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EXPLORING EQUINE ASTHMA: PATHOGENESIS INSIGHTS AND DIAGNOSTIC CHALLENGES

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"A full-grown horse or dog is beyond comparison
a more rational, as well as a more conversible animal,
than an infant of a day, a week or even a month old.
But suppose the case were otherwise, what would it avail?
The question is not, can they reason? Nor, can they talk?
But, Can they suffer?"

Jeremy Bentham, 1748 – 1832

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ABSTRACT

Equine asthma (EA) is a common respiratory syndrome of horses, including a spectrum of chronic inflammatory disorders of the lower airway, which can be classified as mild-moderate (MEA) or severe (SEA), based on clinical severity. Although it is spread worldwide and has been studied for decades, gaps persist in our understanding of EA's pathogenesis. This highlights the need for refined diagnostic procedures to swiftly identify and subcategorize EA, finally enabling targeted therapeutic interventions. This doctoral thesis attempted to fill these gaps through a series of retrospective and prospective studies, aiming to contribute to the EA scientific community with valuable insights.

Notably, our findings based on intradermal testing revealed that SEA-affected horses predominantly exhibit late-phase type I hypersensitivity, whereas MEA-affected horses manifest type IV hypersensitivity. Furthermore, our investigations demonstrated associations between distinct immune response types and various EA severity and cytological subtypes, including innate immunity, Th2, and Th17 responses. In addition to intradermal testing, thoracic ultrasonography showed promise as a diagnostic tool for EA. Indeed, ultrasound alterations were more prevalent in SEA-affected horses than those with MEA, correlating with increased neutrophils in bronchoalveolar lavage fluid. In racehorses, while EA did not appear to be linked with dynamic upper airway obstructions, it did show an association with impaired athletic capacity in cases of neutrophilic inflammation. Oscillometry studies proved to be encouraging, revealing differences in respiratory reactance between healthy and MEA-affected horses in a mixed population. Conversely, among racehorses in training, no differences in lung function were observed at rest,

but horses with MEA experienced increased resistance after intense exercise compared to their healthy counterparts.

In summary, our results contribute to the understanding of key aspects of EA etiopathogenesis, laying the foundations for future research. Additionally, we advocate for the inclusion of intradermal testing and thoracic ultrasonography in a comprehensive diagnostic protocol for EA. Finally, our innovative work with oscillometry underscores its diagnostic potential, opening doors to various fields of application in equine respiratory medicine.

CHAPTER 1.

LITERATURE REVIEW:

EQUINE ASTHMA

1.1 DEFINITION

Equine asthma (EA) is a syndrome which includes non-infectious, inflammatory, and chronic disorders of adult horses, clinically characterized by airflow obstruction, mucus hypersecretion and airway hyperreactivity (Bullone and Lavoie 2020). Based on the severity and recurrence of the condition, it can be classified as mild-moderate equine asthma (MEA) or severe equine asthma (SEA) (Couetil *et al.* 2016).

However, a larger spectrum of EA endotypes and phenotypes is thought to exist. Endotypes describe subtypes of a disease which differ in specific pathogenetic mechanisms at a molecular level, genetic characters, or response to therapy (Lotvall *et al.* 2011, Svenningsen and Nair 2017); phenotypes, instead, represent physical or measurable properties resulting from the expression of the genes in relation to the environment (Ozdemir *et al.* 2018, Agache 2019). Different EA endotypes can be characterized by different underlying expression of cytokines, lymphocytes recruitment and macrophage polarization, and may influence the sensitivity to treatment (Cordeau *et al,* 2004, Debrue *et al.* 2005, Lavoie *et al.* 2011, Murcia *et al.* 2019). Phenotypes may be defined on the basis of the severity of clinical symptoms (mild-moderate or severe), the environmental triggering factors (indoors or outdoors allergens), the entity of mucus accumulation in the trachea, the different proportions of inflammatory cells in the bronchoalveolar lavage fluid (neutrophils, eosinophils, mast cells), the age of appearance (early or late), and specific airway remodeling characteristics (Couetil *et al.* 2020). The ability to identify and distinguish endotypes and phenotypes represents one of the hot topics in the research on equine asthma, as it may open the door to the development of novel diagnostic tools, targeted therapeutic approaches, and prognostic factors.

Although much still needs to be understood about equine asthma, this condition has been recognized for centuries, as the first reports of horses suffering from chronic inflammation of the airways date back to the XVII century. Historically, the severe form of this disease, characterized by reversible bronchospasm in horses fed with hay, has been known as "heaves" or "broken wind". During the XX century, various terms have been adopted to describe this condition, mainly based on similarities with human diseases, including "equine emphysema", "chronic obstructive pulmonary disease", and "chronic bronchiolitis" (Obel and Schmiterlow 1948, Robinson *et al.* 1996, Nyman *et al.* 1991); however, all these terms were later abandoned, as they did not define accurately and correctly the condition. A new classification was introduced in the late 80s, including "recurrent airway obstruction" (RAO) and "inflammatory airway disease" (IAD): RAO describes a clinical condition characterized by recurrent and reversible episodes of bronchoconstriction triggered by the inhalation of environmental antigens, while IAD describes lower airway inflammation in horses not experiencing dyspnea (Derksen *et al.* 1996, Couetil *et al.* 2007). As in some horses the symptoms are triggered by outdoors environmental antigens (i.e., pollens), the term Summer Pasture Associated Obstructive Pulmonary Disease (SPAOPD) has been introduced (Rodrigues Costa *et al.* 2000). During the last decade, several similarities have been encountered between RAO, IAD and different phenotypes of human asthma (Leclere *et al.* 2011, Bullone and Lavoie 2015, Lange-Consiglio *et al.* 2019); therefore, the most recent ACVIM Consensus Statement recognized a novel classification: IAD has been defined as "mild-moderate equine asthma", while RAO as "severe equine asthma" (Couetil *et al.* 2016).

1.2 EPIDEMIOLOGY

Equine asthma is the most common cause of chronic cough in horses (Pirie 2014). In particular, SEA naturally affects from 10% to 17% of adult horses over 7 years of age, living in the Northern Hemisphere). Retired horses seem to be more likely to be affected by SEA, compared to those used for competitions, probably due to their older age (Hotchkiss *et al.* 2007). Evidence about possible sex or breed predisposition is lacking. In most studies, males and females are equally affected, but a large retrospective study conducted in North America suggested that females were predisposed to develop SEA (Couetil and Ward 2003), which has been anecdotally observed since the XIX century (Dun 1859); recently, a preliminary study reported an influence of estrus cycle on lung function in mares with SEA, suggesting a possible role of hormones on this disease (Mainguy-Seers *et al.* 2022). Conflicting results have also been reported regarding breed predilection; in particular, some studies report either Thoroughbreds or ponies as the most likely breed to be affected by SEA (Couetil and Ward 2003), while others as the least likely (McPherson *et al.* 1979). However, in several breeds, SEA has shown to be partly heritable (Ramseyer *et al.* 2007). Among horses with SEA, the proportion of those whose symptoms are triggered by indoors allergen, especially in autumn and winter months, is higher compared to those triggered by outdoors allergens in spring and summer (summer equine pasture asthma, EPA) (Hotchkiss *et al.* 2007).

Conversely, MEA can affect horses of any age but is more commonly reported in young or middle-aged horses (Couetil *et al.* 2016); moreover, it is reported that the incidence of MEA decreases with the increase of age in racehorses (Chapman *et al.* 2000). However, it is possible that the age at first appearance may reflect different phenotypes of MEA (Couetil *et al.* 2020). Horses of any sex and breed can be affected (Couetil *et al.* 2016). Concerning the prevalence,

it has been suggested that MEA affects up to 80% of Thoroughbred racehorses in training (Christley and Rush 2007, Nolen-Walston *et al.* 2013, Ivester *et al.* 2018), representing the second most common cause of poor performance in young racehorses (Wilsher *et al.* 2006). Although some authors reported a prevalence of MEA among other sport and leisure horses between 20% and 31% (Robinson *et al.* 2006, Widmer *et al.* 2009), it is currently unknown; it is estimated to be lower than the percentage of clinically healthy horses with lower airway inflammation detected at bronchoalveolar cytology, but higher than the prevalence of SEA (Boivin *et al.* 2018a).

1.3 ETIOPATHOGENESIS

Equine asthma has been recognized as a multifactorial syndrome, resulting from the interaction between genetic susceptibility and exposure to environmental antigens, determining a hypersensitivity reaction (Leclere *et al.* 2011, Bullone and Lavoie 2015, Bullone and Lavoie 2020).

While no specific genetic risk factors have been investigated for MEA yet, most studies have focused on SEA. First report of SEA familiarity dates back to the '30s (Schaeper 1939) and, throughout the following 80 years, a strong genetic determinant has been observed in several breeds and families (Gerber 1989, Marti *et al.* 1991, Ewart and Robinson 2007, Leclere *et al.* 2011). Indeed, offspring of affected sires have a more than 4-fold increased risk for developing SEA (Ramseyer *et al.* 2007). In high-prevalence Warmblood families, two chromosomes regions have been identified as associated with SEA, which differ between the families (ECA13 and ECA15) (Swinburne *et al.* 2009), while IL-4 receptor, TXNDC11, and CLEC16A have been indicated as candidate gene (Racine *et al.* 2011, Schnider *et al.* 2017, Mason *et al.* 2018). However, to date, no potential genetic markers have been identified, due to the heterogeneity observed between families and the complexity of EA's genetic basis. In fact, it has been hypothesized that EA has a polygenic background, with a large number of genes that contribute to less than 10% to the total genetic effect (Couetil *et al.* 2020). Interestingly, horses with EA seem to be predisposed to allergic skin diseases (Graubner *et al.* 2012, Kehrli *et al.* 2015, Lanz *et al.* 2017, Verdon *et al.* 2018), and to have a reduced susceptibility to intestinal parasites (Neuhaus *et al.* 2010, Brundler *et al.* 2011, Schleuniger *et al.* 2011, Simoes *et al.* 2023).

In susceptible horses, clinical signs of EA are triggered by the inhalation of environmental antigens, which may vary according to environmental exposure (Bullone and Lavoie 2015). The most frequently involved antigens are airborne organic and inorganic dusts present in the stables, such as moulds, spores, mites, plant debris, bacterial endotoxins, peptidoglycans and proteases; moreover, as asthmatic horses have airway hyperreactivity, some toxic gases such as ammonia, ultrafine particles (< 100 nm), cold air, and dry air may further contribute to lower airway inflammation (Pirie *et al.* 2003, Riihimaki *et al.* 2008, Pirie 2014, McGorum and Pirie 2008). Seasonal pollens and other antigens found in high levels outdoors in the spring and summer represent another group of etiological agents, associated with the condition known as "equine summer pasture asthma" (EPA) (Leclere *et al.* 2011, Bullone and Lavoie 2020). In MEA, intense exercise has also been identified as a risk factor: while the exact cause of this phenomenon remains unknown, it has speculated that exercise may lead to the inhalation of particulate matter, exposure of the small airways to cold unconditioned air, and exercise-induced pulmonary hemorrhage (EIPH), which may lead to pulmonary inflammatory response (McKane and Slocombe 2010). The heightened minute ventilation and straightening of airways during exercise reduce particle deposition in the upper and large lower airways, resulting in greater deposition in the smaller airways. The observed increased risk of MEA with the commencement of racing, reported by some studies, supports the hypothesis that both deep inhalation of particulate matter during exercise and EIPH may play roles in the MEA pathogenesis (Christley and Rush 2007).

There is general agreement that EA represents a hypersensitivity response to inhaled antigens, and allergic mechanisms are accepted as an important pathophysiological feature of both human and equine asthma (Pirie 2014). In

particular, various studies suggest that IgE-mediated immediate-type allergic reactions are associated with EA. Indeed, IgEs have shown to be increased in the bronchoalveolar lavage fluid of affected horse, and IgE-positive cells have been detected by immunohistochemistry studies in the lung tissue of SEA horses (Van der Haegen *et al.* 2005, Künzle *et al.* 2007). Some other authors also hypothesized an involvement of delayed-onset type IV hypersensitivity, mediated by type Th-2 response (CD4+ lymphocyte subgroup), with metaplasia of goblet cells, hyperproduction of mucus, neutrophils recruitment and stimulation of IgE production (Anton *et al.* 2005, Horohov *et al.* 2005). However, the efforts to categorize the immune response types involved in EA pathogenesis have not led to a univocal response, but rather to contrasting results. Indeed, while most studies report a predominance of Th2 response (Lavoie *et al.* 2001, Bowles *et al.* 2002, Cordeau *et al.* 2004, Dewachi *et al.* 2006, Beekman *et al.* 2012), the involvement of other immune response types has been recognized, such as innate (Laan *et al.* 2006a, Hughes 2011), Th1 (Ainsworth *et al.* 2003), and Th17 (Debrue *et al.* 2005, Korn *et al.* 2015, Pacholweska *et al.* 2017, Gressler *et al.* 2022). Finally, some authors have hypothesized that the immune response in EA may be mixed (Giguere *et al.* 2002, Horohov *et al.* 2005, Lavoie-Lamoureux *et al.* 2010, Lavoie *et al.* 2011). The discrepancy between results suggest that multiple immunological mechanisms may contribute to different disease phases (Pirie 2014); therefore, differences in sample collection time points in relation to either the time of challenge or the onset of clinical signs may explain the encountered differences (Horohov *et al.* 2005, Pietra *et al.* 2011).

In EA, the activation of T cells leads to the recruitment of neutrophils into the airways (Pirie 2014). However, different cell types have been involved in EA pathogenesis, including macrophages, eosinophils, mast cells, and epithelial

cells (Laan *et al.* 2005, Pirie 2014, Bullone and Lavoie 2015). During acute exacerbations of SEAs, horses experience a pulmonary inflammation dominated by neutrophils (Jean *et al.* 2011), while in MEA the airway lumen may be infiltrated by eosinophils, metachromatic cells, or neutrophils (Bullone and Lavoie 2015). The cardinal clinical feature of EA, reversible airway obstructions, results from the underlying airway inflammatory response, associated with bronchoalveolar inflammation, bronchospasm, excessive mucus secretion, airway hypersensitivity. In SEA, bronchial remodeling has been recognized as a key feature, and has shown to be only partially improved by prolonged period of disease remission induced by therapy or antigen avoidance strategies (Leclere *et al.* 2011, Bullone and Lavoie 2020). Indeed, systemic inflammation (Lavoie-Lamoureux *et al.* 2012) and subclinical airway obstruction (Leclere *et al.* 2012, Van Erck *et al*. 2006) persist also during remission phases; this persistence is, at least partially, attributed to a continuous remodeling of the airways (Leclere *et al.* 2012). Recent studies have documented airway remodeling also in horses affected by MEA, involving the bronchial *lamina propria*, epithelium and smooth muscle (Bessonat *et al.* 2022, Dupuis-Dowd and Lavoie 2022).

Finally, the role of infectious agents in the development of EA has been hypothesized, but remains uncertain. Indeed, the presence of tracheal mucus containing bacteria (such as *Streptococcus zooepidemicus*, *Actinobacillus* spp., and *Pasteurella* spp.) has been observed also in healthy racehorses (Cardwell *et al.* 2014); however, some studies have shown an increased risk of MEA with higher levels of bacterial CFUs in tracheal wash samples (Wood *et al.* 2005a). Similarly, SEA-affected horses commonly have bacteria in their tracheal secretion (Lavoie 2007). However, it is unclear whether bacterial infections directly contribute to increased mucus production, or if bacterial colonization

occurs as a result of impaired mucus clearance (Couetil *et al.* 2016). Similarly, viral infections, such as equine influenza virus and herpes viruses, can lead to temporary inflammation in the lower airways and may potentially increase the risk of MEA and SEA, but their role remains a topic of debate (Newton *et al.* 2003, Christley and Rush 2007, Fortier *et al.* 2009).

1.4 CLINICAL PRESENTATION

The clinical presentation of EA is the consequence of airway inflammation and hyperreactivity, mucus hypersecretion, and airway obstruction (Pirie 2014); the entity of clinical signs varies with disease severity.

