=32) or occasional (n=7). Owners also reported that all 39 animals had required treatment with corticosteroids and bronchodilators in the two weeks prior to the disease diagnosis (table 5).

When asked to identify trigger factors associated with the occurrence of clinical signs 64.1% of owners (n=25) considered hay to be the main trigger factor, followed by season (30.8%, n=12) and exercise (5.1%, n=2).

A year later, owners reported an improvement of clinical signs for some horses (n=25), with 20 horses rarely showing clinical signs, whilst 6 animals continued exhibiting frequent asthma signs. During the year following the implementation of the environmental management protocol almost half of the horses (n=17) did not require any medication. Regarding overall clinical improvement, cough and nasal discharge, no significant statistical difference was found between the proportion of horses medicated and the proportion of horses which were not. However, regarding respiratory effort at rest, a significantly higher proportion ($p<0.05$) of the horses which did not receive medication during this period presented with a normal breathing at rest compared to the proportion of horses which received medication.

Table 5 – Adoption of each environmental management parameter by horse owners and health status of SEA-affected horses, before and after the implementation of the environmental guidelines

Parameter	Before	After	Parameter	Before	After
Ventilation (n=39)			Nasal discharge (n=39)		
Poor	19	16	Yes	22	15
Good	20	23	No	17	24
Housing (n=39)			Breathing effort (n=39)		
Stabled	39	31	Yes	39	14
Pasture	0	8	No	0	25
Time spent outdoors (n=39)			Cough (n=39)		
≤6 hours	31	23	Yes	39	29
>6 hours	8	16	No	0	10
During cleaning (n=39)			Medical treatment (n=39)		
Stays inside	16	12	Yes	39	22
Turned out	23	27	No	0	17
Bed material (n=39)			Frequency of clinical signs (n=39)		
High dust	29	18	Rare	0	20
Low dust	10	21	Occasional	7	13
Hay (n=39)			Frequent	32	6
Dry	39	30	Clinical improvement (n=39)		
Soaked	0	9	Yes	-	25
			No	-	14

3.2. Influence of environmental management on clinical signs

A statistical effect was found between poor ventilation and the presence of increased respiratory effort at rest, cough and the need for medical treatment (table 6). On the other hand, a good ventilation influenced the occurrence of rare clinical signs and overall improvement of the horse.

Table 6 – Influence of the environmental parameters adopted by horse owners on the SEA-affected horses health status

	Nasal discharge		Breathing effort		Cough		Medical treatment		Clinical signs			Improvement	
	Yes	No	Yes	No	Yes	No	Yes	No	Rare	Occasional	Frequent	Yes	No
Ventilation													
Poor	50%	50%	62.5%$_a$	37.5%$_a$	100%$_a$	0%$_b$	81.3%$_a$	18.8%$_a$	25%$_a$	37.5%$_a$	37.5%$_a$	37.5%$_a$	62.5%$_a$
Good	30.4%	69.6%	17.4%$_b$	82.6%$_b$	56.5%$_a$	43.5%$_b$	39.1%$_b$	60.9%$_b$	69.6%$_b$	30.4%$_a$	0%$_b$	82.5%$_b$	17.4%$_b$
Housing													
Stabled	48.4%$_a$	51.6%$_a$	41.9%	58.1%	83.9%$_a$	16.1%$_a$	64.5%$_a$	35.5%$_a$	41.9%$_a$	38.7%$_a$	19.4%$_a$	58.1%	41.9%
Pasture	0%$_b$	100%$_b$	12.5%	87.5%	37.5%$_b$	62.5%$_b$	25.5%$_b$	75%$_b$	87.5%$_b$	12.5%$_a$	0%$_a$	87.5%	12.5%
Time outdoors													
≤6 hours	43.5%	56.5%	52.2%$_a$	47.8%$_a$	91.3%$_a$	8.7%$_a$	69.6%$_a$	30.4%$_a$	39.1%$_a$	34.8%$_a$	26.1%$_a$	47.8%$_a$	52.2%$_a$
>6 hours	31.3%	68.8%	12.5%$_b$	87.5%$_b$	50%$_b$	50%$_b$	37.5%$_b$	62.5%$_b$	68.8%$_a$	31.3%$_a$	0%$_b$	87.5%$_b$	12.5%$_b$
During cleaning													
Stays inside	58.3%	41.7%	58.3%$_a$	41.7%$_a$	91.7%	8.3%	83.3%$_a$	16.7%$_a$	16.7%$_a$	50%$_a$	33.3%$_a$	41.7%	58.3%
Turned out	29.6%	70.4%	25.9%$_a$	74.1%$_a$	66.7%	33.3%	44.4%$_b$	55.6%$_b$	66.7%$_b$	25.9%$_a$	7.4%$_b$	74.1%	25.9%
Bed material													
High dust	47.4%	52.6%	52.6%$_a$	47.4%$_a$	89.5%$_a$	10.5%$_a$	73.7%$_a$	26.3%$_a$	36.8%	36.8%	26.3%	57.9%	42.1%
Low dust	30%	70%	20%$_b$	80%$_b$	60%$_b$	40%$_b$	40%$_b$	60%$_b$	65%	30%	5%	70%	30%
Hay													
Dry	46.7%	53.3%	43.3%	56.7%	93.3%$_a$	6.7%$_a$	70%$_a$	30%$_a$	40%$_a$	40%$_a$	20%$_a$	53.3%$_a$	46.7%$_a$
Soaked	11.1%	88.9%	11.1%	88.9%	11.1%$_b$	88.9%$_b$	11.1%$_a$	88.9%$_b$	88.9%$_b$	11.1%$_a$	0%$_a$	100%$_b$	0%$_b$

Different letters indicate a difference in proportions for a $p<0.05$. Comparisons were made for each variable, by line and for each factor level.