SEA, including EPA, is a recurrent condition, clinically characterized by the alternation of periods of disease exacerbation and remission. Indeed, both forms are commonly seasonal, as they are associated with the exposure to specific environmental antigens. Therefore, while classical SEA exacerbations are more common during the winter, when horses are stabled in a low-ventilated environment and highly exposed to moulds and mites, SPA exacerbations mainly occur during spring-summer months, when they are grazing and exposed to seasonal pollens. However, many SEA horses are hypersensitive to different antigen classes, and may present clinical symptom throughout the whole year (Lavoie 2007). During exacerbations, the main clinical signs of SEA horses include increased expiratory effort at rest with abnormal breathing pattern (dyspnea), chronic cough, and exercise intolerance; moreover, mucopurulent nasal discharge can be observed, but is sometimes absent due to the regular swallowing of secretions that enter the pharynx from the trachea (Robinson *et al.* 1996, Pirie 2014). Dyspnea can be typically observed as increased nasal flaring and abdominal component during exhalation, reflecting obstruction of peripheral airways and respiratory exertion (Van Erck *et al.* 2006). In severely affected horses, this exertion can lead to hypertrophy of the external abdominal oblique muscles resulting in the characteristic "heave line" (Lavoie 2007, Niedzwiedz 2014), and to increased oxygen consumption and greater energy expenditure, potentially leading to cachexia (Mazan *et al*. 2004). Moreover, severely affected horses may present "anus pump" and flatulence as a result of forced expiration and cough (Lavoie

2007). During clinical examination, abnormal findings may be detected at thoracic auscultation. These may include wheezing sounds at the end of expiration, a result of progressive narrowing of airways, as well as early inspiratory crackles resulting from the sudden reopening of airways that had collapsed during exhalation. Furthermore, in specific cases, the lung auscultation fields may be enlarged due to lung hyperinflation, resulting from significant air entrapment (Pirie 2014). However, horses in remission of the symptoms may exhibit little to no clinical signs, and be indistinguishable from healthy or MEA horses. However, due to progressive lung remodeling, persistent low-grade airway inflammation and mucus accumulation, horses may still present poor athletic performance or exercise intolerance, and present occasional cough, especially at the beginning of exercise (Lavoie 2007).

Horses with MEA, instead, only show subtle and non-specific clinical signs, including decreased performance and chronic, intermittent cough (Couetil *et al.* 2016). In particular, poor racing performance and decreased willingness to perform in saddle horses seem to be associated with tracheal mucus accumulation and increase of neutrophils in the bronchoalveolar lavage fluid (MacNamara *et al.* 1990, Fogarty and Buckley 1991, Holcombe *et al.* 2006, Widmer *et al.* 2009, Richard *et al.* 2010a). Cough can be observed at rest or, more commonly, during exercise (Wasko *et al.* 2011); however, some MEA-affected horses never show chough. Occasionally, young horses with MEA may also show serous to mucopurulent nasal discharge (Wood *et al.* 2005a, Cardwell *et al.* 2011), which seems to be associated with increased tracheal mucus (Cardwell *et al.* 2014). Interestingly, MEA horses with cough and/or nasal discharge seem to be at increased risk of later developing SEA (Bosshard and Gerber 2014). Finally, at thoracic auscultation, horses with MEA are frequently normal, while some of them may exhibit increased breath sounds

or subtle wheezes during rebreathing maneuvers (Lavoie *et al.* 2011, Nogradi *et al.* 2015). Due to the lack of specific and obvious clinical signs, ancillary diagnostic procedures are needed for a definitive diagnosis of MEA.

1.5 DIAGNOSIS

The diagnosis of SEA is usually easy to obtain during exacerbation, because history and clinical examination are often sufficient to diagnose, and the visible relief in respiratory effort after the administration of a bronchodilator can represent a further diagnostic confirmation (Hoffman *et al.* 2007). However, diagnosis can be complex in SEA-affected horses during clinical remission and in MEA-affected horses, that are usually almost normal at physical examination and only present mild clinical or subclinical signs (i.e., poor performance). Therefore, ancillary diagnostic procedures are needed to reach a definitive diagnosis. The diagnostic protocol for EA can include collection of history, clinical examination, airway endoscopy, analysis of airway secretions, radiographs, ultrasonography, lung function testing, blood biomarkers and clinical pathology (Couetil *et al.* 2020).

1.5.1 History

A thorough collection of history is essential for the diagnosis of EA. In case of SEA, a presumptive diagnosis can be commonly made on history alone, when the recognition of episodes of reversible dyspnea associated with the exposure to specific antigens is reported (Couetil *et al.* 2020). In subclinical or mild cases, history alone is not sufficient, but still of primary importance. Important information to collect include the type of feed and feeding practices (Ivester et al. 2018), stabling, type of bedding, barn environment (Bullone *et al.* 2016b), travel history, vaccinations, past respiratory infections, familiarity, etc. (Couetil *et al.* 2020). Moreover, history of poor performance or exercise intolerance, cough, and nasal discharge may support the tentative diagnosis of EA; however, these signs are quite non-specific, and their absence does not rule out the disease (Rettmer *et al.* 2015). In 2007, Ramseyer and colleagues

developed a 1 to 4 scoring system based on a questionnaire on history, called HOARSI (Horse Owner Assessed Respiratory Signs Index) (Table 1) (Ramseyer *et al.* 2007). However, in a following study, it did not show to be sensitive enough to distinguish between horses with SEA in remission, horses with MEA, and normal horses (Laumen *et al.* 2010).

Score	Clinical history
1	No episodes of coughing or nasal discharge
2	Mucous nasal discharge, occasional coughing, or both
3	Abnormal breathing, regular or frequent coughing, or both
4	Abnormal breathing, regular/frequent coughing and poor performance, or both

Table 1. HOARSI scoring system (Ramseyer *et al.* 2007).

1.5.2 Clinical examination

The detection of the clinical signs described in Paragraph 1.4 can contribute to the diagnosis of EA. In order to distinguish between healthy and asthmatic horses, various clinical scoring systems have been proposed; however, they are useful for the identification of SEA during exacerbation, but are not sensitive enough to recognize MEA or SEA in remission (Robinson *et al.* 2000, Tilley *et al.* 2012). Among the most useful scoring systems, two deserve to be mentioned: the adapted 23-point scoring system and the IDEASS scoring system. The 23-point scoring system is considered the most useful for the categorization of airway obstruction from mild to severe, showing a sensitivity of 82% and a specificity of 70% (Table 2) (Lavoie *et al.* 2019a). It was developed by adapting a previous clinical score proposed by Hoffman and colleagues (1992) and consequently applied to EA by Tesarowski and colleagues (1996).

Variable	Description	Score
Respiratory rate (breaths/min)	< 16	0
	16 – 20	1
	21 – 25	2
	26 – 30	3
	> 30	4
Nasal discharge	None	0
	Serous	1
	Mucopurulent	3
Abdominal lift	None	0
	Mild (perceptible heave line)	1
	Pronounced (abdomen, thorax and anal movement)	3
Nasal flaring	None	0
	Present	1
Tracheal sounds	Normal (tubular sound)	0
	Increase in intensity	1
	Mucus movement	3
Bronchial tones	Normal	0
	Audible ventral and dorsal sounds	2
Crackles	None	0
	Present	2
Wheezes	None	0
	Present	2
Cough	None	0

	Inducible by tracheal massage	1
	Intermittent	2
	Paroxysmal	3
Total (maximum)		23

Table 2. Adapted 23-point clinical scoring system for airway obstruction (Lavoie *et al.* 2019a).

The IDEASS (Improved clinically Detectable Equine Asthma Scoring System) scoring system is an algorithm developed on the basis of a meta-analysis for the quantification of EA severity, relying on a 8-point scale (Figure 1). In this scale, a change in one point represent the minimally clinical detectable difference and EA clinical signs (Calzetta *et al.* 2018).

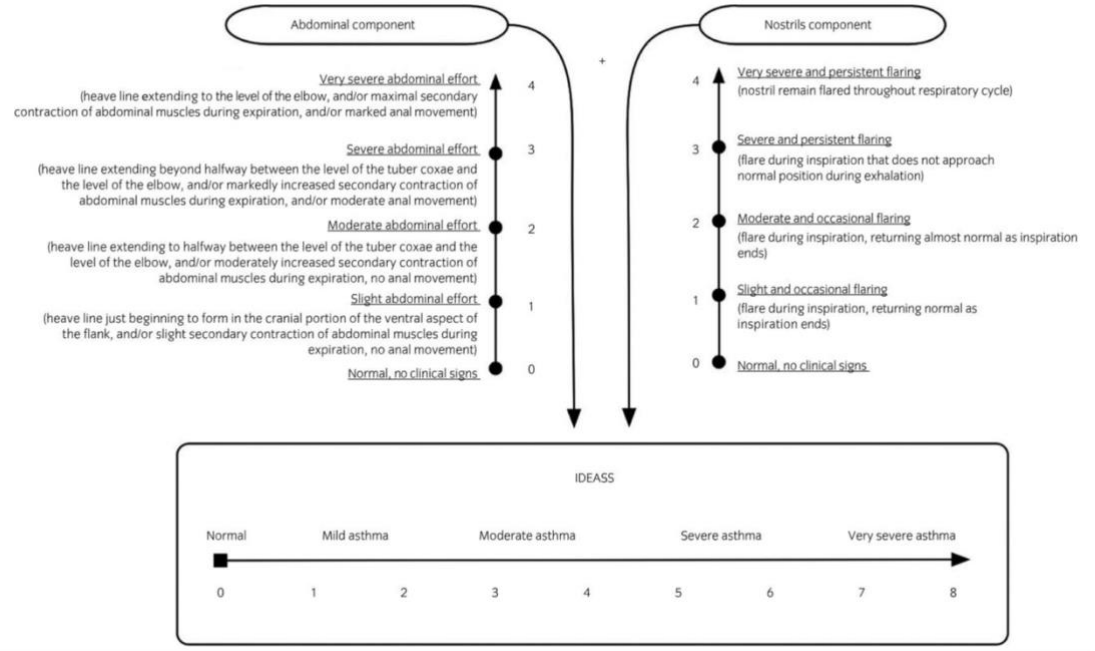

Figure 1. Improved clinically Detectable Equine Asthma Scoring System (IDEASS) (Calzetta *et al.* 2018).

1.5.3 Airway endoscopy

Airway endoscopy is an essential ancillary test for the diagnostic confirmation of EA; in particular, the detection of excessive mucus in the tracheobronchial tree is suggestive of lower airway inflammation (Koblinger *et al.* 2011, Cardwell *et al.* 2014, Couetil *et al.* 2016, Rossi *et al.* 2018). The accumulation of mucus in the trachea can be scored from 0 to 5, based on its quantity and distribution (Table 3) (Gerber *et al.* 2004). In MEA, the detection of mucus grade ≥ 2/5 for racehorses and ≥ 3/5 for sports and pleasure horses is considered sufficient for the diagnosis (Couetil *et al.* 2016). However, the absence of mucus cannot rule out EA (Rettmer *et al.* 2015).

Score	Endoscopic findings
0	None: clean trachea
1	Little: singular to multiple small blobs
2	Moderate: large blobs, not confluent
3	Marked: confluent to stream forming
4	Large: pool forming
5	Extreme: profuse amounts

Table 3. Tracheal mucus scoring system (Gerber *et al.* 2004).

Other information that can be obtained by airway endoscopy include the presence of inflammation or obstructions of the upper airways, the presence of blood in the trachea, and the presence of edema of the tracheal bifurcation. In particular, at the nasopharyngeal level, pharyngeal lymphoid hyperplasia (PLH) can be observed and graded based on its severity from 0 to 4 (Table 4) (Holcombe and Ducharme 2007). Although PLH has not been directly associated with EA (Holcombe *et al.* 2000), its detection has been associated with cough and tracheal mucus accumulation in Thoroughbred racehorses (Christley *et al.* 2001a, Couetil and Hawkins 2013a), and with stabling and exposure to dust, which represent risk factors also for EA (Holcombe *et al.* 2000).

Score	Endoscopic findings
0	Normal
1	Presence of few small white follicles over the dorsal pharyngeal walls
2	Numerous small follicles interspersed with occasional hyperemic follicles on the dorsal pharyngeal wall and extending ventrally over the lateral nasopharyngeal walls
3	More hyperemic follicles coalesce over the entire dorsal and lateral walls of the nasopharynx
4	Large, edematous, hyperemic follicles that frequently coalesce into broad- based and polypoid aggregates

Table 4. Pharyngeal lymphoid hyperplasia scoring system (Holcombe and Ducharme 2007).

The evaluation of upper airway obstructions may be useful because some authors have hypothesized that a cause-effect relationship between EA and dorsal displacement of the soft palate or other pharyngeal dynamic abnormalities (Courouce-Malblanc *et al.* 2010, Van Erck 2011, Wysocka and Klucinski 2018, Joo *et al.* 2021), although no consensus has been reached yet.

The detection of blood in the trachea, especially if the endoscopy is performed after exercise, allows the diagnosis of EIPH, which has been associated with MEA in racehorses (Christley and Rush 2007). The detection of tracheal bifurcation edema, observed as blunting or thickening of the carina, can represent a further sign of lower airway inflammation; a 0 to 4 scoring system has been proposed for its classification (Table 5) (Ferrucci *et al.* 2008). However, no direct association between tracheal bifurcation score and EA has been detected (Koch *et al.* 2007). Finally, airway endoscopy allows the execution of further diagnostic procedures, such as tracheal wash, bronchoalveolar lavage, endobronchial biopsies, and endobronchial ultrasonography.

Score	Endoscopic findings
0	Normally sharp carina
1	Little mucosal swelling of the carina and main bronchi
2	Moderate mucosal swelling
3	Marked mucosal swelling
4	Severe mucosal swelling

Table 5. Tracheal bifurcation blunting scoring system (Ferrucci *et al.* 2008).

1.5.4 Airway secretions analysis

During airway endoscopy, respiratory secretions should be collected to confirm the diagnosis of EA; in particular, cytological examination of the bronchoalveolar lavage (BAL) represents the diagnostic gold standard for the diagnosis of both SEA and MEA (Couetil *et al.* 2016). Another useful type of airway secretion may be tracheal wash (TW), although its role in the diagnosis of EA is still debated (Couetil *et al.* 2020).

The BAL should be performed by infusing a volume between 250 and 500 mL of 0.9% saline solution, through a videoendoscope or a BAL sterile tube, and re-aspirating it; generally, the 50-70% of the infused volume is expected to be re-collected (Couetil *et al.* 2016). The collected fluid should be subjected to cytological examination for differential cell count. It has been largely discussed on when a BAL cytology should be considered as normal, without reaching a univocal consensus (Hare and Viel 1998, Nolen-Walston *et al.* 2013, Couetil *et al.* 2016, Hermanage *et al*. 2019, Couetil *et al.* 2020); in fact, it has been observed that various factors influence BAL cytology, including collection technique, fluid volume, collection from one or both lungs, staining used, number of counted cells, environment, and type of population (Depecker *et al.* 2014, Couetil *et al.* 2020). However, the Consensus Statement on Equine Asthma developed by the American College of Veterinary Internal Medicine,

proposes the following cut-off values: total nucleated cell count ≤ 530 cells/μL, neutrophils ≤ 5%, eosinophils ≤ 1%, and mast cells ≤ 2% (Couetil *et al.* 2016). Horses with SEA typically show neutrophils > 25% and decrease lymphocytes counts (Derksen *et al.* 1985, Couetil *et al.* 2001); however, a paucigranulocytic phenotype has recently been described, due to the mucus sequestering neutrophils, with neutrophils representing just > 5% of total nucleated cells (Bullone and Lavoie 2017a). Conversely, horses with MEA show a mild to moderate increase in neutrophils, eosinophils, and/or mast cells percentages (Fogarty and Buckley 1991, Moore *et al.* 1995, Couetil *et al.* 2001, Richard *et al.* 2009, Depecker *et al.* 2014). In particular, independently from the procedure used for cytological examination, BAL cytology is considered as diagnostic of MEA when consisting of neutrophils > 10%, and/or mast cells > 5%, and/or eosinophils > 5%; values between normal and MEA cut-offs are considered as equivocal and should be interpreted based on the applied technique (Couetil *et al.* 2016).

The role of TW in the diagnosis of EA, instead, is quite debated. Indeed, previous studies reported either a 17.5% (Rossi *et al.* 2018) or 37% disagreement (Malikides *et al.* 2003), or even no correlation at all (Derksen *et al.* 1989), between BAL and TW cytology. Certainly, it has been recognized that TW is not appropriate for mast cells count, as they are quite rare in the trachea (Rossi *et al.* 2018). However, in the ideal scenario, both BAL and TW should be performed, as TW also allows the execution of bacterial culture and identification of concurrent bacterial colonization or infection (Couetil *et al.* 2020).

1.5.5 Bronchial biopsies

During endoscopy, endobronchial biopsies may be easily collected using a transendoscopic forceps. A semi-quantitative histological score has been recently developed in SEA, revealing a good correlation between severity of central airway remodeling and airway obstruction (Bullone *et al.* 2014, Bullone *et al.* 2016a). However, this score did not allow the distinction between healthy horses and those with SEA in remission (Bullone *et al.* 2015a).

1.5.6 Diagnostic imaging

At thoracic radiographic examination, horses with EA show an increased bronchointerstitial pattern and, during the exacerbation phase, a flattening of the diaphragm due to alveolar hyperinflation (Seahorn and Beadle 1993). However, it is a poorly sensitive procedure for the diagnosis of EA and is not associated with BAL cytology or pulmonary function in MEA (Mazan *et al.* 2005) but may be useful to rule out differential diagnoses (Couetil *et al.* 2016).

Thoracic ultrasonography is usually considered unrewarding in EA (Couetil *et al.* 2020). Indeed, only a small preliminary study reported more intense ultrasound changes in horses with SEA compared to healthy and MEA-affected horses (Siwinska *et al.* 2019).

Recently, transendoscopic endobronchial ultrasonography (EBUS) has been validated in horses, showing promising results for the estimation of airway smooth muscle remodeling in SEA (Bullone *et al.* 2015a). However, its use is still limited to research purposes.