Whilst stabling influenced the presence of cough, pasture influenced the absence of nasal discharge and cough, as well as reports of rare clinical signs, absence of pharmacological treatment and overall clinical improvement.

The effect of the amount of time spent outdoors on breathing effort, cough, medical treatment and overall clinical improvement was statistically significant. A statistically significant proportion of horses spending more than 6 hours outdoors had normal breathing effort at rest, did not require administration of corticosteroids and bronchodilators and showed clinical improvement. On the other hand, horses spending 6 hours or less outdoors was reflected by on the presence of cough.

Also, remaining indoors during cleaning of the stables influenced owner-reporting signs of cough and the need for pharmacological treatment to manage clinical signs. There was a significant difference ($p<0.05$) between the proportion of horses which were turned out during this period of increased stable activity and the horses which remained indoors.

The effect of low dust bedding materials was statistically significant ($p<0.05$), regarding the absence of increased respiratory effort and reports of no need for medical treatment as well as overall clinical improvement. A statistically significant difference was observed between the proportion of horses with high dust bedding material, such as sawdust and straw, and those with low dust bedding materials. Reporting of the presence of cough was influenced by the use of materials with high dust content.

Lastly, a significant difference between the proportion of horses fed dry hay and those fed soaked hay was observed. The effect of dry hay feeding on cough, need for pharmacological treatment and frequency of clinical signs was statistically significant ($p<0.05$). On the contrary, properly soaked hay (between 20-30 minutes) was related to the reports of absence of cough, of lack of medical treatment, of infrequent occurrence of clinical signs and of overall clinical improvement.

3.3. Owner compliance to environmental management guidelines

The overall compliance to the environmental management guidelines was deemed poor in 51.3% of the cases (n=20), acceptable in 33.3% (n=13) and good for the remaining 15.4% (n=6). Of this last group only 3 interviewees (7.7%) managed to adopt all the six environmental parameters suggested (table 7).

Also, four owners failed to adopt any of the environmental management parameters recommended to them and reported no clinical improvement, as well the need to use corticosteroids and bronchodilators to manage the disease.

The two parameters which were most unpopular among the owners were feeding soaked hay (23.1%) and keeping the horse at pasture (20.5%). In contrast, the change of bedding material was the most adopted environmental parameter (53.8%) (table 5).

Increased compliance had an effect on the absence of respiratory effort at rest, absence of cough, decreased frequency of clinical signs, non-administration of pharmacological treatment and a clinical improvement of the horse.

Table 7 – Influence of owner compliance on SEA-affected horses health status

	Owner compliance		
	Bad (n=20)	Acceptable (n=13)	Good (n=6)
Nasal discharge			
Yes	55%	23.1%	16.7%
No	45%	76.9%	83.3%
Breathing effort			
Yes	55% a	23.1%	0% b
No	45% a	76.9%	100% b
Cough			
Yes	100% a	69.2% b	25.6% c
No	0% a	30.8% b	100% c
Treatment			
Yes	80% a	46.2% b	0% c
No	20% a	53.8% b	100% c
Clinical signs			
Rare	25% a	69.2% b	100% b
Occasional	45% a	30.8% a, b	0% b
Frequent	30% a	0% b	0% a, b
Improvement			
Yes	45% a	76.9% a, b	100% b
No	55% a	23.1% a, b	0% b

Different letters indicate a difference in proportions for a $p<0.05$. Comparisons were made for each variable and for each column.

4. Discussion

In this study, SEA was diagnosed using a clinical staging method. The discriminant analysis of this method showed very good results, with 92.5% of original cases correctly classified and 85% of cases correctly classified through cross validation, thus presenting a viable discrimination between groups (Tilley, Sales Luís & Branco Ferreira, 2012).

In order to evaluate owner compliance, owners were interviewed to ascertain the adherence to environmental management parameters, before and one year after disease diagnosis.

At the time of diagnosis all horses presented with cough and increased respiratory effort at rest and all required treatment with corticosteroids and bronchodilators for the control of airway inflammation and obstruction. Furthermore, 22 horses showed signs of nasal discharge, which may indicate that this clinical sign is not always present in SEA-affected horses or that owners tend to neglect its occurrence (Rettmer, Hoffman, Lanz, Oertly & Gerber, 2015; Couëtil et al., 2016).

The assessed overall compliance to environmental management was extremely low, with more than half the owners (51.3%) adopting only two or less of the suggested parameters (poor compliance). The compliance could only be considered good in 6 cases (15.4%), in which 5 or 6 of the suggested parameters were adopted.