Finally, computed tomography and magnetic resonance are not useful for the diagnosis of EA, as their application is limited to the upper respiratory tract (Kozlowska *et al.* 2022).

1.5.7 Lung function testing

While lung function testing is considered the gold standard for the diagnosis of human asthma (GINA Report 2023), its application in equine medicine is still limited to research settings (Couetil *et al.* 2020). Different techniques have been investigated in equine medicine, including conventional lung mechanics, forced expiration, spirometry, oscillometry, and electrical impedance tomography.

Conventional lung mechanics consists of the measurement of pleural pressure changes in relation to airflow during tidal breathing and provides information about pulmonary resistance and dynamic compliance: the first suggests airway narrowing or obstruction, while the latter reflects the elastic properties of the lungs (Marlin and Deaton 2007). Currently, it is considered the gold standard for the identification of airway obstruction in horses with SEA, but its sensitivity for MEA is low (Couetil *et al.* 2001, Bedenice *et al.* 2008). In one study, no differences were observed between MEA-affected horses and healthy controls at rest under eupneic conditions, while significant differences were identified during hyperventilation induced by a CO2 rebreathing test (Pirrone *et al.* 2007). However, it is a complex, time-consuming, and invasive technique, requiring the use of a facemask, a flowmeter and an esophageal balloon connected to a pressure transducer. Therefore, its use in clinical settings is very limited (Couetil *et al.* 2020).

Forced expiration consists of a measurement of airflow during late forceful expiration, which may detect a reduction in the expiratory flow as an early indicator of mild airflow obstruction. However, it requires deep sedation, the use of a nasotracheal tube, and mechanical ventilation, which makes this technique not applicable in clinical settings (Couetil *et al.* 2001).

Spirometry has also been applied to horses, providing data on tidal volume, peak expiratory and expiratory flows, time to peak flow, and forced vital capacity (Moore 2012); however, the use of general anesthesia or sedation is usually necessary, which limits its clinical application and the interpretation of the results (Moens 2010, Raidal *et al.* 2017, Kozlowska *et al.* 2022). Only in one study, the use of spirometry in EA showed promising results: indeed, an association between neutrophils percentages in the TW and lower flowtime curved in the second half of inspiration and expiration was found (Evans *et al.* 2011).

Oscillometry has the advantage of being a non-invasive technique, which does not require the active collaboration of the patient (Smith *et al.* 2005). It evaluates the answers of the respiratory system to external superimposed forces, the oscillations, filtered by the signals obtained from tidal breathing (Hoffman 2002). In particular, it measures impedance, which is composed by airway resistance (Rrs) and reactance (Xrs) (Smith *et al.* 2005). While Rrs represents the resistance to the flow in the airways, depending on caliber and architecture of the airways, Xrs represents the distensibility and elasticity of the lung tissues (Oostveen *et al.* 2003). Besides the total respiratory Rrs and Xrs, technology implementation has allowed to differentiate between the inspiratory and the expiratory phases of the breath, obtaining the so-called "within-breath" parameters, which improves the sensitivity to detect airway obstruction (Dellacà *et al.* 2004, Klein *et al.* 2006); indeed, the differences between expiratory and inspiratory phases may further provide useful information on airway obstruction (Richard *et al.* 2009, Stucchi *et al.* 2022). Based on the method used to generate the oscillations, oscillometry techniques can be divided into forced oscillation technique (FOT) and impulse oscillometry system (IOS): the first uses monofrequency sinusoidal waves

measured at multiple separated frequencies, while the second one consists of a train of pulses that decomposes into a continuous frequency spectrum (Hoffman 2002). In equine medicine, the first studies date back to 1989, when oscillometry proved to be well-tolerated and feasible for the measurement of total impedance in healthy ponies, and a resonance frequency of 3 Hz was identified (Art *et al.* 1989, Young and Hall 1989). Later, multiple studies on EA were conducted. In SEA, oscillometry reveals higher values of R and lower values of X compared to healthy horses at low frequencies, reflecting lower airways obstruction (Young et al. 1997, Hoffman and Mazan 1999, Van Erck *et al.* 2004a, Van Erck *et al.* 2004b, Stucchi *et al.* 2022); moreover, R and X at 3 Hz frequency have been correlated with airways remodeling (Bullone *et al.* 2014). Interestingly, during clinical remission of SEA, some authors reported no differences in R and X compared to healthy horses (Deaton *et al.* 2007), while another study including within-breath analysis reported lower values of mean and inspiratory X at low frequencies (Stucchi *et al.* 2022). In MEA horses, less consistent results have been obtained: two studies reported higher values of R compared to healthy horses, both in the inspiratory and expiratory phases, while no differences in X values were observed at frequencies representative of distal lower airways (Hoffman and Mazan 1999, Richard *et al.* 2009). However, despite the many advantages, oscillometry is currently restricted to research institutions, as it is still at an early stage of clinical development, and protocols, interpretation, and clinical application in horses still need to be defined (Van Erck *et al.* 2010, Kozlowska *et al.* 2022).

Finally, electrical impedance tomography (EIT) is a non-invasive, radiation-free, real-time imaging technique which allows the assessment of continuous lung ventilation and perfusion (Frerichs *et al.* 2017). It has the advantage of being affordable, easy to implement, well-tolerated by non-sedated horses,

and portable (Secombe *et al.* 2019a), but its applicability in equine medicine is still at an early stage, especially due to the complexity of data and susceptibility to artifacts (Kozlowska *et al.* 2022). In horses, EIT recently showed to be capable of identifying bronchoconstriction and bronchodilation after histamine provocation and subsequent bronchodilator administration, showing a potential for the evaluation of airway obstruction (Secombe *et al.* 2021). Moreover, a recent study showed no differences in EIT parameters at rest between healthy and asthmatic horses but showed higher peak expiratory and inspiratory flows in horses with MEA and SEA compared to healthy horses; in particular, the expiratory phase showed to be more affected than the inspiratory phase in asthmatic patients (Herteman *et al.* 2021).

Regardless of the lung function testing technique, sensitivity is often too low to detect mild or subclinical obstructions in MEA or during the remission phase of SEA. Therefore, the use of bronchoprovocation or bronchodilation tests are commonly recommended to identify low-grade obstruction (Bedenice *et al.* 2008). Among bronchoprovocation tests, histamine has been widely used in EA studies of all severity (Couetil *et al.* 2001, Bedenice *et al.* 2008, Richard *et al.* 2009, Tilley *et al.* 2012, Wichtel *et al.* 2016, Cullimore *et al.* 2018); however, the association between histamine bronchoprovocation, BAL cytology, and lung function has been questioned (Couetil *et al.* 2020). In cases of SEA, also hay challenge has been proposed, but is considered inappropriate in clinical settings (Couetil *et al.* 2016). Moreover, in SEA, bronchodilation tests may be more appropriate, consisting of the evaluation of airflow variability after the administration of bronchodilators, such as systemic N-butylscopolammonium bromide or inhaled albuterol or ipratropium bromide (Mazan *et al.* 2004, Couetil *et al.* 2020).

1.5.8 Clinical pathology and blood biomarkers

In EA-affected horses, hematology variables are usually within normal ranges (Riihimaki *et al.* 2008).

At arterial blood gas analysis, SEA horses usually present with marked hypoxemia during the exacerbation phase, due to the increased dead space during ventilation, resulting from hyperinflation and compression on pulmonary capillaries preventing adequate perfusion (Nyman *et al.* 1991). Conversely, in horses with MEA, the PaO_2 is usually within normal limits at rest, while the $PaCO_2$ may be slightly increased, suggesting an alteration in ventilation/perfusion ratio (Couetil and Denicola 1999). Interestingly, during exercise, MEA horses present a more pronounced hypoxemia compared to healthy controls (Sanchez *et al.* 2005).

Various serum proteins have been studied as potential predictors of MEA. In particular, surfactant protein D showed to be higher in racehorses with MEA than healthy ones both at rest and after exercise, but it was not correlated with BAL cytology (Richard *et al.* 2012). Acute phase proteins such as serum amyloid A and haptaglobin were not increased in racehorses with MEA (Richard *et al.* 2010a, Leclere *et al.* 2015), while they were higher in MEA compared to healthy horses in another study (Bullone *et al.* 2015b).

1.6 TREATMENT

The therapeutic approach to Equine Asthma is based on management strategies, to reduce or avoid antigens exposure, and pharmacological interventions, to decrease lung inflammation and bronchoconstriction. The majority of the scientific literature is focused on the treatment of SEA, which is commonly translated to MEA management (Couetil *et al.* 2016).

1.6.1 Management strategies

Antigen avoidance is considered the most important measure for the successful management and prevention of EA (Rush and Mair 2004). Indeed, in SEA horses, it showed to allow clinical signs improvement within 3 days, together with a progressive improvement of lung function, regression of airway neutrophilic inflammation, and partial reversion of bronchial smooth muscle remodeling (Leclere *et al.* 2012). Similarly, in horses with MEA, decreased exposure to dust leads to an improvement of clinical signs, including cough and poor performance (Nogradi *et al.* 2014)

In general, maintaining asthmatic horses at a pasture with fresh grass represents the most effective antigen avoidance strategy, except for horses suffering from EPA, that benefit from stabling (Rush and Mair 2004, Lavoie 2007). However, also when kept on pasture, round bale hay should be avoided, due to its particular allergenicity (Rush and Mair 2004). When horses are stabled, multiple strategies may be implemented to reduce antigen exposure. In particular, management strategies can either consist of reducing airborne particles generation, by using low-dust feedstuff and bedding, or of increasing their eliminations, by improving barn ventilation (Couetil *et al.* 2016). Dry hay represents the primary responsible of respirable dust high levels (Wasko *et al.* 2011). Therefore, while changing bedding from straw to

low-dust cardboard material alone is able to halve respirable dust levels (Kirshvink *et al.* 2002), the combination of replacing straw and dry hay with wood shavings and pelleted or cubed hay or haylage leads to a 2-3 fold decrease or respirable dust (Woods *et al.* 1993, Rush and Mair 2004, Clements and Pirie 2007a). In alternative, hay may be immersed in water, reducing dust by 60% (Clements and Pirie 2007b); however, this is an acceptable strategy only in mildly affected horses (Rush and Mair 2004). Besides dust, changing feed and bedding materials may also help reducing endotoxin concentration, directly contributing to airway inflammation improvement (Pirie *et al.* 2002); indeed, a hay- and straw-free environment showed to have 10-fold less respirable endotoxin concentrations (Rush and Mair 2004). Regarding barn ventilation, keeping doors open, independently from the season, showed to decrease exposure to dust (Rosenthal *et al.* 2006, Millerick-May *et al.* 2013, Ivester *et al.* 2014). Moreover, barn mechanical ventilation may be useful to decrease ultrafine particles, bacteria and fungi, and seems to aid tracheal mucus reduction; however, its effect on lower airway inflammation in debated (Walinder *et al.* 2011). Indeed, it has been shown that ventilation is not able to reduce the aeroallergens load in the "breathing zone" of horses when eating hay, when dust concentrations are 35-fold higher (Woods *et al.* 1993, Rush and Mair 2004).

1.6.2 Pharmacological treatment

The pharmacological treatment of EA is based on the administration of corticosteroids, reducing airway inflammation, and bronchodilators, providing symptomatic relief from airway obstruction (Rush and Mair 2004). They are commonly used in association, are effective in leading to pulmonary function improvement (Calzetta *et al.* 2017), and can be administered by systemic (intravenous, intramuscular or oral) or topical (inhalation) routes

(Couetil *et al.* 2016); while some studies report a quicker and greater improvement after systemic administration (Couetil *et al.* 2005, Couetil *et al.* 2006, Robinson *et al*. 2009), other authors and a recent meta-analysis proved no differences between systemic or inhalation routes (Ivester and Couetil 2014, Calzetta *et al.* 2017). In general, long-term treatments are more effective for EA management compared to short-term administrations (Calzetta *et al.* 2017).

1.6.2.1 Corticosteroids

Corticosteroids are mainly used for lower airway inflammation control and lung function improvement. Multiple studies showed that corticosteroid treatment, in association with management strategies, was effective in the control of clinical signs, airway neutrophilic inflammation, and inflammatory cytokines expression (Leclere *et al.* 2012, Couetil *et al.* 2005, DeLuca *et al.* 2008); however, when administered without concurrent air quality improvement, its effect of neutrophils reduction lacks at both short- and long-term (Lapointe *et al.* 1993, Gerber *et al.* 2011, Leclere *et al.* 2012). For the treatment of MEA, corticosteroids are usually empirically used by analogy with SEA; indeed, they seem to be effective in decreasing airway hypersensitivity, but not in reducing neutrophilic inflammation (Couetil *et al.* 2016). Among corticosteroids, dexamethasone is the most commonly used for the systemic treatment of EA; its administration by parenteral and oral routed has shown improvement in symptoms relief, improvement of lung function, and reduction of inflammation (Rush *et al.* 1998, Lavoie *et al*. 2002, Cornelisse *et al.* 2004, Grady *et al.* 2010, Leclere *et al.* 2010). Other useful systemic corticosteroids for the treatment of EA include oral prednisolone, intramuscular triamcinolone acetonide, and intramuscular isoflupredone acetate (Lapointe *et al.* 1993, Picandet *et al.* 2003, Leclere *et al.* 2010). Many studies have also investigated the efficacy of inhaled corticosteroids in SEA

therapy, having the advantage of delivering high drug concentrations locally to the airways while concomitantly minimizing systemic side effects, such as laminitis and adrenal suppression (Duvvier *et al.* 1997, Durham 2001, Cornelisse and Robinson 2003, Niedermaier and Gehlen 2009). Among the corticosteroids reported for inhalation administration, fluticasone propionate is the most used (Robinson *et al.* 2009), inducing a partial reversion of airway remodeling, but not a neutrophilic inflammation reduction (Leclere *et al.* 2012); interestingly, it showed to be (Dauvillier *et al.* 2011). Other reported inhaled corticosteroids showing a good efficacy in SEA are beclomethasone dipropionate (Rush *et al.* 2000, Couetil *et al.* 2006), budesonide (Kampmann *et al.* 2001, Lavoie *et al.* 2019b) and, more recently, ciclesonide (Lavoie *et al.* 2019a, Pirie *et al.* 2021).

1.6.2.2 Bronchodilators

Besides corticosteroids, the use of bronchodilators has been deeply investigated in horses with SEA, while in the treatment of MEA it is empirical, as the bronchoconstriction at rest is too low to induce clinical signs; however, bronchodilators may be effective in reducing cough and increasing mucociliary clearance (Norton *et al.* 2013, Couetil *et al.* 2016). In SEA, bronchodilators can help to relieve severe airway obstruction, but also to enhance the deposition and efficacy of inhaled corticosteroids (Rush *et al.* 1999). Bronchodilator drugs used in EA can be classified based on their mode of action as sympathomimetics (β2-agonists) or parasympatholytics (Pirie 2013). The most used bronchodilator is clenbuterol, a β2-adrenergic agonist which can be administered systemically, either by intravenous route or, more commonly, orally (Sander *et al.* 2002); however, to obtain a satisfactory effect in severely affected horses, doses should exceed the recommended one, or incrementally increasing doses may be needed to maintain treatment efficacy

(Erichsen *et al.* 1994). Indeed, the prolonged administration of sympathomimetics may lead to a drug-induced β2 adrenergic receptors down-regulation; however, the concomitant administration of corticosteroids is able to prevent this phenomenon (Abraham *et al.* 2002). Moreover, besides bronchodilator effects, clenbuterol may also play an anti-inflammatory role, demonstrated both *in vitro* and *ex vivo* (Laan *et al.* 2006b, Laan *et al.* 2006c). Other molecules are used by inhalation administration, including albuterol, salmeterol, pirbuterol, fenoterol, and trimetoquinol (Derksen *et al.* 1999, Henrikson and Rush 2001, Mazan *et al.* 2003, Camargo *et al.* 2007, Bertin *et al.* 2011). Recently, the long-term combined administration of inhaled salmeterol and fluticasone showed to be more effective than fluticasone alone in improving clinical signs and controlling airway neutrophilic inflammation (Bullone *et al.* 2017). Similarly, a meta-analysis on the treatment of SEA, showed that the first therapeutic choice for EA should include long-term treatments with inhaled corticosteroids (fluticasone) and long-acting β2-adrenergic agonists (salmeterol) (Calzetta *et al.* 2017).

Other bronchodilator classes that proved to be effective in SEA include anticholinergics and antimuscarinics. Among anticholinergic drugs, N-butylscopolammonium bromide proved a strong and rapid bronchodilator effect after intravenous administration (Couetil *et al.* 2012), similarly to atropine; however, their use should be limited to emergency cases, as a rescue therapy, due to the associated side effects (Pirie 2013). Finally, ipratropium bromide is a commonly used antimuscarinic agent, which showed efficacy in inducing bronchodilation in SEA horses, with a longer duration of action compared to β2-adrenergic drugs (Robinson *et al.* 1993, Duvivier *et al.* 1999). A similar action has been reported for glycopyrrolate and revatropate (Art *et al.* 2003, McGorum *et al.* 2013). Interestingly, although the mechanism of action

has not been clarified yet, furosemide seems to have a bronchodilator effect in SEA horses when administered by intravenous or inhalation routes (Broadstone *et al.* 1991, Aguilera-Tejero *et al*. 1993), but its efficacy has been questioned (Bayly *et al.* 2001).

1.6.2.3 Other medical treatments

Among other medications proposed for EA, other molecules with anti-inflammatory effects and mucoactive agents have been investigated. In case of mastocytic MEA, sodium cromoglycate has been used to improve clinical signs and decrease airway hyperreactivity in racehorses (Hare *et al.* 1994). In horses with neutrophilic MEA, low dose interpheron alpha seemed to reduce airway inflammation and inflammatory mediator concentrations in the BAL (Moore *et al.* 1996, Moore *et al.* 1997, Moore *et al.* 2004). Recently, the administration of immunostimulatory agents, such as cytosine-phosphate-guanosine-oligodeoxynucleotides, showed promising results in ameliorating lung function, clinical signs and mucus secretion in SEA (Klier *et al.* 2015, Klier *et al.* 2019, Klier *et al.* 2022). Some antimicrobial macrolides, such as azithromycin and clarithromycin, are sometimes used in human asthma due to their immunomodulatory properties, in cases that cannot be controlled with corticosteroids and bronchodilators alone (Gibson *et al.* 2017); however, a randomized controlled crossover study in SEA horses failed to detect any improvement (Mainguy-Seers *et al.* 2019). In human allergic asthma, allergen-specific immunotherapy has also proven to be helpful in alleviating symptoms and reducing medication, according to systematic reviews and meta-analyses (Kim *et al.* 2013, Erekosima *et al.* 2014); however, in EA, it has not been widely used to date, probably because the causative allergens are often not identified (Mueller *et al.* 2018). In the last years, the administration of inhaled lidocaine has been studied for the treatment of EA (Minuto *et al.* 2022), showing to be

effective in ameliorating clinical score, and significantly decreasing BAL neutrophil percentages and tracheal mucus accumulation (Mahalingam-Dhingra *et al.* 2022). Among mucolytic and mucokinetic agents, acetylcysteine, bromhexine, ammonium chloride, and potassium iodide infusions have been used, but no scientific evidence on their utility in EA treatment has been reached yet (Pirie 2013, Couetil *et al.* 2016). Several nutraceutical supplements have also been investigated, including polyunsaturated omega-3 fatty acids (Kohl-Parisini *et al.* 2007, Nogradi *et al.* 2015), curcumin derivate (Sandersen *et al.* 2011), and caffeic acid (Rutledge *et al.* 2022).

1.7 PROGNOSIS

The prognosis of EA depends on the severity and phenotype of the disease, and few studies tried to assess the long-term effects of this condition. Concerning SEA, a median survival time of 8 years after the diagnosis has been reported, with an 87% of survivors at three years from the diagnosis (Aviza *et al.* 2001). SEA per se rarely leads to death, but severely affected horses may be euthanized due to chronic progression of the disease and resistance to standard therapies (Lavoie 2007, Calzetta *et al.* 2017, Mainguy-Seers and Lavoie 2021). After treatment, the owner report apparent healing in 21% of horses, and repeated episodes of disease exacerbations in the remaining 78-79%; the progression of SEA can lead to weight loss in 19% of affected horses (Ainsworth *et al.* 1998, Aviza *et al.* 2001). Indeed, chronic inflammations persists also during remission phases, and airway remodeling is only partially reversible (Leclere *et al.* 2011, Lavoie-Lamoureux *et al.* 2012, Bullone and Lavoie 2020). Concerning their athletic use, 21% of affected horses have to be retired from any athletic activity, 64% may be used for pleasure riding, and only 10% can go on with competing riding (Aviza *et al.* 2001); in general, the usefulness of the horse is considered as negatively affected by SEA in 41-52% of horses (Ainsworth *et al.* 1998, Aviza *et al.* 2001).

Data on the prognosis of MEA are scarce. Its prognosis is good for health and for return to previous level of athletic performance after pharmacological treatment and the environmental management (Couetil 2014). However, recent studies reported the occurrence of progressive airway remodeling also in MEA horses, which long-term effects have not been clarified yet (Bessonat *et al.* 2022, Dupuis-Dowd and Lavoie 2022).

CHAPTER 2.
AIM OF THE RESEARCH

2.1 EQUINE ASTHMA: WHAT IS MISSING?

Although Equine Asthma has been known for centuries, and more than 1200 scientific papers have been published on this disease, much knowledge is still missing concerning its pathophysiology, diagnosis, and therapeutic approach. The Consensus Statement of the American College of Veterinary Internal Medicine on lower airway inflammation (Couetil *et al.* 2016), and the Equine Asthma Group reunited at the 2019 Havemeyer Equine Asthma Workshop (Couetil *et al.* 2020), have identified some suggestions for future research directions, classified by topic groups. Concerning the pathophysiology of EA, the main proposed topics include the role of environmental pollutants and infectious agents, the identification of genetic risk factors, the better understanding of immune responses associated with different subtypes of EA, and the identification of factors associated with the progression of milder forms of EA to more severe forms. For the diagnosis of EA, the main identified needs are the development of portable and sensitive devices for lung function testing in the field, and the identification of systemic non-invasive biomarkers. Suggestions regarding therapeutic approach include the development of pharmacological treatments alternative to corticosteroids, immunological treatments, respiratory nutraceuticals, and precision medicine approaches personalized on the basis of different endotypes and phenotypes.

Besides the above-mentioned topics, other ideas proposed throughout the years by different researchers still need to be explored. First, a better understanding of the hypersensitivity response and the environmental antigens triggering EA symptoms would open the door on the possible use of allergen-specific immunotherapy in asthmatic horses, and would allow a more targeted allergen avoidance strategy, whenever possible. Moreover, the application of thoracic ultrasonography for the diagnosis of EA has been only

recently investigated by one study and deserves further research, as it would represent a non-invasive technique easily appliable to field settings. Regarding the pathogenesis of EA, different authors have hypothesized a cause-effect association with other respiratory disorders, including upper airway inflammation, upper airway obstructions, and exercise-induced pulmonary hemorrhage, but no evidence has been reached yet. Moreover, although MEA is widely recognized as a cause of poor performance, its role in fitness impairment has only scarcely been investigated.

2.2 DESCRIPTION OF THE RESEARCH

Based on these premises, the present research aims to address some of the questions still existing within the EA scientific community, and to contribute to a broader understanding of this complex syndrome. Once identified possible fields of investigation, seven different studies were conducted, either retrospectively (presented in Chapter 3) or prospectively (presented in Chapter 4). The retrospective studies were performed by analyzing the data of the clinical equine patients referred to the Equine Unit of the Veterinary Teaching Hospital of the University of Milan, throughout a period of about 20 years. For these horses, owners had given their consent for the use of clinical data for research purposes, and all the described clinical procedures were performed for diagnostic purposes. In the prospective studies, horses were selected among a population of horses referred to the Equine Unit of the Veterinary Teaching Hospital, or to the Equine Sports Medicine Laboratory "Franco Tradati" of the University of Milan. All the prospective studies were non-experimental, and previously approved by the Animal Care Committee of the University of Milan (Organismo Preposto al Benessere degli Animali);

moreover, informed consent was given by all horses' owners or delegated holders. The hypotheses and objectives of each study are presented in the next paragraph.

2.3 HYPOTHESES AND OBJECTIVES OF THE RESEARCH

The first study (presented in Paragraph 3.1) is entitled "Intradermal Testing Results in Horses Affected by Mild-Moderate and Severe Equine Asthma" and was retrospectively conducted.

Hypothesis: Intradermal testing may be useful to better understand the role of different allergens in the hypersensitivity responses involved in EA pathogenesis.

Objective: To evaluate the intradermal testing reactions at different timepoints, reflecting different types of hypersensitivity responses, and to measure the prevalence of positive reactions induced by different allergens in horses affected by SEA or MEA.

The second study (presented in Paragraph 3.2) is entitled "The role of thoracic ultrasonography and airway endoscopy in the diagnosis of equine asthma and exercise-induced pulmonary hemorrhage" and was retrospectively conducted.

Hypothesis: A complete ultrasonographic and endoscopic assessment of the respiratory tract may be useful for the diagnosis of SEA, MEA and concomitant MEA and EIPH in horses.

Objective: To evaluate whether the findings of thoracic ultrasonography and airway endoscopy may help to rapidly distinguish between horse affected by SEA, MEA, or concurrent MEA and EIPH.

The third study (presented in Paragraph 3.3) is entitled "Upper and lower airways evaluation and its relationship with dynamic upper airway obstruction in racehorses" and was retrospectively conducted.

Hypothesis: Racehorses showing anatomical-functional abnormalities or inflammation of the upper and lower airways may be predisposed to the development of dynamic upper airway obstruction.

Objective: To evaluate if the findings of the diagnostic procedures performed at rest on the upper airway, and endoscopic and cytological results of the lower airway, are associated with the occurrence of dynamic upper airway obstruction during high-speed treadmill exercise.

The fourth study (presented in Paragraph 3.4) is entitled "Impact of lower airway inflammation on fitness parameters in Standardbred racehorses" and was retrospectively conducted.

Hypothesis: Mild-moderate equine asthma causes poor racing performance by impairing the fitness and the aerobic capacity of affected horses.

Objective: To measure objectively the impact of the inflammation of the lower airways on the fitness parameters of Standardbred racehorses measured through a standardized incremental exercise test on treadmill.

The fifth study (presented in Paragraph 4.1) is entitled "Cytokine mRNA expression in the bronchoalveolar lavage cells from horses affected by different equine asthma subtypes" and was prospectively conducted.

Hypothesis: Different severity and cytological subtypes of EA may be associated with the involvement of different cytokines, reflecting different immunological pathways.

Objective: To investigate the associations between the mRNA expression of a series of cytokines (IL-1β, IL-2, IFN-γ, IL-4, IL-17), involved in different immune response types, in the cells isolated from the BAL and EA severity, cytological profile, and lung function parameters.

The sixth study (presented in Paragraph 4.2) is entitled "Differences in lung function between horses affected by mild-moderate equine asthma and healthy controls measured by a novel portable oscillometry device" and was prospectively conducted.

Hypothesis: Lung function parameters, measured by a novel oscillometry device, may differ between healthy horses and those affected by MEA.

Objective: To detect the differences in respiratory resistance and reactance, measured by oscillometry, between healthy and MEA-affected horses.

The seventh study (presented in Paragraph 4.3) is entitled "Respiratory oscillometry testing in relation to exercise in healthy and asthmatic Thoroughbreds" and was prospectively conducted.

Hypothesis: Oscillometry may detect changes in lung function induced by intense exercise in racehorses, which may differ between healthy and MEA-affected subjects. Moreover, a better lung function may be predictive of enhanced racing performance.

Objective: To evaluate the variations in resistance and reactance at 15 and 45 minutes after exercise compared to baseline values, to compare the lung function parameters at rest and after exercise between healthy and MEA horses, and to evaluate whether horses with better lung function at rest have a better aerobic capacity.

CHAPTER 3.
RESEARCH:
RETROSPECTIVE STUDIES

3.1 INTRADERMAL TESTING RESULTS IN HORSES AFFECTED BY MILD-MODERATE AND SEVERE EQUINE ASTHMA

The present study has already been published in the following manuscript:

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3.1.1 Introduction

Equine asthma (EA) is a syndrome including inflammatory, recurrent, and chronic lower airway disorders of adult horses, sharing several similarities with human asthma (Lange-Consiglio *et al.* 2019, Bullone and Lavoie 2020). It is clinically characterized by airflow obstruction, mucus hypersecretion and airway hyperreactivity. Based on the severity and recurrence of the condition, it is classified as mild, moderate or severe equine asthma (Bullone and Lavoie 2020). Severe equine asthma (SEA) is the most common cause of chronic cough in horses (Pirie 2014), naturally affecting 10–15% of adult horses (typically over seven years of age) living in the northern hemisphere (Hotchkiss *et al.* 2007) where horses are stabled indoors during most of the year (Leguillette 2003). It is characterized by recurrent and reversible episodes of disease exacerbations, consisting of increased respiratory effort at rest, coughing and exercise intolerance (Leclere *et al.* 2011). Mild or moderate equine asthma (MEA), in contrast, can affect horses of any age but is more commonly reported in young horses; poor performance can be the only symptom, generally associated with occasional coughing and mucus hypersecretion, with normal breathing at rest (Couetil *et al.* 2016).

In asthmatic horses, clinical signs are triggered by the inhalation of antigens present in the stables, such as moulds, spores and mites (Pirie *et al.* 2003), which may vary according to environmental exposure and geographical area (Bullone and Lavoie 2015). Seasonal pollens and other antigens found in high levels outdoors in spring and summer represent another group of etiological agents able to cause exacerbations in susceptible horses: this condition is known as summer pasture asthma (Leclere *et al.* 2011, Bullone and Lavoie 2020). Although it is generally agreed upon that EA represents a hypersensitivity response (HR) to inhaled antigens, a precise pathogenetic

mechanism among susceptible horses has not yet been identified (Hotchkiss *et al.* 2007).

Various studies suggest that IgE-mediated immediate-type allergic reactions are associated with SEA (Halliwell *et al.* 1979, McPherson *et al.* 1979, Evans *et al.* 1992, McGorum *et al.* 1993). In fact, the increase in IgEs detected in the bronchoalveolar lavage fluid of the SEA horses is typical of a type I HR (Marti *et al.* 2003). Moreover, histamine release from pulmonary mast cells in response to allergens exposure in vitro is significantly greater in asthmatic horses compared to healthy horses, suggesting the involvement of IgE-mediated reactions leading to increased mast cell degranulation (Hare *et al.* 1999). Mast cell mediators, such as histamine and leukotrienes, induce smooth muscle contraction and may contribute to bronchospasm in SEA (Olszewski *et al.* 1999, Marti *et al.* 2003). Also, a delayed-onset type-IV hypersensitivity seems to be involved in SEA, mediated by Th-2 lymphocytes (CD4+ lymphocyte subgroup) (Lavoie *et al.* 2001, Giguere *et al.* 2002, Cordeau *et al.* 2004). Therefore, the identification of specific allergens that induce hypersensitivity reactions could represent an important tool for diagnosis, prognosis and choice of the most appropriate treatment (Chan *et al.* 2005, GINA Report 2023).

For this purpose, in human medicine, the gold standard of allergy tests is considered the skin prick test (SPT), while in atopic horses, intradermal testing (IDT) is the preferred method used to confirm the diagnosis of immediate and delayed hypersensitivity reactions and to select avoidance measures and/or allergen-specific immunotherapy (ASIT) (Antunes *et al.* 2009, Scott and Miller 2011, Klier *et al.* 2021). After the allergen is inoculated, allergen-specific IgE binds to the high-affinity IgE receptor (FcεRI) on the surface of mast cells, activating a signal transduction cascade, which finally induces mast cell degranulation: consequently, various preformed inflammatory mediators are

released, and a wheal-and-flare reaction develops (Antunes *et al.* 2009). Up to 85% of human asthmatic patients show positive reactions to SPT for common aeroallergens (Bobb and Ritz 2003). However, positivity is not specific for asthma nor is it present in all asthma phenotypes: in fact, it identifies atopic status, which increases the probability that a patient with respiratory symptoms has allergic asthma. Thus, it is essential to interpret positive skin test reactions in light of the patient's history (GINA Report 2023).

Most healthy patients could have one or more positive reactions to skin tests; however, atopic horses have a significantly greater number of positive reactions (Scott and Miller 2011). In one study including 86 horses, SEA horses seem to be at least six times as likely to respond to one or more allergens compared to healthy horses, and the mean number of allergens provoking a positive reaction in SEA horses is approximately two to 10 times greater than in healthy horses (Jose-Cunilleras *et al.* 2001). Moreover, the mean wheal diameter in SEA horses seems to be significantly greater than in control horses, indicating a greater reactivity (Wong *et al.* 2005): in one study including 40 horses, 98% of the diameters of the wheals in the SEA group were larger than 1 cm, contrasting with 27% in the healthy group (Tilley *et al.* 2010).

However, contrasting results have been obtained from different studies, creating a lack of understanding of IDT as an additional diagnostic tool for equine asthma (Eyre 1972, Halliwell *et al.* 1979, McPherson *et al.* 1979, Evans *et al.* 1992, McGorum *et al.* 1993, Jose-Cunilleras *et al.* 2001, Lorch *et al.* 2001, Lebis *et al.* 2003, Wong *et al.* 2005, Tilley *et al.* 2010, Klier *et al.* 2021). However, it is difficult to compare findings of different investigations, since IDT and the allergens used in the different studies are not standardized for the horse (Tahon *et al.* 2009, Klier *et al.* 2021). The present study aims to evaluate IDT reactions and to measure the prevalence of positivity induced by different allergens in horses affected by either SEA or MEA.