Compliance was based on the number of parameters adopted, and although the adoption of one parameter might influence the occurrence of others (e.g. a horse in pasture will have a good ventilation), care has been taken to evaluate each parameter individually. In fact, even though some of our studied population was turned out to pasture, they were still being fed dry hay, which would result in a high concentration of airborne dust particles around the horse's nostrils when eating (Clements & Pirie, 2007).

Furthermore, four of the interviewed owners chose not to adopt any of the provided environmental recommendations, which can be considered very worrying. These particular animals did not show any signs of clinical improvement, required pharmacological treatment to control clinical signs, and the owners continued to report frequent signs of nasal discharge, cough and increased breathing effort at rest one year after the initial diagnosis of the disease at the time of the second questionnaire.

Of all the environmental recommendations, the type of bedding was the most easily modified (n=11). Still, the use of straw and saw dust bedding was associated with cough, which can be related to the high content of respirable dust particles found in these materials (Vandenput, Duvivier, Votion, Art & Lekeux, 1998).

Despite ventilation being essential for air renewal and, for eliminating some of the ammonia and dust particles found in stables (MacGorum, Ellison & Cullen, 1998), at least 16 asthmatic horses were continually maintained in stables with poor ventilation.

At the time of the initial evaluation all 39 asthmatic horses were stabled and owners reported that they frequently manifested clinical signs (Couëtil et al., 2016). At the second interview, the horses, which were kept at pasture, presented with rare clinical signs of nasal discharge and cough and showed an overall improvement associated with lack of need for pharmacological treatment.

Turning the asthmatic horses out for more than 6 hours per day was insufficient to ensure disease remission. This could be related to the fact the owners may have included, here, the time spent riding the horse, which may further contribute to hyperreactiveness and inflammation, caused either by dusty riding arenas or by the air flow velocity and temperature during exercise (Davis et al., 2006).

It is possibly easier for owners to adopt a change in protocol which solely involves a new purchase (e.g. wood shavings) rather than one that requires a change in everyday habits (e.g. turning out the horses or taking them from the stables during periods of increased activity) or modification of infrastructures (e.g. ventilation).

The fact that the horse should not be inside during cleaning and mucking out is an established husbandry practice mostly related to the risk of injuring the horse whilst cleaning the bed. The potential respiratory hazard for the horses associated with the increased concentration of respirable dust particles is usually neglected, although owners seem aware of the health risk it poses for humans. Some owners claimed it was unpractical to remove the horses, thus these animals tended to present cough and required medical treatment.

Furthermore, although hay was identified as a trigger factor in 64.1% of the cases, owners proved reluctant in changing their horse's forage. To significantly reduce the concentration of respirable dust, hay should be soaked (completely immersed) in water for 20 to 30 minutes (Blackman & Moore-Colyer, 1998; Vandenput et al., 1998; Swain, 2004; Clements & Pirie, 2007; Hotchkiss, Reid & Christley, 2007b). Most interviewees simply sprinkled the hay surface with water, which caused its centre to remain dry and was therefore ineffective (Clarke & Madelin, 1987). Hay soaking is a time-consuming procedure and this might be one of the reasons why so many owners refused to adopt this practice. Dry hay feeding was related to reports of cough and soaked hay feeding to clinical improvement.

In this study, overall compliance was rather poor, and many horses required medical treatment, which provides an immediate relief of clinical signs, allowing the horse to assume a more comfortable breathing pattern. Nonetheless, airway inflammation persists if the horse's environment is not addressed (Robinson et al., 2001).

It is important to note some confounding factors, namely the frequency of pharmacological administration. Although, in the present study, medication was used as a mean of controlling clinical signs for a short of period time, there is the possibility that owners relied on medication and opted for a suboptimal environmental management. Also, because we did not enquire about the last time the horse was medicated prior to the second interview, there is also the possibility that at this time point some horses were under the influence of medication. However, we found no significant difference in overall clinical improvement, cough and nasal discharge, between the proportion of horses which received treatment and those which did not, which supports the use of bronchodilators and corticosteroids as a temporary means of reducing airway inflammation.

A good owner compliance was associated with rare clinical signs, lack of pharmacological treatment to maintain respiratory function and overall clinical improvement of the SEA-affected horses.

Owner compliance is therefore pivotal and persistence in maintaining the newly adopted environmental changes is essential for the horse's long-term well-being (Hotchkiss, Reid & Christley, 2007; Hotchkiss, Reid & Christley, 2007b). The poor compliance observed in the present study reinforces the need to alert veterinarians of this reality. The authors believe it is of utmost relevance that enough time is taken during consultation to increase owner awareness of the risks that are taken by lack of compliance.

In fact, in clinical trials in humans, there has been a switch from compliance to adherence, in order to decrease dropout rates (Fregni & Illigens, 2018). Therefore, maybe in veterinary medicine we should not be talking about compliance, which is based on the owner following the suggestion of the veterinarian as a form of authority, leading to a passive behaviour. Instead, maybe we should work on adherence, which is based on the owner being better informed in order to increase trust, leading to active choices on the owner's behalf.