3.1.2 Materials and Methods

3.1.2.1 Horses

For this retrospective study, clinical records of all horses affected by SEA or MEA for which an IDT was performed between 2008 and 2020 at the Equine Unit of the Veterinary Teaching Hospital of the University of Milan (Italy) were reviewed. To be enrolled, all horses had to be vaccinated against influenza and tetanus and regularly dewormed. Each patient underwent a complete diagnostic evaluation, including clinical history, general physical examination, airway endoscopy and assessment of tracheal mucus, and bronchoalveolar lavage (BAL) cytology (Couetil *et al.* 2020). For SEA horses, inclusion criteria were history of exercise intolerance, dyspnea, coughing, normal rectal temperature, absence of alterations at thoracic ultrasound consistent with pneumonia, detection of tracheobronchial mucus grade >2/5 and neutrophils count >25% in BAL cytology (Couetil *et al.* 2016). MEA horses were selected on the basis of history of poor performance and occasional cough, eupnea at rest, normal rectal temperature, detection of tracheobronchial mucus grade >2/5 (Couetil *et al.* 2016) and BAL cytology consisting of neutrophils >5%, and/or mast cells >2% and/or eosinophils >1% (Couetil 2014). Whenever glucocorticoids were recently administered, they were withdrawn for a minimum of three weeks for oral or topical administration, or eight weeks for injective administration; similarly, antihistamines were withdrawn for a minimum of 10 days because these drugs could cause false negative IDT results (Scott and Miller 2011). Moreover, none of the horses included in this study had history of dermatological diseases nor had ever received ASIT.

3.1.2.2 Intradermal Testing

Fifty allergens, consisting of individual allergens or allergen mixes, were used for IDT, including eight moulds/fungi, five mites, six insects, 20 plants, three animals and eight feedstuffs (Artuvetrin® Skin test, Nextmune, Lelystad, Netherlands; Prick test, Lofarma SPA, Milan, Italy). Mould allergens included *Alternaria alternata*, *Candida albicans*, *Aspergillus fumigatus*, *Cladosporium spp.*, *Helmintosporium sativum*, Mycophytes mix, Trichoepidermophytes mix, and *Penicillium* mix. Mite allergens included *Acarus siro*, *Gliciphagus domesticus*, *Tyrophagus putrescentiae*, *Lepidogliphus destructor* and *Dermatophagoides* mix. Insect allergens included *Culicoides spp.*, *Culex spp.*, *Aedes spp.*, *Tabanus spp.*, *Chrysops spp.* and *Musca domestica*. Plant allergens included *Olea europea*, *Cupressus sempervirens*, *Corylus avellana*, *Cynodon dactylon*, *Artemisia vulgaris*, *Pinus silvestris*, *Plantago lanceolata*, *Holcus lanatus*, *Ostrya virginiana*, *Lolium perenne*, *Poa pratensis*, *Populus nigra*, *Robinia pseudoacacia*, *Pyrethrum spp.*, *Ulmus campestris*, *Parietaria* mix, *Poaceae* mix, *Compositae* mix, *Aceraceae-Fagaceae* mix, and *Betullaceae* mix. Animal allergens included dog epithelium, cat epithelium and feathers mix. Food allergens included wheat flour, barley flour, cornflour, rice flour, soy flour, rye flour, fibers mix and milk proteins. Saline was used as a negative control and histamine chlorhydrate, 10 mg/mL, as a positive control. Since the availability of the different allergens varied during the period of the study depending on the supplier company, not all horses were tested with the same allergens and only 27 allergens were common to every patient.

The IDT was performed on the lateral aspect of the neck. A rectangular area measuring 20 cm × 40 cm was clipped and cleaned to eliminate gross contamination. A permanent black marker was used to draw circles of 2.5 cm diameter, located 1.5 cm apart from each other, in order to indicate injection sites (Figure 2). A 0.1 mL volume for each allergen, including negative and

positive controls, was injected intradermally using a 25-gauge needle. No sedation was necessary for any horse. Injection sites were evaluated 30 min (immediate), 4 h (late-phase), 24 h and 48 h (delayed) after injections, in order to detect different hypersensitivity reaction types. Reactions were judged subjectively by two equally experienced operators through visual inspection and digital palpation and were graded from 0 (no reaction) to 4 (maximum reaction) on the basis of wheal diameter, thickness, turgidity, warmth and achiness (Lorch *et al.* 2001). All reactions of grade ≥1 were considered as positive responses.

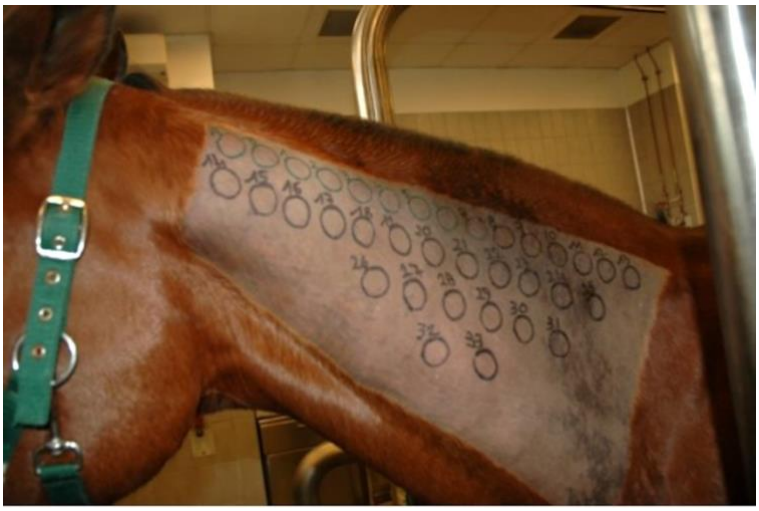

Figure 2. The lateral aspect of the neck has been clipped and circles drawn with a black marker to indicate injection sites.

3.1.2.3 Statistical Analysis

Horses were divided on the basis of their diagnosis into the SEA and MEA groups. All data were evaluated for normality using a Shapiro–Wilk test. Data for weight were normally distributed, while data for age were normalized by means of logarithmic transformation. The grades of IDT reactions were not normally distributed. Weight and normalized age between groups were compared using an unpaired Student's *t*-test. The differences in sex distribution between groups were evaluated by means of a Fisher's exact test.

A Fisher's exact test was used to compare the number of horses having positive reactions to any allergen in total and at each evaluation time between groups. The total grades of the reactions, divided into mild-moderate reactions (grades 1–2) or strong reactions (grade 3–4), from any allergen were compared between groups with a Fisher's exact test. Besides, the number of allergens provoking positive reactions in total and at each evaluation time was compared between groups using a Fisher's exact test.

The prevalence of positive reactions and the medians of the grades of the reactions provoked by each specific allergen at every evaluation time were calculated in both groups. The frequency of positive and negative reactions induced by each specific allergen at every evaluation time was compared between groups by means of a Fisher's exact test. The grades of the reactions provoked by each allergen at every evaluation time were compared between groups using a Mann–Whitney test.

Data are presented as mean ± standard deviation (SD) if normally distributed and as median and interquartile ranges if not normally distributed. Statistical significance was set at $p < 0.05$. Data were analyzed using a commercially available statistical software package (GraphPad Prism 9.1.0 for MacOS; GraphPad Software, San Diego, CA, USA).

3.1.3. Results

3.1.3.1 Horses

Thirty-eight horses met the inclusion criteria and were divided into a SEA group (26 horses) and a MEA group (12 horses). The study population consisted of 13 females and 25 males, aged from 2 to 22 years (mean ± SD: 10.79 ± 4.91 years), weighing from 297 to 642 kg (mean ± SD: 498.13 ± 78.61 kg). There was no difference in weight between groups (SEA 512.8 ± 85.79 kg, MEA 466.3

± 49.35 kg; p = 0.09); in contrast, a statistically significant difference was observed for age between groups (SEA 12.77 ± 3.8 years, MEA 6.5 ± 4.34 years; p < 0.0001). Even if a greater percentage of males was present in the MEA group (10 males, 2 females) compared to the SEA group (15 males, 11 females), there was no significant difference in sex distribution between groups (p = 0.158).

3.1.3.2 Intradermal Testing

At 30 min, all horses of both groups had a grade three or four reaction to intradermal injection of histamine (positive control); instead, no reaction to saline (negative control) was observed in 19 horses (12 SEA, 7 MEA), while a grade 1 reaction was detected in 19 patients (14 SEA, 5 MEA).

The frequencies of positive reactions to any allergen and the allergens inducing positive reactions in the SEA and MEA groups at every evaluation time are reported in Table 6. No significant difference was observed in the total number of horses having positive reactions to any allergen between groups. However, at 30 min and 4 h, significantly more horses in the SEA group had positive reactions compared to the MEA group (p < 0.0001); in contrast, at 24 h and 48 h, more horses in the MEA group had positive reactions compared to the SEA group (p < 0.0001). No significant difference was observed in the total grades of the reactions at any allergen between groups (p = 0.563). In total, there was no difference between groups in the number of allergens provoking positive reactions (p = 0.348), nor at 4 h (p = 0.751) and 48 h (p = 0.734). Instead, at 30 min, a significantly higher number of allergens gave positive reactions in the SEA group compared to the MEA group (p < 0.0001); at 24 h, more allergens induced positive reactions in the MEA group compared to the SEA group (p = 0.0002).

	Total Time	30 min	4 h	24 h	48 h
Positivities SEA	48.98%	98.34%	76.63%	15.09%	5.84%
Positivities MEA	50%	84.12%	47.64%	49.34%	18.90%
	ns	$p < 0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Allergens SEA	49.05%	98.73%	76.73%	15%	5.74%
Allergens MEA	50.33%	93.95%	77.50%	22.91%	6.24%
	ns	$p < 0.0001$	ns	$p = 0.0002$	ns

Table 6. Frequencies of positive reactions to any allergens and of the allergens inducing positive reactions at each evaluation time in the severe equine asthma (SEA) and mild-moderate equine asthma (MEA) groups.

The frequencies of positive reactions to each allergen at different evaluation times in SEA and MEA horses are reported in Table 7. At 30 min, 36/50 allergens (72%) induced a positive reaction in 100% of the SEA horses, while 28/50 allergens (56%) gave positive responses in 100% of the MEA horses. Reactions of moderate intensity (median grade 2) were induced by 45/50 allergens (90%) in the SEA group, and by 46/50 allergens (92%) in the MEA group.

At 4 h, 100% of the SEA horses had positive reactions to *Culex spp.*, *Aedes spp.*, *Tabanus spp.* and *Chrysops spp.*, while more than 90% of the SEA horses responded positively to dog epithelium, *Cupressus sempervirens* and *Dermatophagoides* mix. In the MEA group, 100% of the horses had positive reactions to *Aedes*, *Tabanus*, dog epithelium and *Dermatophagoides* mix; more than 90% responded positively to *Parietaria* mix, wheat flour, cornflour, rice flour, soy flour, *Plantago lanceolata*, *Penicillium* mix, *Culex* and *Lepidogliphus destructor*. In the SEA group, strong reactions (median grade 3) were provoked by *Aedes* and dog epithelium, and moderate reactions (median grade 2) were induced by *Tabanus spp.*, *Culex spp.*, *Chrysops spp.* and wheat flour. In the MEA group, strong reactions (median grade 3) were provoked by *Aedes spp.*, *Tabanus spp.* and dog epithelium, and moderate reactions (median grade 2) were

induced by *Alternaria alternata*, *Dermatophagoides* mix, *Candida albicans*, *Artemisia vulgaris*, *Plantago lanceolata*, *Robinia pseudoacacia*, wheat flour and soy flour.

At 24 h, *Aedes spp.* induced positive reactions in 100% of the SEA horses, and *Candida albicans* in 81% of the SEA horses; in the MEA group, *Candida albicans* provoked positive responses in 83% and *Tabanus spp.* in 82% of the horses. At 48 h, *Candida albicans* induced positive reactions in 85% of the SEA horses and 92% of the MEA horses. At 24 h and 48 h, *Candida albicans* induced strong reactions (median grade 3) in both the SEA and MEA groups.

No significant differences were observed between groups for the frequency of positive and negative reactions induced by each specific allergen at every evaluation time, with some exceptions: *Alternaria alternata* at 24 h ($p = 0.022$), barley flour at 24 h ($p = 0.03$) and *Penicillium* mix at 4 h ($p = 0.03$) gave significantly more positive reactions in MEA horses compared to the SEA horses, while *Culex spp.* at 30 min ($p = 0.016$) and milk proteins at 48 h ($p = 0.035$) induced significantly more positive reactions in SEA horses compared to the MEA horses. Moreover, no significant differences were observed between groups for the grade of the reactions provoked by each allergen at every evaluation time, except for *Alternaria alternata* at 4 h ($p = 0.014$) and 24 h ($p = 0.017$), *Candida albicans* at 4 h ($p = 0.04$), corn flour at 30 min ($p = 0.021$) and 4 h ($p = 0.046$), which provoked more intense reactions in the MEA group compared to the SEA group, and milk proteins at 48 h ($p = 0.035$), which induced stronger reactions in the SEA group compared to the MEA group.

ALLERGEN	30 min		4 h		24 h		48 h	
	SEA	MEA	SEA	MEA	SEA	MEA	SEA	MEA
Acarus siro	92%	100%	81%	75%	8%	0%	4%	0%
Aceraceae-Facaceae mix	96%	92%	65%	58%	0%	8%	0%	0%
Aedes spp.	67%	50%	100%	100%	100%	67%	33%	17%
Alternaria alternata	100%	100%	69%	83%	8%	42%	4%	17%
Artemisia vulgaris	100%	92%	85%	75%	12%	33%	0%	0%
Aspergillus fumigatus	100%	92%	81%	67%	12%	17%	4%	0%
Barley flour	100%	92%	80%	83%	4%	33%	4%	0%
Betullaceae mix	96%	92%	77%	67%	8%	8%	0%	0%
Candida albicans	96%	92%	85%	83%	81%	83%	85%	92%
Cat epithelium	100%	100%	85%	67%	8%	17%	4%	8%
Chrysops spp.	100%	100%	100%	60%	36%	0%	21%	0%
Cladosporium spp.	100%	92%	73%	75%	4%	8%	4%	0%
Compositae mix	100%	100%	88%	82%	17%	27%	0%	9%
Corn flour	100%	100%	65%	92%	15%	8%	4%	0%
Corylus avellana	100%	100%	65%	75%	4%	8%	0%	0%
Culex spp.	100%	64%	100%	91%	35%	45%	0%	9%
Culicoides spp.	100%	82%	82%	73%	29%	18%	6%	18%
Cupressus sempervirens	100%	92%	92%	67%	12%	8%	0%	0%
Cynodon dactylon	96%	92%	69%	67%	8%	0%	0%	0%
Dermatophagoides mix	100%	100%	92%	100%	19%	42%	4%	0%
Dog epithelium	100%	100%	96%	100%	31%	50%	19%	25%
Feathers mix	100%	100%	67%	43%	33%	14%	0%	0%
Fibers mix	100%	100%	67%	57%	10%	0%	0%	0%
Gliciphagus domesticus	100%	90%	65%	60%	4%	10%	0%	0%
Helmintosporium sativum	96%	100%	73%	83%	8%	25%	0%	0%
Holcus lanatus	100%	100%	65%	75%	10%	8%	0%	0%
Lepidogliphus destructor	100%	90%	80%	90%	15%	20%	4%	0%
Lolium perenne	100%	100%	73%	75%	60%	25%	0%	8%
Milk proteins	100%	100%	60%	75%	33%	25%	0%	0%
Musca domestica	67%	50%	67%	33%	8%	0%	60%	0%
Mycophytes mix	100%	100%	79%	82%	0%	27%	0%	0%
Olea europea	96%	92%	77%	67%	4%	8%	0%	0%

Ostrya virginiana	96%	92%	65%	67%	19%	17%	0%	0%
Parietaria mix	100%	100%	85%	92%	15%	17%	0%	17%
Penicillium mix	96%	100%	54%	92%	8%	25%	4%	8%
Pinus silvestris	96%	100%	67%	82%	17%	27%	0%	0%
Plantago lanceolata	100%	100%	73%	92%	19%	25%	4%	0%
Poa pratensis	100%	100%	58%	67%	8%	17%	0%	8%
Poaceae mix	96%	100%	81%	75%	4%	17%	0%	0%
Populus nigra	100%	100%	80%	88%	20%	25%	20%	0%
Pyrethrum spp.	100%	86%	67%	71%	33%	0%	0%	0%
Rice flour	100%	100%	88%	92%	12%	42%	0%	8%
Robinia pseudoacacia	100%	88%	60%	75%	20%	0%	0%	0%
Rye flour	100%	88%	60%	75%	60%	13%	40%	13%
Soy flour	100%	100%	85%	92%	12%	25%	4%	8%
Tabanus spp.	100%	73%	100%	100%	47%	82%	29%	27%
Trichoepidermophytes mix	88%	100%	75%	70%	10%	10%	5%	0%
Tyrophagus putrescentiae	100%	91%	75%	73%	17%	18%	8%	0%
Ulmus campestris	100%	100%	44%	25%	11%	0%	0%	0%
Wheat flour	100%	100%	77%	92%	15%	25%	0%	0%

Table 7. Frequencies of positive reactions to each allergen at different evaluation times in the SEA and MEA groups.