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Study Limitations

This study used a population of only 39 SEA-affected horses and their corresponding owners. It would be pertinent to evaluate a larger population to obtain more insight on the difficulties associated with owner compliance. Also, the frequency of pharmacological treatment varied significantly between horses, and this might have had an impact on the data obtained.

Conflict of interest statement

None

Ethical animal research

Horses described in this work where clinical cases referred to the FMV-UL Equine Teaching Hospital for respiratory evaluation. Owner's signed an informed consent form, specific for this work, which was also approved by the Ethics and Animal Welfare Comity of the Faculty of Veterinary Medicine of the University of Lisbon, under project reference 007/2014.

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Authorship

All authors contributed to the study design, execution, interpretation of findings and approved the final manuscript.

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CHAPTER 4

Discussion

1. Contribution of lung function tests to the staging of severe equine asthma syndrome in the field

The clinical signs associated with severe EAS have been thoroughly described in the scientific literature and are fairly easy to recognise during disease exacerbation. Affected horses usually present cough, increased respiratory effort at rest, nasal discharge and exercise intolerance (Bond et al., 2018).

It is, however, the mild cases, where low grade inflammation is present, that pose a diagnostic challenge (Robinson et al., 2000). Under such circumstances the use of ancillary diagnostic tools is essential for detecting the small changes in the lung function of asthmatic horses (Hoffman, Kuehn, Riedelberger, Kupcinkas & Miskovic, 2001; Miskovic, Couëtil & Thompson, 2007; Rettmer, Hoffman, Lanz, Oertly & Gerber, 2015).

Furthermore, clinical staging is fundamental when addressing insidious diseases, such as severe EAS. Because it is a chronic and debilitating condition, affecting all types of horses from leisure to high-performance equine athletes, correct therapeutic management, continuous monitoring of disease progression and an accurate prognosis are essential. Staging methods may also prove useful in convincing less cooperative tutors of the importance of environmental management in accomplishing disease remission, since they provide an objective measurement of the severity of the disease.

With such goals in mind, Tilley and co-workers developed a clinical staging method based on physical examination findings, endoscopic mucus characterization, thoracic x-ray changes and BALF neutrophil percentage. This clinical staging method allowed the characterization of severe EAS into four categories according to the severity of changes observed in each parameter evaluated (Tilley, Sales Luís & Branco Ferreira, 2012b).

Because of the type of equipment required for this clinical staging, namely the specifications of the x-ray generator for the correct visualisation of the pulmonary parenchyma, this methodology, was more suited for diagnostic centres and had a limited field application. It also didn’t include information pertaining to the patients’ lung function, which more accurately portraits lower grade inflammation characteristic of the milder forms of severe EAS (Robinson et al., 2000; Hoffman et al., 2001).

Nevertheless, in equine practice, the vast majority of clinical procedures are done in an ambulatory setting, mostly due to the complicated logistics associated with transporting a horse to a veterinary hospital or clinic. For this reason, it is important that a staging method for severe EAS may be easily used in equine ambulatory practice. This requires the use of portable equipment, which can be operated easily by the practitioner in the field.

Consequently, we proposed the development of an alternative method for severe EAS staging, which contemplated lung function measurements and could ultimately be used in ambulatory practice. The result was the development of a discriminant model for severe EAS staging which included the parameters used in the clinical staging method (physical examination, endoscopic mucus score, thoracic x-ray score and BALF neutrophil percentage) (Tilley et al., 2012b) and the data obtained from arterial blood gas analysis, pleural pressure measurement, flowmetric plethysmography and histamine bronchoprovocation.

Both the thoracic x-ray and endoscopic mucus score where not included in the final model, since these parameters did not possess a normal distribution, a requirement for the discriminant analysis. Also, both parameters fail to identify cases of low-grade inflammation when parenchymal lung changes are still very subtle and there hasn't been a change in mucus rheology and an increase in its production (Gerber, Lindberg, Berney & Robinson, 2004a; Gerber et al., 2004b; Kutasi, Balogh, Lajos, Nagy & Szenci, 2011; Rettmer et al., 2015; Couëtil et al., 2016;).

Although some authors have reported that clinical scores alone lack the ability to distinguish between healthy animals and those with mild stages of severe EAS (Robinson et al., 2000; Miskovic et al., 2007), we have found significant differences between the group means pertaining to the degree of cough, nasal flare and abdominal lift observed during the physical examination. Because these clinical signs are related to the degree of airway inflammation and obstruction, horses in remission tend to present discreet clinical signs (Rettmer et al., 2015).

Unsurprisingly, the neutrophil percentage found in the BALF cytology was a good indicator of airway inflammation and portrayed significant differences between group means (Tilley et al., 2012b). This parameter is commonly used in the diagnosis of severe EAS, where the cut off value of 20% neutrophils in the differential cytological count is considered an indicator o severe EAS (Couëtil et al., 2016).

As previously mentioned, for the lung function assessment we elected diagnostic tests which could be employed in ambulatory setting and were therefore portable. These included, arterial blood gas (ABG) analysis, indirect pleural pressure measurement, flowmetric plethysmography and evaluation of airway reactivity through histamine bronchoprovocation.

It has been previously described that the onset of hypoxaemia is associated with more advanced stages of severe EAS (Nuytten, Deprez, Picvet, Van Den Hende & Muylle, 1988; Couëtil & DeNicola, 1999, Sánchez, Couëtil, Ward & Clark, 2005). In agreement with this published data, the only parameter of the ABG analysis included in our model, which tended towards statistical significance and described some of the variability observed in the studied population, was P_aO_2

For the indirect pleural pressure (ΔPpl) evaluation we used the handheld Dppl device N V1.01, with an operating battery, allowing measurements in the field. The ΔPpl is used in the evaluation of conventional lung mechanics and a value of ΔPpl>12 cm H_2O is considered indicative of severe EAS (Pirie, Pirie, Cranston & Wright, 1990). However, in a more recent consensus a ΔPpl>15 cm H_2O was specified as having diagnostic value for severe EAS

(Couëtil et al., 2016). Some of the group means obtained in this study were lower than the reference value referred in the 2016 consensus, although they were in accordance with the data published by Pirie and its team. This parameter was also statistically significant and thus was included in our model. We hypothesized that the use of the α2-agonist detomidine for the sedation protocol may have resulted in airway smooth muscle relaxation and therefore a decrease in airway resistance, which explains the lower values of ΔPpl obtained in this population. The smooth muscle relaxant effect of detomidine has been previously described by Broadstone and colleagues (Broadstone, Gray, Robinson & Derksen, 1992). While, the ΔPpl measurement explained most of the variability observed inside the studied population, it is important to remember that this is an invasive test, requiring oesophageal intubation and placement of a balloon catheter for conducting the necessary measurements and some tutors may be a little reluctant to allow this procedure to be performed on their horses. Nevertheless, the manufacturers of the Dppl used in our study, the University of Vienna, were very interested in the field based staging method proposed by us and therefore offered to develop a new Dppl devise, which was sent to our team for testing, and is in fact much more practical.

The Open Pleth™ system allows the study of airway obstruction through flowmetric plethysmography. It can also be associated with histamine bronchoprovocation in order to evaluate airway hyperresponsiveness. This is another portable diagnostic tool, that although slightly more sensitive due to the wires attached to the various components of the equipment (bands, pneumotachograph, RIP control box and computer) may be safely used in the field. It usually requires the usage of a head stand to prevent development of nasal oedema in sedated animals, which would interfere in the measurements of flow by the pneumotachograph.

We found that, in our sample of horses, the degree of airway obstruction (Δflow) did not differ significantly between groups. This could be related to the size of our study population, which resulted in lack of power for this variable, but it could also be associated with the fact that this test is not highly sensitive for detecting low grade airway obstruction (Hoffman et al., 2001; Rettmer et al 2015). The association with histamine bronchoprovocation usually increases the test sensitivity and allows detection of milder cases of severe EAS (Rettmer et al., 2015; Wichtel, Gomez, Burton, Wichtel & Hoffman, 2016). In this study we found significant differences between groups concerning the maximum concentration of histamine ([Hist]) tolerated during the bronchoprovocation test. Nonetheless, we found that both stage 1 asthmatic horses and the healthy controls, tolerated the maximum histamine concentration administered (32mg/ml), indicating that histamine concentration alone would prove insufficient for distinguishing between these two groups. It may also indicate that in sub-clinical cases, in this study represented as stage 1 – latent severe EAS, airway hyperresponsiveness is not present although airway inflammation may be observed in the BALF cytology, where neutrophil differential counts are higher than the 20% cut off value.

Finally, using the discriminant analysis, we obtained two significant canonical functions, which included the parameters clinical score, BALF neutrophil percentage, ΔPpl, arterial blood P_aO_2 and maximum histamine concentration ([Hist]). Together the two functions explained 99.3% of the data variability found in this equine population and cross-validation resulted in a correct allocation 86.8% of the cases. This result indicates that a high number of cases were correctly identified and further supports the new staging method developed.

The findings obtained resulted in four linear functions, which may be used to correctly identify the severity of the disease. These functions are to be introduced in a spread sheet and through the input of the results obtained from the diagnostic tests, a final value should is calculated. The function which portraits the highest value indicates the correct staging of the horse.

The results obtained suggest that, despite the limitations of this study, namely its small sample and the lack of representation of stage 4 asthmatic horses, the linear functions can be used to correctly classify severe EAS-affected horses. Because the experimental protocol required that the horses should be tested at the veterinary school equine hospital, some tutors were reluctant to transport and test a horse with overt respiratory distress, which is why it proved very difficult to include stage 4 asthmatic horses in our study. In fact, the six most severe cases of severe EAS referred to the equine hospital were not included in this study due to the refusal of the horses' tutors. This method now requires further validation in a larger population of asthmatic horses.

Nonetheless, the results obtained were very promising, further contributing to the validation of some of the variables used in the diagnosis of severe EAS, thus helping to shed more light on the diagnosis and staging of this debilitating disease.

2. Intestinal parasitic infection in horses with severe Equine Asthma Syndrome

The association between allergic diseases, atopy and asthma, and the resistance to parasitic infection has been reported both in equine and human medicine (Lynch et al., 1998; McKay, 2006; Hopkin, 2009; Medeiros et al., 2011; Bründler et al., 2011). The exact mechanism by which this relationship occurs remains unclear.