3.1.4 Discussion

To the authors' knowledge, this is the first study comparing results of intradermal testing between horses affected by severe and mild-moderate equine asthma, even though no healthy horses were included in the present study. The SEA and MEA groups were weight- and sex-matched; however, the MEA horses were significantly younger compared to the SEA horses. In fact, SEA usually affects adult horses over seven years of age, while MEA can affect horses of any age, but it is more commonly reported in young horses (Couetil *et al.* 2016).

SEA horses showed a higher probability to develop positive reactions to an allergen at 30 min and 4 h, compared to the MEA horses; this result is in accordance with a previous study, in which SEA horses developed more positive reactions at 30 min and 4 h, compared to healthy horses (Halliwell *et al.* 1979). This suggests that a type-I hypersensitivity response may be involved in the pathogenesis of the SEA, based on IgE-mediated mast cell degranulation. Interestingly, in our study, at 24 and 48 h, the MEA horses had more positive reactions compared to the SEA horses: this could suggest that delayed type-IV hypersensitivity could be more implicated in the pathogenesis of mild forms of EA compared to more severe phenotypes. This hypothesis is further supported by the fact that the SEA horses had positive reactions to a greater number of allergens at 30 min, compared to the MEA horses, while MEA horses responded to more allergens at 24 h compared to the SEA horses.

However, in total time, there was no difference nor in the frequency of positivity nor in the number of allergens provoking positive reactions between the SEA and MEA horses. Previous studies reported that SEA horses develop overall more positive reactions compared to healthy horses (Evans *et al.* 1992, Wong *et al.* 2005) and respond to a greater number of allergens (Jose-Cunilleras *et al.* 2001, Wong *et al.* 2005). In contrast, in one study, more positive reactions were observed among horses without atopy, compared to the SEA horses: however, most environmental allergens used in that investigation included grasses, weeds, trees and insects, which are mostly present outdoors, where non-atopic horses had been kept for prolonged periods; therefore, non-atopic horses had been previously more exposed and sensitized to those environmental allergens compared to the SEA group (Lorch *et al.* 2001). The authors of another study comparing different allergy tests in asthmatic horses observed no significant difference between the IDT results of the healthy and

the asthmatic group: however, it included a small number of patients, and only type I hypersensitivity responses were evaluated (Klier *et al.* 2021). In our study, no difference in the grades of the reactions was observed between the SEA and MEA groups; previous studies showed that SEA horses were likely to develop not only a greater number of reactions, but also of a stronger intensity compared to healthy horses, suggesting that asthmatic horses have an increased sensitivity to specific antigens (Jose-Cunilleras *et al.* 2001, Tilley *et al.* 2010).

In the present study, at 30 min, all the SEA horses responded to 72% of the allergens, while all the MEA horses responded to 56% of the allergens with a moderate intensity grade, suggesting that asthmatic horses have a tendency to develop immediate hypersensitivity responses to most common allergens. However, an evaluation of the IDT reactions at 30 min only allows to identify the tendency of the patient to overreact to common allergens, without providing useful information about the specific antigens that could be considered as triggers of the allergic response. An evaluation of the response at 4 h seems to be more useful in identifying specific allergens in SEA horses. In this experiment it was found to correspond to the late-phase of type I hypersensitivity, which is likely involved in the pathogenesis of allergic reactions and is more allergen-selective compared to the immediate type (30 min).

Previous studies also suggested that the observation time point at 4 h is the most significative when performing an IDT (Lorch *et al.* 2001, Wong *et al.* 2005), with optimal sensitivity and specificity (Wong *et al.* 2005). In our study, positive reactions at 24 h significantly decreased in the SEA group, suggesting less involvement of type-IV hypersensitivity, compared to type I; however, in the MEA group, positivities at 24 h were slightly more than those at 4 h, indicating that delayed response could be implicated in mild forms of asthma

and, therefore, this evaluation time should be taken into consideration for the identification of the most allergenic antigens in MEA horses. A previous study suggested that observation of IDT responses at 24 h is pivotal in SEA horses, since some positive delayed reactions could not be preceded by a type I response, even if it rarely occurs (Jose-Cunilleras *et al.* 2001). The present study is the first one evaluating IDT reactions also at 48 h; however, positive reactions were rarely observed and the only allergen provoking intense responses was *Candida albicans* in both groups.

Nevertheless, the role of *Candida albicans* in the IDT is controversial: in fact, this allergen commonly induces delayed reactions also in healthy non-atopic horses (Lorch *et al.* 2001) and, in human medicine, it has been used to evaluate the overall capacity of a subject to develop delayed hypersensitivity responses (Meizel *et al.* 1984). Therefore, positive reactions to *Candida albicans* at 24 or 48 h should be considered of scarce clinical significance; *Candida albicans* could rather be used as a positive control for delayed type-IV hypersensitivity reactions, as well as histamine is utilized for immediate responses. In conclusion, it is reasonable that a complete evaluation of IDT results should include observation of the responses at 30 min, 4 h and 24 h: a reading at 30 min allows to evaluate whether the patient is hyperreactive to common allergens. At 4 h, it is possible to identify specific allergens responsible for type-I hypersensitivity reactions; at 24 h, eventual delayed responses (which may or may not be preceded by an immediate or late-phase reaction) could be observed. Finally, an evaluation at 48 h in the present study did not provide any adjunctive relevant information.

Among our patients, at 4 h, 100% of the SEA horses developed positive reactions to *Culex spp.*, *Aedes spp.*, *Tabanus spp.* and *Chrysops spp.*, suggesting that hypersensitivity to the class of allergens "insects" could be common among asthmatic horses; also 100% of the MEA horses reacted to *Aedes spp.*

and *Tabanus spp.*, while more than 90% responded to *Culex spp. Aedes spp.* induced strong reactions in both the SEA and MEA groups, and also provoked delayed reactions in 100% of the SEA horses at 24 h; this insect, commonly known as "tiger mosquito", has spread worldwide during the past few decades (Goubert *et al.* 2016) and our results suggest that its role as an allergen deserves to be taken much into consideration among EA patients.

Tabanus spp. provoked moderate-intensity responses in the SEA group and strong reactions in the MEA group; however, the role of this allergen in IDT has been questioned since it seems to induce too many positive reactions also among healthy non-atopic horses (Lebis *et al.* 2003, Wong *et al.* 2005). Interestingly, a correlation between insect bite hypersensitivity (IBH) and airways hyperreactivity has been recently demonstrated (Lanz *et al.* 2017): in fact, horses affected by IBH seem to have a higher risk of being concurrently asthmatic, and vice versa (Kehrli *et al.* 2015). This suggests that multiple hypersensitivities could share a common immunogenetic background or route of sensitization; concurrent different hypersensitivity manifestations are also observed among human patients affected by asthma and atopic dermatitis (Spergel and Paller 2003), dogs with allergic dermatitis, conjunctivitis and rhinitis (Olivry *et al.* 2001), and cats with feline asthma and pruritic dermatosis (Diesel 2017). Considering the high allergenic potential that insect antigens seem to have in horses, protecting them from insect bites becomes pivotal in the control of allergic manifestations, including asthma.

In addition to insects, the present study identified other relevant allergens which induced moderate-strong reactions in many of our patients of both the SEA and MEA groups, including dog epithelium and *Dermatophagoides* mix. In addition, some pollens frequently provoked positive reactions: in the SEA group *Cupressus sempervirens*, while in the MEA group *Parietaria* mix, *Artemisia vulgaris*, *Robinia pseudoacacia* and *Plantago lanceolata*. In MEA horses, a high

hypersensitivity to different food flours was detected too. Previous investigations showed a high allergenicity of *Aspergillus fumigatus*, *Alternaria alternata*, *Tyrophagus putrescentiae*, *Micropolyspora faeni*, *Saccharopolyspora rectivirgula*, *Aspergillus terreus*, *Eurotium amstelodami*, *Geotrichum candidum*, *Wallemia sebi* and *Dermatophagoides spp.* in asthmatic horses (McPherson *et al.* 1979, Derksen *et al.* 1988, Halliwell *et al.* 1993, Schmallenbach *et al.* 1998, Eder *et al.* 2000, Jose-Cunilleras *et al.* 2001, Lebis *et al.* 2003, Künzle *et al.* 2007, Niedzwiedz *et al.* 2015, White *et al.* 2019).

The present study confirms a great reactivity to *Dermatophagoides spp.*, formerly reported as one of the allergens inducing the strongest reactions (Lebis *et al.* 2003) in both the SEA and MEA horses. Moreover, a moderate hypersensitivity to *Alternaria alternata* was shown in our study among MEA horses: this allergen has atmospheric peaks during the seasons in which the majority of exacerbations of SEA occur and, in a study about skin test using 16 relevant aeroallergens, it induced the largest wheal diameter in SEA horses (Tilley *et al.* 2010). In human medicine, it is regarded as one of the most clinically important moulds worldwide, playing a major role in the pathogenesis of allergic asthma: sensitization against *Alternaria alternata* is considered an important risk factor for greater asthma severity and acute asthma exacerbations of affected patients (Bush and Prochnau 2004, Pulimood *et al.* 2007, Lehmann *et al.* 2017).

The allergenic role of *Aspergillus fumigatus* and *Tyrophagus putrescentiae* has been questioned; in fact, in different studies, they have shown to induce positive reactions also in healthy horses (McGorum *et al.* 1993, Jose-Cunilleras *et al.* 2001, Tilley *et al.* 2010). However, in our study they did not appear to be particularly allergenic in the SEA or the MEA horses. The different results observed between our study and previous analogous investigations could be explained by the fact that they were performed in different geographic areas,

characterized by different climates and concentrations of airborne allergens. Moreover, to date, the studies about IDT in SEA horses lack standardization and IDT reactions are generally subjectively evaluated: some studies proposed to measure the diameter of the wheals and to establish cut-off values to distinguish positive and negative results (Lebs *et al.* 2003, Wong *et al.* 2005, Tilley *et al.* 2010). Though the warmth of the wheals could be estimated by means of thermography, the assessment of turgidity and achiness relies on the operator's sensitivity and cannot be objectively measured. The lack of objective measurements represents one of the main limitations of IDT. Furthermore, in our study, all IDT responses of grade ≥1 were considered as positive reactions, while some previous studies only considered reactions of grade ≥1.5 (Tallarico and Tallarico 1998) or even ≥3 (Wong *et al.* 2005). In the present work, it is reasonable that subclinical hypersensitivities were also included; therefore, the evaluation of the intensity of the reactions by assignment of a score was essential in order to interpret positive results.

Since positivity is not specific for asthma but only identifies atopic status, confirmation of the clinical significance of the identified allergens in the pathogenesis of asthma should be pursued. To this aim, allergen avoidance is indicated (Tillet *et al.* 2012) to observe the eventual remission of symptoms and prevention of exacerbations. However, avoidance strategies are not always feasible, since some aeroallergens are ubiquitous—in this case, the most accurate evaluation of the diagnostic value of IDT may be to compare these tests with the gold standard of induction of clinical disease by a provocative inhalation challenge using the identified allergens, which would provide more substantial evidence of their role as causative antigens (Jose-Cunilleras *et al.* 2001, Wong *et al.* 2005).

When the confirmation of the pathogenic role of specific allergens is obtained, selected patients may be treated with ASIT (Jose-Cunilleras *et al.* 2001), which

is defined as the administration of gradually increasing quantities of an allergen extract to an allergic subject to ameliorate the symptoms associated with subsequent exposure to the causative allergen (Bousquet *et al.* 1998). Today, immunotherapy is, according to systematic reviews and meta-analyses, an accepted treatment for human allergic asthma, helpful in alleviating symptoms and reducing the needing for medication (Kim *et al.* 2013, Erekosima *et al.* 2014). There is also some evidence for the successful treatment of feline asthma with immunotherapy, but studies to date are limited to experimentally sensitized cats (Reinero *et al.* 2011). In equine medicine, there are only few studies about immunotherapy for the treatment of asthma (Loewenstein *et al.* 2009), which were not controlled and included only small numbers of horses; therefore, it has not been widely used to date (Mueller *et al.* 2018). However, based on the similarities with human asthma (Lange-Consiglio *et al.* 2019, Bullone and Lavoie 2020), it is reasonable that ASIT could also be effective for the treatment of EA and future investigations should be addressed to verify this hypothesis.

3.1.5 Conclusions

In conclusion, mostly type-I hypersensitivity response seems to be involved in the pathogenesis of SEA, and the evaluation of IDT responses at 4 h is essential for the identification of specific antigens responsible for allergic reactions. Insects, *Dermatophagoides spp.* and dog epithelium, when induced in the MEA and SEA horses of this study, provided the most significant hypersensitivity responses and could therefore be considered as the main allergenic antigens in our geographic area. Future investigations are required to evaluate clinical significance of IDT and the efficacy of ASIT for the treatment of EA patients.

3.2 THE ROLE OF THORACIC ULTRASONOGRAPHY AND AIRWAY ENDOSCOPY IN THE DIAGNOSIS OF EQUINE ASTHMA AND EXERCISE-INDUCED PULMONARY HEMORRHAGE

The present study has already been published in the following manuscript:

Lo Feudo CM, Stucchi L, Alberti E, Stancari G, Conturba B, Zucca E, Ferrucci F (2021) The role of thoracic ultrasonography and airway endoscopy in the diagnosis of equine asthma and exercise-induced pulmonary hemorrhage. Vet Sci, 8:276.

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Ethical statement: For the present study, no ethical approval was required, as all the procedures were performed on clinical horses for diagnostic purposes and included informed owner consent for the use of clinical data.

3.2.1 Introduction

Equine asthma (EA) is a chronic and recurrent respiratory syndrome characterized by airflow obstruction, mucus hypersecretion and airway hyperreactivity (Bullone and Lavoie 2020). Although some clinical presentations, such as chronic cough and poor performance, are common to asthmatic horses of all severities (Couetil *et al.* 2020), EA can be classified as mild-moderate (MEA) or severe (SEA) (Bullone and Lavoie 2020); while SEA typically affects adult horses over 7 years of age (Hotchkiss *et al.* 2007), MEA can affect horses of any age but is more commonly reported in young horses (Couetil *et al.* 2016). A diagnosis of EA can be reached by collection of history, clinical examination, airway endoscopy and cytology of the bronchoalveolar lavage fluid (BALf) and/or tracheal wash (TW) (Couetil *et al.* 2020). Some authors suggest that MEA could be associated with exercise-induced pulmonary hemorrhage (EIPH) (Newton and Wood 2002), while other studies observed no significant correlation between these disorders (Chapman *et al.* 2000, Sanchez *et al.* 2005, Hinchcliff *et al.* 2010, Da Silva *et al.* 2017). Therefore, this represents a controversial topic. EIPH is a common disorder in racehorses and other sport horses subjected to strenuous exercise, defined as the detection of blood in the trachea at endoscopic examination after exercise (Hinchcliff *et al.* 2015) or the presence of hemosiderin in alveolar macrophages in BALf (Doucet and Viel 2002a). Some studies suggest that, in EIPH-affected horses, imaging may aid to diagnosis. Thoracic radiographs could show the presence of densities in the caudodorsal areas of the lungs; however, in many EIPH-affected horses only minimal radiographic abnormalities are observed and non-EIPH horses could have marked abnormalities too (O'Callaghan and Goulden 1982, Pascoe *et al.* 1983, O'Callaghan *et al.* 1987a, Doucet and Viel 2002b). In one study, thoracic ultrasonography proved to be highly sensitive in detecting the presence of fluid accumulation in the caudodorsal lung fields

in EIPH-affected horses; however, it has a low specificity, not allowing to distinguish between the presence of blood or inflammatory fluid (Ferrucci *et al.* 2009). In EA, imaging is not considered part of the minimum diagnostic protocol (Couetil *et al.* 2020). Radiographic abnormalities do not seem to be associated with BALf cytology or pulmonary function in MEA horses (Mazan *et al.* 2005). Endobronchial ultrasound seems to be promising in identifying airway smooth muscle remodeling in SEA-affected horses (Bullone *et al.* 2015a), but its use is still limited to research purposes. Only one preliminary study describes the use of thoracic ultrasonography for the diagnosis of EA, reporting that SEA-affected horses show more intense ultrasound changes compared to healthy horses and to MEA-affected horses; however, it only included a small number of patients (Siwinska *et al.* 2019).

Airway endoscopy is pivotal for the diagnosis of both EA and EIPH; in EA, the presence of mucus in the trachea is likely to predict airway inflammation (Rossi *et al.* 2018) and, in particular, the detection of mucus grade ≥2/5 in racehorses or ≥3/5 in other sport or pleasure horses may be sufficient to diagnose EA (Couetil *et al.* 2016). The presence of pharyngeal lymphoid hyperplasia (PLH), visible at endoscopy, is associated with stabling and exposure to dust and other antigens, which are also associated to EA (Holcombe *et al.* 2000). Moreover, in Thoroughbreds in training, an association between coughing, tracheal mucus accumulation and PLH has been observed (Couetil and Hawkins 2013a). However, no direct relationship between PLH and EA has been demonstrated yet (Holcombe *et al.* 2000). Although it is reported that EA-affected horses may show thickening and blunting of the tracheal bifurcation (Robinson 2003), the results of a study about tracheal septum scoring revealed no differences between asthmatic and healthy horses (Koch *et al.* 2007); therefore, the clinical relevance of this finding remains unclear. While the detection of blood in the airways after exercise is considered

the gold standard for the diagnosis of EIPH (Hinchcliff *et al.* 2015), contrasting results have been obtained regarding the association between EIPH and tracheal mucus accumulation (Chapman *et al.* 2000, Newton and Wood 2002, Hinchcliff *et al.* 2010).