Amongst the most popular explanations found in the scientific community are the hygiene hypothesis and the genetic hypothesis.

The hygiene hypothesis theorises that chronic infectious diseases, such as intestinal parasitism, influences cellular and humoral immunoregulation and thus prevents the development of allergic diseases (Strachan, 1989; Von Mutius, 2007; Tilley, Sales Luís & Branco Ferreira, 2012a; Lambrecht & Hammad, 2017). Thus, the enforcement of hygiene habits in the modern western society resulted in an increase on the prevalence of allergies.

The other hypothesis states that the genetic makeup of allergic individuals confers them the advantage of being more resistant to parasitic infections while at the same time conferring an increased vulnerability to environmental allergens, since the same genes are responsible for both the allergic susceptibility and resistance to parasites (Neuhaus et al., 2010; Lanz et al., 2013).

Severe EAS, along with insect bite hypersensitivity, both considered a multiple hypersensitivity (MHS) diseases, result in airway hyperreactivity on affected animals. Also, both these diseases have been associated with the absence of nematode eggs in the faeces of the affected horses, which may indicate that MHS contributes to the parasitic resistance, by the same mechanisms described above, and that the presence of one of this diseases may predispose to the occurrence of the other (Kehrli, Jandova, Fey, Jahn & Gerber, 2015; Lanz, Brunner, Graubner, Marti & Gerber, 2017).

In the present study, we focused on a thoroughly characterized population of severe EAS-affected Lusitano horses with the goal of verifying if the asthmatic animals of this particular breed also presented a resistance to gastrointestinal helminth infection, as describe in other horse breeds (Neuhaus et al., 2010; Bründler et al., 2011). To our knowledge no previous study on this subject had been conducted solely in Lusitano horses kept in Mediterranean conditions. It was our goal to further investigate this subject and verify if this phenomenon was transversal to this Portuguese horse breed. Although, not being able to clearly define the exact mechanism responsible for allergy and parasitic resistance Also, we set out to determine if the strongyle species identified in the asthmatic horses differed from the ones found in healthy controls housed under the same conditions and in the same horse farms, in order to minimise confounding variables.

Our results indicated that, similarly to what has been previously reported by other authors, the Lusitano asthmatic horses bred in a Mediterranean climate also presented an increased resistance to gastrointestinal helminth infection (Neuhaus et al., 2010; Bründler et al., 2011; Medeiros et al., 2011). We observed that the asthmatic horses had significantly lower Egg Per Gram (EPG) and Larvae Per Gram (LPG) counts than the healthy controls housed in the same farms and under the same environmental and feeding management as the severe EAS group. On average the animals in the control group shed nine times more eggs and ten times more infective larvae then their asthmatic counterparts. We observed that some of the healthy controls presented an alarmingly high parasitic burden, which could be caused by reinfection or more concerningly be due to anthelmintic resistance or inadequate therapy.

Nonetheless, because the frequency of the deworming program could not be fully controlled in this study, since some of the horses which were included were competing in international dressage and show jumping events and thus high performance athletes, a few of the animals, both from the asthmatic and control groups, had null EPG and LPG counts.

Both our groups presented a similar population of gastrointestinal nematodes, with strongyle type eggs and *Cyathostomun sensu latum* larvae being the most prevalent. This data is in agreement with other published studies, which report an increase in the prevalence of cyathostomin nematodes in adult horse infections (Klei & Chapman, 1999; Kaplan & Nielsen, 2010; Kaplan & Vidyashankar, 2012).

We did, however, identify a high prevalence of *Strongyloides westeri* in the egg and larvae counts of one of the non-asthmatic horses. This nematode is usually found in younger animals and the cases are seldom asymptomatic (Miller, Bellaw, Lyons & Nielsen, 2017). We can only theorise about the reason for this occurrence, which could possibly be related to some degree of immunosuppression, although we have no means of confirming this theory.

As such, we did observe that although Lusitano asthmatic horses appear to share an increased resistance to gastrointestinal nematode infection, the infectious species did not differ from those found in their healthy counterparts.

3. Owner compliance to an environmental management protocol for severe Equine Asthma Syndrome

In the management of severe EAS it is essential to minimise horses' exposure to dusty environments which are rich in aeroallergens (Robinson, Derksen, Jackson, Peroni & Gerber, 2001). As such, owner compliance is fundamental and is therefore the key to successful disease remission (Hotchkiss, Reid & Christley, 2007a, b).

Due to its significance, ascertaining owner compliance was the final goal of this study. For this purpose, we focused on six characteristics of each horses' environment (ventilation, type of housing, time spent outdoors, procedure during stable cleaning, bedding material and forage fed), as well as on clinical signs and their evolution as reported by their owners, before and one year after diagnosis of severe EAS. At the time of diagnosis, a written environmental protocol was given to the owners.

Our findings were in agreement with other published works, which described that these six environmental parameters influenced severe EAS exacerbation (Blackman & Moore-Colyer, 1998; Vandeput, Duvivier, Votion, Art & Lekeux, 1998; Robinson et al., 2001; Clements & Pirie, 2007; Hotchkiss et al., 2007b).

Stabling of horses in facilities with bad ventilation during most of the day may increase their exposure to high respirable dust concentrations. In addition, the choice of a bedding material with a high concentration of respirable particles, like saw dust of poor-quality straw, and the permanence of the horse indoors during periods of increased stable activity, further contributes to airway inflammation and obstruction. Horses exposed to these husbandry conditions were more likely to frequently exhibit clinical signs of severe EAS, require treatment with corticosteroids and bronchodilators and show no signs of overall improvement when maintained in this type of environment.

In our studied asthmatic population, we found that the report of nasal discharge during disease exacerbation was not as frequent as other clinical signs (cough and increased respiratory effort at rest), which may indicate that owners tend to neglect this clinical finding, or this sign may not always be present during disease exacerbation.

When determining the overall owner compliance to the environmental management protocol, the results were surprisingly low. More than half the interviewees (51.3%) were allocated in the 'poor compliance' category, since they only managed to apply two or less of the suggested environmental modifications. The remaining 33.3% were assigned to the 'adequate compliance' group and a smaller percentage of only 15.4% to the 'good compliance' cluster. Of the 39 interviewed owners, only 3 were able to complete all the recommended husbandry guidelines in order to ensure the respiratory health of the asthmatic horses.

Also, 4 owners failed to adopt any of the recommended guidelines, which resulted in no improvement of the clinical status in the year following the initial diagnosis.

On the initial interview, owners were asked to identify the trigger factor responsible for their horses' clinical signs and hay feeding was commonly reported. Despite this information, only 9 owners opted to feed their horses soaked instead of dry hay, which is known to reduce respirable dust concentration significantly (Clements et al., 2007).

The parameter with the highest compliance was the use of a bedding material with low dust concentration, such as dust free wood shaving, cardboard or rubber, in detriment of saw dust or straw.

The parameters associated with infrastructures, such as ventilation and type of housing had a lower compliance, most likely due to the need for financial investment in creating new or improving existent infrastructures.

Since the amount of time spent outdoors is largely influenced by the type of housing, most animals which were housed in a paddock or kept at pasture tended to spend more time outside than those which were stabled.

Pharmacological treatment provides relief of airway inflammation and obstruction and is useful for rapidly improving respiratory function. However, the drug effects are transitory an may even prove insufficient if the asthmatic horse is maintained in the offending environment. Because owner compliance was so low, horses required pharmacological treatment to improve clinical status, although this was unsuccessful in eliminating clinical signs.

This study indicates that ensuring owner compliance is fundamental for severe EAS remission and, that although corticosteroids and bronchodilators may provide instant symptomatic relief, only persistent aeroallergen avoidance can ensure the respiratory health of asthmatic horses.

As a take home message, we believe that veterinarians should ideally spend more time during consultation ensuring that environmental management guideline are well understood and these practical issues resulting from implementing these guidelines are seen through together. Only this way can owner compliance be improved.

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CHAPTER 5

Conclusion

Severe EAS is the most prevalent respiratory disease affecting adult horses in the Northern hemisphere. It is a recurrent condition, which requires life-long environmental management of the affected animals, incurring significant losses in the equine industry.
Early diagnosis is essential to ensure that the animals remain in remission and no permanent pathological changes occur in the affected horses' airway.
A correct diagnosis and staging of severe EAS requires integration of several diagnostic ancillary methods, specially in cases where low grade inflammation is present and clinical signs become more discreet. Staging can be a challenging when it must be performed in an ambulatory setting. To overcome this limitation, we developed a staging method based not only on clinical information but also on lung function results, using only portable diagnostic equipment. For this purpose, a discriminant analysis of several variables which could be used in severe EAS staging was conducted and four linear functions were obtained which could be used to characterise each disease stage. The variables clinical score (nasal flare, abdominal lift and cough), BALF neutrophil percentage, differential pleural pressure, arterial blood oxygen partial pressure and maximum histamine concentration tolerated during bronchoprovocation testing were included in our staging model. Furthermore, the discriminant linear functions could be used to correctly identify and stage severe EAS-affected animals. However, this method should be further validated in a larger number of asthmatic horses both in disease exacerbation and remission, as well as on healthy horses.
It has been reported that asthmatic horses tend to present a lower gastrointestinal burden than healthy animals. We found this to be true in Lusitano breed horses kept in a Mediterranean climate, since the horses presented lower EPG and LPG counts than the control horses, which could indicate an increased resistance to gastrointestinal nematode infection. In a more practical sense this finding could indicate that due to a natural resistance, severe EAS-affected horses could require fewer anthelmintic treatments to control parasitic infections. On a further note, we found that the presence of an allergic condition, like severe EAS, doesn't seem to influence the nematode species which will infect the horse. In this particular study we reported that the most prevalent nematodes encountered were cyathostomins, which reflects the parasitology trends reported in Portugal.
As previously mentioned, a correct environmental management is fundamental to ensure disease remission in severe EAS-affected horses. Aeroallergen avoidance ensures control of airway inflammation, obstruction and hyperreactivity and should be the primary goal of owners and veterinary practitioners, in detriment of medical treatment with corticosteroids and bronchodilators, which serves as an aid to rapidly, but often temporarily, improve lung function. Most of the interviewees in our study were unable to adopt all the recommended environmental changes, which significantly dropped the overall compliance of the studied population. Only 15.4% of the interviewed owners were grouped in the 'good compliance' category, having

adopted ≥5 of the suggested environmental parameters. These owners reported that their horses improved, had rare manifestations of clinical signs and did not require any pharmacological treatment.