Since no consistent and solid results are reported about ultrasonographic and endoscopic findings in asthmatic and EIPH-affected horses, the present study aims to evaluate whether a complete ultrasonographic and endoscopic assessment could aid in the diagnosis of SEA, MEA and concomitant MEA and EIPH in horses.

3.2.2 Materials and Methods

3.2.2.1 Horses

For this retrospective study, the clinical records of the horses affected by SEA or MEA hospitalized at the Equine Unit of the Veterinary Teaching Hospital of the University of Milan (Italy) between 2001 and 2020 were reviewed. The patients that underwent a complete diagnostic evaluation, including clinical history, general physical examination, thoracic ultrasonography, airway endoscopy and BALF and/or TW cytology, were included in the study and divided into three groups: SEA group, MEA group and MEA + EIPH group. SEA horses were selected on the basis of history of exercise intolerance, dyspnea and coughing, normal rectal temperature and neutrophils count > 5% in BALF or >20% in TW cytology (Malikides *et al.* 2003). For MEA horses, inclusion criteria were history of poor performance and occasional coughing, eupnea at rest, normal rectal temperature and BALF cytology consisting of neutrophils > 5%, and/or mast cells > 2% and/or eosinophils > 1% (Lo Feudo *et al.* 2021a). Among the MEA horses, whenever hemosiderophages were observed in the BALF cytology and represented ≥10% of total alveolar

macrophage count, the patients were considered as EIPH positive (Ferrucci *et al.* 2009) and therefore included in the MEA + EIPH group.

3.2.2.2 Thoracic Ultrasonography

A thoracic ultrasonographic examination was performed on every horse before sedation and endoscopy. The ultrasound machine (Technos MPX, Esaote, Firenze, Italy; MyLab ClassC, Esaote, Firenze, Italy) was used with a 3.5 to 5.0 MHz convex transducer. In order to obtain high quality images, the image parameters were adjusted to every single patient. To increase acoustic coupling between the skin and the transducer, the skin was cleansed with alcohol and ultrasound gel was applied on the transducer. The ultrasound examination was performed bilaterally, from the 3rd intercostal space (ICS) to the 17th ICS, moving the transducer in a dorsoventral direction. The pleural surface was observed and eventual pathological alterations, such as comet-tail artifacts and wider focal lesions, were detected. To assign precise scores to the ultrasonographic findings, the lung area was divided into cranial, middle and caudal (Siwinska *et al.* 2019): the cranial area included 3rd–7th ICSs, the middle area included 8th–12th ICSs and the caudal area included 13th–17th ICSs. A score was assigned to each area, on the left and on the right side, on the basis of the presence of comet-tail artifacts, using a specifically developed scoring system based on the one created by Siwinska and colleagues (2019) (Table 8). The scores assigned to each area on one side were summed to those assigned to the same areas on the opposite side of the thorax. In this way, scores for cranial, middle and caudal areas were obtained. Then, the scores for each area were summed to calculate the total lung ultrasonographic score. Moreover, the number of focal lesions in each area was recorded. Similarly to the ultrasonographic scores, the numbers of focal lesions on both sides were

summed to obtain the number for cranial, middle and caudal areas, and then the total number of focal lesions.

Score	Ultrasonographic Findings
0	Normal pleural surface, no comet-tail artifacts
1	Single comet-tail artifacts in 1 intercostal space
2	Numerous comet-tail artifacts in 1–2 intercostal spaces or single comet-tail artifacts in 2–3 intercostal spaces
3	Numerous comet-tail artifacts in 3–4 intercostal spaces
4	Numerous comet-tail artifacts in 5 intercostal spaces

Table 8. Thoracic ultrasonography scoring system (Siwinska *et al.* 2019).

3.2.2.3 Airway Endoscopy, BAL and TW

To perform airway endoscopy and collect BALf or TW samples, a flexible videoendoscope (EC-530WL-P, Fujifilm, Tokyo, Japan) was used. The horses were previously sedated with detomidine hydrochloride (0.01 mg/kg IV; Domosedan; Vetoquinol, Italy) and restrained with a twitch. The endoscope was passed through the nasal passages and the upper and lower tracts of the respiratory system were examined. Endoscopic findings were graded using three scoring systems: a 0–4 score for pharyngeal lymphoid hyperplasia (Burrell 1985), a 0–5 score for tracheal mucus accumulation (Gerber *et al.* 2004) and a 0–4 score for edema of the tracheal bifurcation (Ferrucci *et al.* 2008). After the visualization of upper and lower airways, BALf and TW samples were collected. Whenever the SEA horses presented severe dyspnea at the moment of the examination, only TW was performed. To perform the BAL, 60 mL of a 0.5% lidocaine hydrochloride solution was sprayed at the level of the tracheal bifurcation to inhibit coughing reflex. Then, the endoscope was passed into the bronchial tree until it was wedged firmly within a segmental bronchus based on the diameter of the endoscope, selected on the basis of the ultrasonographic findings. Here, a 300 mL pre-warmed sterile saline 0.9% was instilled and the fluid immediately aspirated (Pirie 2014). The BALf sample was then stored in sterile ethylenediaminetetraacetic acid (EDTA) tubes and

processed within 90 min. To perform the cytological examination, a few drops of pooled BALf were cytocentrifugated (ROTOFIX 32, Hettich Cyto-System, Germany) at 26× g for 5 min. The slides were air dried, stained with May-Grünwald Giemsa and Perl's Prussian blue and observed under a light microscope at 400× and 1000× for 400-cell leukocyte differential count (Stucchi *et al.* 2020). To perform the TW, a 60 mL pre-warmed sterile saline 0.9% was flushed into the intrathoracic portion of the tracheal lumen, re-aspirated and transferred into sterile plain tubes for microbiological evaluation (Ferrucci *et al.* 2008). To this aim, 10 microliters of pooled TW were cultured on blood agar plates 5% and incubated at 37°C; after 48 h, bacterial species identification and UFC/mL count were performed (Ferrucci *et al.* 2013). In the SEA-affected patients that did not undergo the BAL, a cytological examination of the TW was performed.

3.2.2.4 Statistical Analysis

The data of the three groups (SEA, MEA, MEA + EIPH) were analyzed using descriptive statistics and evaluated for normality by means of the Shapiro–Wilk test. As data were not normally distributed in every group, non-parametric statistics was applied. The Kruskal–Wallis test, with Dunn's multiple comparison test, was used to compare age, BALf leukocyte differential count, endoscopic scores, ultrasonographic scores (total lung area, cranial area, middle area and caudal area) and number of lung focal lesions (total and in every lung field) between groups. The differences between the ultrasonographic scores and the number of focal lesions on the left and right sides of the thorax were evaluated by means of the Mann–Whitney test. The association between age and BALf leukocyte differential count, endoscopic scores, ultrasonographic scores and number of lung focal lesions was evaluated using the Spearman correlation. The Spearman correlation was also

used to evaluate the relationship between BALf leukocyte differential count and endoscopic scores, ultrasonographic scores and number of lung focal lesions. The Kruskal–Wallis test was used to evaluate the association of the endoscopic scores with the ultrasonographic scores and the number of lung focal lesions. The frequencies of positive results in the microbiological examination were compared between groups by means of Fisher's exact test. The relationship between microbiological findings and age, BALf leukocyte differential cell count, endoscopic scores, ultrasonographic scores and number of lung focal lesions was evaluated using the Mann–Whitney test. Data are presented as median and interquartile ranges (IQR). Statistical significance was set at $p < 0.05$. Data were analyzed using a commercially available statistical software package (GraphPad Prism 9.1.0 for MacOS; GraphPad Software, San Diego, CA, USA).

3.2.3. Results

3.2.3.1. Horses

A total of 303 horses met the inclusion criteria and were divided into SEA group (62 horses), MEA group (118 horses) and MEA+EIPH group (123 horses). The study population consisted of 83 mares, 80 geldings and 140 stallions, aged from 2 to 28 years (median 5, IQR 3–9) and weighing from 272 to 680 kg (median 464 kg, IQR 434–507 kg). The most represented breeds were Standardbreds (180 horses), followed by Warmbloods (58 horses), Thoroughbreds (17 horses) and Quarter Horses (14 horses); the remaining 34 horses belonged to different mixed breeds. A significant difference was observed for age between the SEA group and the MEA group (SEA median 13 years, IQR 10–17 years; MEA median 4 years, IQR 3–8 years; $p < 0.0001$) and between the SEA group and the MEA+EIPH group (MEA+EIPH median 4

years, IQR 3–5 years; $p < 0.0001$), while no difference was detected between the MEA group and the MEA+EIPH group.

3.2.3.2. Cytological Findings

Since 26 SEA horses were dyspnoic at the time of the examination, BAL and its cytology were performed in 277 horses (91.42%). Leukocyte differential cell counts of the BALfs of the SEA, MEA and MEA + EIPH horses are shown in Table 9.

	SEA	MEA	MEA + EIPH
Macrophages	32.95% ± 13.18%	45.33% ± 10.17%	45.67% ± 8.82%
Lymphocytes	18.7% ± 10.68%	33.38% ± 12.78%	33.14% ± 10.84%
Neutrophils	44% ± 19.16%	14.44% ± 10.1%	13.4% ± 7.98%
Eosinophils	1.24% ± 2.24%	2.23% ± 3.63%	2.95% ± 4.32%
Mast cells	3.11% ± 1.85%	4.62% ± 2.47%	4.84% ± 2.39%
Hemosiderophages	0.62% ± 1.8%	2.49% ± 3.2%	24.26% ± 13.59%

Table 9. Leukocyte differential counts, expressed as mean ± standard deviation, of the bronchoalveolar lavage fluids of the SEA, MEA and MEA + EIPH horses. Note: the percentages of the hemosiderophages are calculated out of the total of macrophages.

When compared to the MEA and the MEA + EIPH groups, the SEA group had significantly greater percentages of neutrophils ($p < 0.0001$) and lower counts of macrophages ($p < 0.0001$), lymphocytes ($p < 0.0001$) and mast cells (SEA vs. MEA: $p = 0.0035$; SEA vs. MEA + EIPH: $p = 0.0002$). Moreover, the percentages of eosinophils were lower in the SEA group compared to the MEA + EIPH group ($p = 0.0159$). Significantly more hemosiderophages were observed in the MEA+EIPH group compared to the MEA and the SEA groups ($p < 0.0001$). No differences were observed between the MEA and the MEA + EIPH horses concerning the counts of macrophages, lymphocytes, neutrophils, eosinophils

and mast cells, nor between the SEA and the MEA horses concerning the counts of eosinophils and hemosiderophages.

Age was associated to the percentages of all classes of BALf leukocytes ($p < 0.0001$). In particular, a positive correlation was observed with neutrophils ($r = 0.5878$), while an inverse correlation was detected with macrophages ($r = −0.3394$), lymphocytes ($r = −0.4260$), eosinophils ($r = −0.2667$), mast cells ($r = −0.229$) and hemosiderophages ($r = −0.3969$).

3.2.3.3 Microbiological Findings

A TW and its microbiological examination were performed in all horses. The bacterial culture was positive in 97 horses (32.01%) and negative in 206 horses (67.99%); in particular, the most frequently isolated bacteria were *Streptococcus* spp. (61 horses), *Pasteurella*/*Actinobacillus* spp. (16 horses) and *Klebsiella* spp. (7 horses). In the SEA group, positive results were observed in 20.97% of the horses, 36.44% in the MEA group and 33.33% in the MEA+EIPH group.

The frequencies of positive results in the MEA groups were significantly higher compared to the SEA group ($p = 0.0418$), while no significant differences were detected between the MEA + EIPH group and the SEA nor the MEA groups. The median age of horses having positive bacterial cultures was 4 (IQR 3–7) years old, while that of those having negative results was 5 (IQR 3–10) years old; horses with positive results were significantly younger than those having negative results ($p = 0.0156$). There was no association between the results of microbiology and BALf cytology.

3.2.3.4 Ultrasonographic Findings

At ultrasonographic examination, the pleural surface was clearly identifiable, and scores could be assigned to every patient. Twenty-five horses (8.25%) showed a normal pleural surface, visible as a hyperechogenic line with

underlying reverberation artifacts, while 278 horses (91.75%) presented some alterations. In particular, comet-tail artifacts were observed in 278 horses (91.75%), while focal lesions were present in 93 horses (30.69%). In total, 159 focal lesions were observed; of these, 86 were on the right side (54.09%), while 73 were on the left side (45.91%). However, no significant difference in the ultrasonographic score and number of focal lesions was detected between the two sides of the thorax. The ultrasonographic scores of the SEA, MEA and MEA+EIPH groups are shown in Figure 3. The median number of lung focal lesions was 0 in every group, in each area and in total. The IQR ranged from 0 to 1 in the cranial area and in total in the SEA and the MEA + EIPH groups; instead, it was 0–0 in the middle and caudal areas in every group, and in the cranial area and in total in the MEA group.

The MEA + EIPH group had significantly higher ultrasonographic scores of the cranial and caudal lung areas when compared to the MEA group ($p < 0.0001$), while no differences were detected between the SEA and the MEA nor the MEA + EIPH groups. In the middle area, no differences were detected between any groups. The MEA group showed a significantly lower total lung ultrasonographic score compared to the MEA + EIPH group ($p < 0.0001$) and to the SEA group ($p = 0.0287$); instead, no differences were observed between the SEA and the MEA + EIPH groups. The MEA + EIPH group showed significantly more focal lesions in the cranial area of the lung compared to the MEA group ($p = 0.0019$), while no difference was observed between the SEA and the MEA nor the MEA + EIPH groups; in the middle and the caudal areas, no differences were detected between any groups. The MEA group presented significantly less lung focal lesions compared to the MEA+EIPH group ($p = 0.0019$) and to the SEA group ($p = 0.0475$), while no differences were observed between the SEA and the MEA + EIPH groups.

Age was not significantly correlated with the ultrasonographic scores nor with the number of lung focal lesions in any areas and in total.

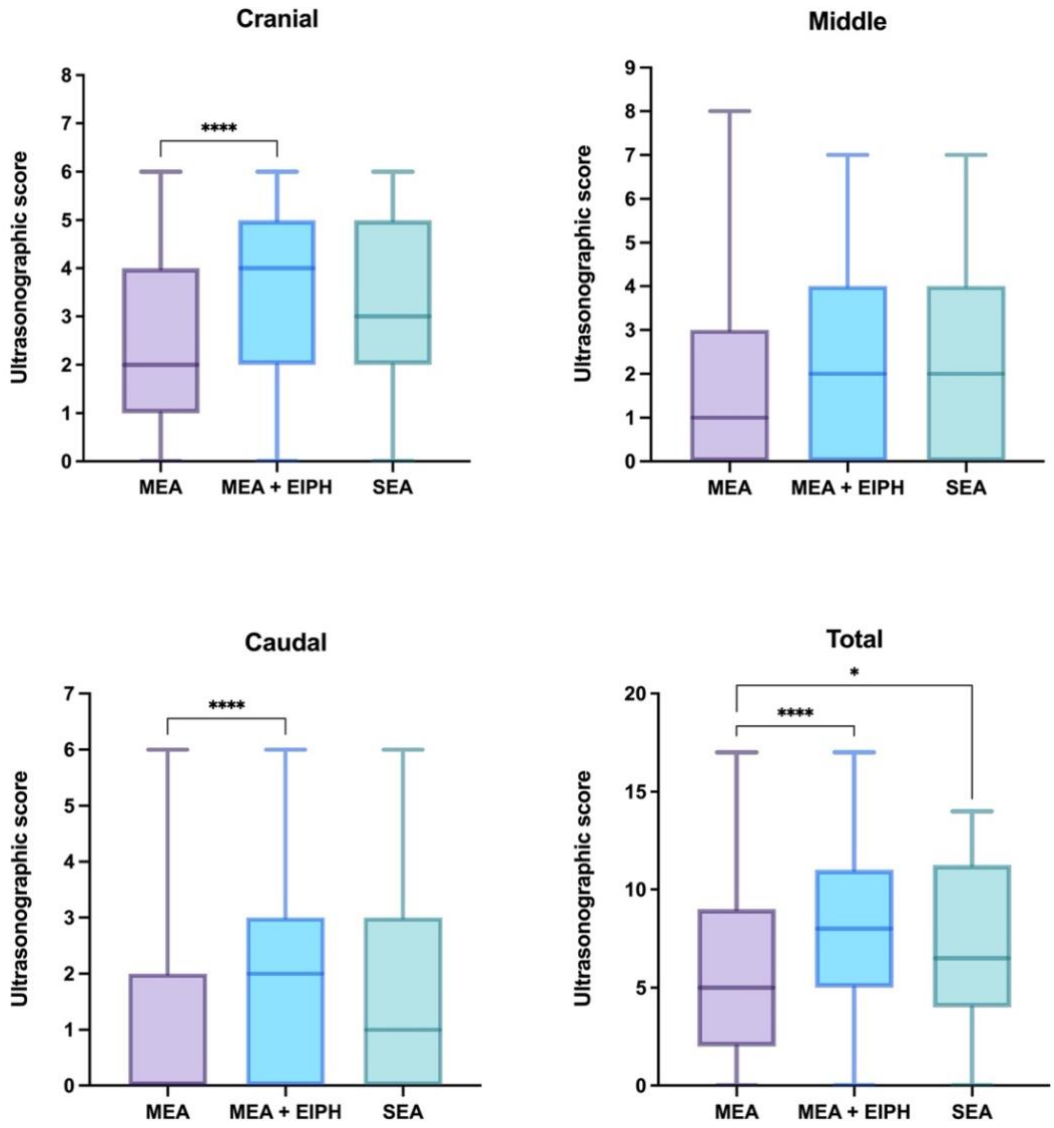

Figure 3. Box plot showing ultrasonographic scores of the cranial, middle and caudal lung fields, and the total ultrasonographic scores in the SEA, MEA and MEA + EIPH groups. The statistical significance is shown as * ($p < 0.05$) and **** ($p < 0.0001$).