Hay was identified as an important trigger factor for the disease, but only a small percentage of the owners opted for feeding soaked hay to their horses, instead of dry hay, which contains a large concentration of respirable dust particles.

Because traditional husbandry practices are culturally ingrained, horse owners may be reluctant to modify their routines. The veterinary practitioner has the imperative role of educating horse owners on the importance of continuous environmental management to ensure the welfare of the asthmatic horse. We believe that this should occupy a significant portion of the consultation time.

CHAPTER 6

Future work

1. Clinical staging of severe equine asthma syndrome

The ambulatory staging method here discussed appears to be a promising tool to help clinicians provide better patient care for asthmatic horses. However, since it was not possible to validate the method in stage 4 horses, this process should be carried out to ensure the method's discriminant power. Furthermore, the method should now be tested in a larger population of asthmatic horses. With technological advancements it may become possible to find suitable alternatives to some of the invasive diagnostic tests that were employed in severe EAS staging.

Recently, a new version of a portable pleural pressure measurement device has been developed named Ventiplot®. Following the population of our EAS staging method, the manufacturers of the pleural pressure device contacted our team in order to develop a new version of the device with our collaboration. The new interface is far smaller and user friendly and may be attached to the horse's halter minimising the risk of hazard for the equipment. The data collected is analysed using the app "Bluefruit" available for iphone, smartphones and tablets. Our team was asked to test this new model before it became commercially available and the pleural pressure readings obtained were consistent with those recorded using the older model. Also, the new design vastly aids its use in ambulatory practice. As such, the Ventiplot^{TM} may be a great alternative for the measurement of pleural pressure.

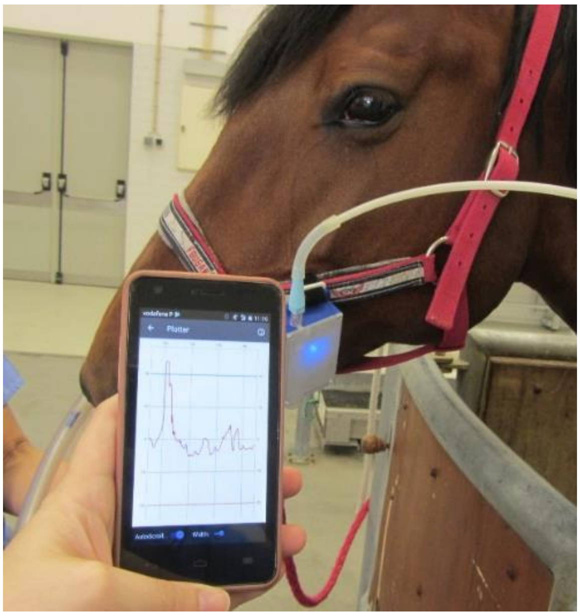

Fig. 17 – Use of the Ventiplot^{TM} for transpulmonary pressure measurement

2. Link between equine asthma and parasite resistance

Because the horses studied in the present study had no common ancestry, it is not possible to infer whether a genetic component may be an important determinant in our results. It would however prove interesting to conduct a large-scale evaluation of the association between EAS and intestinal helminth infection resistance in families of Pure Bred Lusitano horses, thus investigating the mechanism behind this occurrence.

Also, although most studies have researched the link between parasitic resistance and severe EAS, none up to date has investigated if the same link is present in cases of mild and/or moderate EAS (formerly inflammatory airway disease).

3. Disease immunology

In the last decades, few advances have been made pertaining the treatment of severe equine asthma syndrome. Medical treatment is mainly symptomatic and disease remission largely depends on allergen avoidance. This may be due to the fact that the exact immunological mechanism responsible of the pathological changes associated with this disease, namely airway inflammation, hyperresponsiveness and obstruction, are still not fully understood. In fact, has stated in chapter one, the nomenclature of this disease is relatively new, and was adopted to encompass some of the traits it has in common with human asthma. A better understanding of the immunological mechanisms and cells involved in severe EAS pathogenesis, will ensure that drugs which specifically target these pathways can be developed and used to further minimise clinical signs associated with the disease.

4. Management

It is also essential to further investigate the major reasons for the lack of owner compliance to environmental management and to educate the public on the importance of choosing stabling conditions that ensure the long-term respiratory health of their horses. This may be particularly important in high performance equine athletes, which in spite of representing a significant economic investment, tend to be stable for the vast majority of the day and thus exposed to high concentrations aeroallergens.

Also, since severe EAS poses such an economic and management issue for equine professionals, genetic mapping may be a useful tool to screen potentially asthmatic horses and either ensure proper environmental management from an early age or to prevent the reproduction of susceptible individuals.