3.2.3.5. Endoscopic Findings

Airways endoscopy was performed in all horses and endoscopic scores (pharyngeal lymphoid hyperplasia, tracheal mucus, tracheal bifurcation) were

assigned to every patient. The endoscopic scores of the SEA, MEA and MEA + EIPH groups are shown in Figure 4.

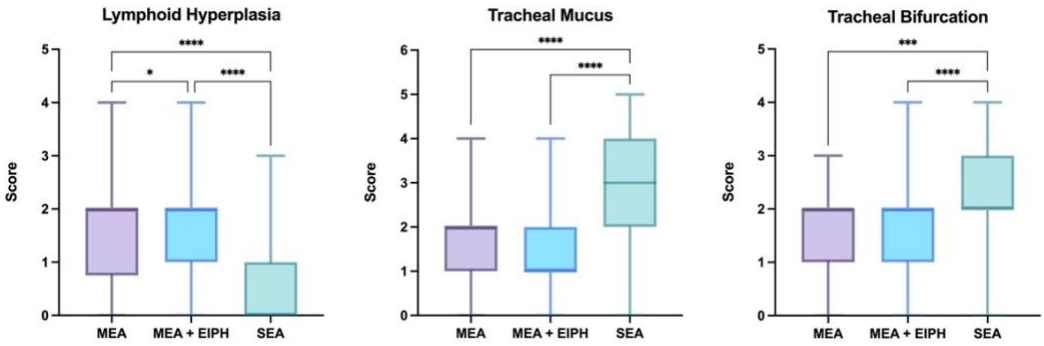

Figure 4. Box plot showing endoscopic scores of pharyngeal lymphoid hyperplasia, tracheal mucus and tracheal bifurcation in the SEA, MEA and MEA + EIPH groups. The statistical significance is shown as * ($p < 0.05$), *** ($p < 0.001$), **** ($p < 0.0001$).

The MEA and MEA + EIPH groups had significantly higher scores of pharyngeal lymphoid hyperplasia compared to the SEA group ($p < 0.0001$); moreover, the MEA + EIPH group had slightly higher PLH scores compared to the MEA group ($p = 0.0451$). The SEA group had significantly higher tracheal mucus scores compared to the MEA and MEA + EIPH groups ($p < 0.0001$); in contrast, no difference was observed between the MEA and the MEA+EIPH groups. The scores of edema of the tracheal bifurcation were significantly higher in the SEA group compared to the MEA ($p = 0.0004$) and the MEA + EIPH ($p < 0.0001$) groups; instead, no difference was observed between the MEA and the MEA + EIPH groups.

Age was positively correlated with tracheal mucus score ($p < 0.0001$, $r = 0.37$) and tracheal bifurcation score ($p = 0.0004$, $r = 0.20$) and inversely correlated with pharyngeal lymphoid hyperplasia score ($p < 0.0001$, $r = -0.66$).

3.2.3.6 BALf Cytology and TW Microbiology vs. Ultrasonographic Findings

The total lung ultrasonographic scores were positively correlated with the percentages of BALf macrophages ($p = 0.0110$, $r = 0.1525$), neutrophils ($p = 0.0009$, $r = 0.20$) and hemosiderophages ($p < 0.0001$, $r = 0.25$) and negatively correlated with the percentages of lymphocytes ($p = 0.0001$, $r = -0.23$). The ultrasonographic scores of the cranial area were positively correlated with the percentages of BALf macrophages ($p = 0.0008$, $r = 0.20$), neutrophils ($p = 0.0014$, $r = 0.19$) and hemosiderophages ($p < 0.0001$, $r = 0.26$) and negatively correlated with the percentages of lymphocytes ($p < 0.0001$, $r = -0.25$). The ultrasonographic scores of the middle area were positively correlated with the percentages of BALf neutrophils ($p = 0.0095$, $r = 0.16$) and negatively correlated with the percentages of lymphocytes ($p = 0.0021$, $r = -0.18$). The ultrasonographic scores of the caudal area were positively correlated with the percentages of BALf macrophages ($p = 0.0162$, $r = 0.14$) and hemosiderophages ($p < 0.0001$, $r = 0.27$).

The total number of lung focal lesions was positively correlated with the percentages of BALf macrophages ($p = 0.0275$, $r = 0.13$), neutrophils ($p = 0.0104$, $r = 0.15$) and hemosiderophages ($p = 0.0251$, $r = 0.13$) and negatively correlated with the percentages of lymphocytes ($p = 0.0032$, $r = -0.18$). The number of lung focal lesions in the cranial area was positively correlated with the percentages of BALf macrophages ($p = 0.0170$, $r = 0.14$), neutrophils ($p = 0.0307$, $r = 0.13$) and hemosiderophages ($p = 0.0128$, $r = 0.15$) and negatively correlated with the percentages of lymphocytes ($p = 0.0103$, $r = -0.15$). No association was observed between the number of lung focal lesions in the middle and caudal areas and the BALF cytology.

Microbiological examination results were not correlated with the ultrasonographic scores nor with the number of lung focal lesions in any areas and in total.

3.2.3.7 BALf Cytology and TW Microbiology vs. Endoscopic Findings

Pharyngeal lymphoid hyperplasia scores were positively correlated with the per-centages of BALf macrophages ($p = 0.0019$, $r = 0.19$), eosinophils ($p = 0.0005$, $r = 0.21$) and hemosiderophages ($p < 0.0001$, $r = 0.29$) and negatively correlated with the percentages of neutrophils ($p < 0.0001$, $r = -0.25$).

Tracheal mucus scores were positively correlated with the percentages of BALf neutrophils ($p < 0.0001$, $r = 0.36$) and negatively correlated with the percentages of lymphocytes ($p < 0.0001$, $r = -0.33$), mast cells ($p = 0.0486$, $r = -0.12$) and hemosiderophages ($p < 0.0001$, $r = -0.28$).

Tracheal bifurcation scores were positively correlated with the percentages of BALf neutrophils ($p = 0.0231$, $r = 0.14$) and negatively correlated with the percentages of lymphocytes ($p = 0.0255$, $r = -0.13$) and hemosiderophages ($p = 0.0002$, $r = -0.22$).

Microbiological examination results were not correlated with any endoscopic scores.

3.2.3.8. Ultrasonographic vs. Endoscopic Findings

Pharyngeal lymphoid hyperplasia scores were not associated with the ultrasonographic scores nor with the number of lung focal lesions in any areas and in total.

The ultrasonographic scores of the caudal area were significantly higher in horses having lower tracheal mucus and bifurcation scores (tracheal mucus: $p = 0.0134$; tracheal bifurcation: $p = 0.0218$), while no association was observed with the ultrasonographic scores of the cranial area, the middle area and the total lung ultrasonographic scores. A greater total number of lung focal lesions was observed in horses showing higher tracheal mucus scores ($p = 0.0498$); in contrast, tracheal bifurcation scores were not associated with the number of lung focal lesions in any areas and in total.

3.2.4 Discussion

The current study presents and compares the findings of thoracic ultrasonography and airway endoscopy in 303 horses affected by SEA, MEA or concomitant MEA and EIPH. The SEA horses were significantly older compared to the MEA and the MEA + EIPH horses. In fact, SEA usually affects adult horses older than 7 years (Hotchkiss *et al.* 2007), MEA is more commonly reported in young horses (Couetil *et al.* 2016) and EIPH typically affects racehorses in career (Hinchcliff *et al.* 2015).

In our study, BALf neutrophils percentages were greater in the SEA group, while eosinophils and mast cells counts were higher in the MEA and the MEA + EIPH groups. In fact, SEA is typically neutrophilic (Couetil *et al.* 2020), while different cytological phenotypes of MEA are reported (eosinophilic, mastocytic, neutrophilic) (Couetil *et al.* 2016). Moreover, younger horses had greater percentages of eosinophils and mast cells, while neutrophils increased with age. This finding is in accordance with what was previously reported. In fact, the different MEA phenotypes have been associated with age (Couetil *et al.* 2016); in particular, the eosinophilic and mastocytic forms are more commonly observed in young horses (<5 years old) (Ivester *et al.* 2014, Couetil *et al.* 2016), while the neutrophilic form is particularly encountered in adult horses (>7 years old) (Couetil *et al.* 2016). Hemosiderophages were more numerous in younger horses, which can be easily explained by the fact that racehorses are the most frequently EIPH-affected patients (Hinchcliff *et al.* 2015) and usually have a short career (Barrey 2014).

Bacterial culture was positive in about one third of the examined horses and the most frequently isolated bacteria were *Streptococcus* spp., *Pasteurella*/*Actinobacillus* spp. and *Klebsiella* spp.; β-haemolytic *Streptococci* and, in particular, *Streptococcus equi* subs. *zooepidemicus* are the most common opportunistic pathogens of equine airways, followed by *Pasteurella* spp.,

Actinobacillus equuli, *Klebsiella pneumoniae* and *Bordetella bronchiseptica*, which are frequently encountered in adult horses (Ferrucci *et al.* 2008, Hodgson and Hodgson 2007). The role of bacterial infection or colonization in the pathogenesis of MEA has been widely discussed and investigated; however, variable results have been obtained by different studies. Multiple authors have observed an association between isolation of bacteria from the TW and MEA (Wood *et al.* 1993, Burrell *et al.* 1996, Newton *et al.* 2003, Wood *et al.* 2005b), presence of coughing (Christley *et al.* 2001a) and tracheal mucus grade (Wood *et al.* 2005b, Cardwell *et al.* 2014). In particular, although *Streptococcus equi zooepidemicus* is the most strongly and consistently MEA-associated infectious agent (Wood *et al.* 1993, Burrell *et al.* 1996, Chapman *et al.* 2000, Christley *et al.* 2001a, Newton *et al.* 2003, Wood *et al.* 2005b, Cardwell *et al.* 2014), evidence of association with MEA has also been observed for *Actinobacillus*/*Pasteurella* spp. (Wood *et al.* 1993, Chapman *et al.* 2000, Christley *et al.* 2001a, Newton *et al.* 2003, Wood *et al.* 2005b, Cardwell *et al.* 2014), *Streptococcus pneumoniae* (Wood *et al.* 1993, Christley *et al.* 2001a, Newton *et al.* 2003) and *Bordetella bronchiseptica* (Christley *et al.* 2001a). However, different studies reported that the association between bacteria and MEA was significant only when total bacterial counts were greater than 103 (Wood *et al.* 1993, Christley *et al.* 2001a) or 104 cfu/mL (Burrell *et al.* 1996). In fact, low bacterial counts could result from contamination of the samples, which is more likely in MEA horses that could cough during endoscopy and have more tracheal mucus, making the TW procedure longer and more complicated (Christley *et al.* 2001a). Even if an association between bacteria and MEA seems to exist, the causative role of infectious agents remains unclear. In fact, the prolonged colonization of the airways by these bacteria could be a consequence, rather than the cause, of the increased mucus accumulation in the trachea (Newton *et al.* 2003, Cardwell *et al.* 2014). Moreover, the previously mentioned studies were mainly performed

on young racehorses, while a recent investigation only including adult horses (over 8 years of age) observed no association between bacterial overgrowth and airway inflammation (Manguin *et al.* 2020). In our study, the MEA horses had more positive results to bacterial culture compared to the SEA horses. An explanation for this could be that MEA horses are younger than SEA horses and the isolation of bacteria was more frequent in young horses; this finding is in agreement with previous studies, reporting a decrease in the incidence of infections with age due to the development of immunity (Wood *et al.* 1993, Newton *et al.* 2003, Wood *et al.* 2005a, Wood *et al.* 2005b). Even though we could have expected that horses with positive bacterial culture could show a higher BALf neutrophilia, compared to horses with negative microbiological results, no association was observed between the results of microbiology and cytology in our study; however, in the previous studies associating positive cultures to neutrophilia, cytology was performed on the TW (Chapman *et al.* 2000), while in the current study it was performed on the BALf. This finding could suggest that BALf cytology, in the absence of pneumonia, is not influenced by bacterial colonization.

Ultrasonographic examination revealed that more than 91% of the horses included in this study presented comet-tail artifacts, similarly to what was observed in a previous study (Siwinska *et al.* 2019); therefore, it is reasonable to consider that the detection of these alterations at ultrasonography could represent a highly sensitive tool to identify airway inflammation. However, the comet-tail artifacts lack specificity as they usually suggest the presence of a small amount of fluid surrounded by aerated lung (Couetil and Hawkins 2013b), without allowing to distinguish the nature of the fluid (inflammatory fluid, edema, blood, etc.) (Ferrucci *et al.* 2009, Siwinska *et al.* 2019). Moreover, single comet-tail artifacts (less than 5) could also be observed in healthy patients, particularly in the lung periphery and the most cranioventral areas

of the lung (Couetil and Hawkins 2013b), as a result of previous asymptomatic infections or exposure to non-infectious inflammatory factors (Siwinska *et al.* 2019). In our study, the MEA horses had less comet-tail artifacts and focal lung lesions compared to the SEA horses and those concomitantly affected by MEA and EIPH; therefore, the ultrasonographic appearance of the lung may provide fast and easy information on the severity of the inflammatory status of the lung. The current study did not include healthy horses, so it did not allow us to compare thoracic ultrasonography between healthy and asthmatic horses; however, a preliminary study on ultrasound changes in asthmatic horses observed a significant difference between SEA and healthy horses, while no differences were detected between MEA and healthy horses. Moreover, similarly to our study, it reported a significant difference between ultrasonographic findings in SEA and MEA horses (Siwinska *et al.* 2019). Regarding the presence of EIPH, a previous investigation reported that ultrasonography had a high sensitivity but a low specificity; in particular, in EIPH horses the comet-tail artifacts were mainly located in the dorsocaudal lung portions (Ferrucci *et al.* 2009), which are the areas most commonly affected by EIPH (O'Callaghan *et al.* 1987b, Derksen *et al.* 2009, Derksen *et al.* 2011, Williams *et al.* 2013). As expected, also in the present study, horses affected by MEA and EIPH showed more comet-tail artifacts in the caudal lung fields compared to the horses only affected by MEA. However, also in the cranial portions, the number of comet-tail artifacts and focal lesions was greater in the MEA + EIPH group compared to the MEA group. This finding could be explained by the fact that blood, even if it originates from the caudal areas of the lung, may accumulate for gravity in the cranioventral regions.

In our study, although no significant difference was observed between the left and right side of the thorax for ultrasonographic score or number of focal lesions, it must be noticed that 54.01% of the focal lesions were observed on

the right side. This finding is in agreement with the results of a previous study, where more advanced lesions were identified in the right lung fields (Siwinska *et al.* 2019). In fact, it is reported that lung lesions are more common in the right side of the thorax (Turner *et al.* 1968, Reef 1998). This could be due to the fact that the right main bronchus is slightly wider and has a smaller bifurcation angle compared to the left bronchus, making the access of allergens and pathogens easier (Siwinska *et al.* 2019).

In this study, we found no association between age and ultrasonographic appearance. To date, the hypothetical relationship between thoracic ultrasound changes and ageing has not been studied in the horse. In human medicine, aging is related to decreased lung elasticity and compliance due to loss of elastin and collagen in the lung tissue (Rantanen 1986, Verleden *et al.* 2021). In contrast, in the horse, alveolar walls do not seem to show any decrease in maximal ex-tensibility in older patients (Martin *et al.* 1977).

The total ultrasonographic score proved, in our study, to be positively correlated with BALf neutrophils counts and percentages of hemosiderophages. In particular, neutrophils were correlated with the ultrasonographic scores in the cranial and middle lung fields, while hemosiderophages to the ultrasonographic scores in the cranial and caudal portions. Moreover, the total number of focal lesions was positively correlated with neutrophils and hemosiderophages counts, mostly in the cranial portions of the lung. These results are in accordance with the finding of more comet-tail artifacts and focal lesions in the SEA horses, in which neutrophilia is observed, and in the MEA + EIPH horses, which present higher counts of hemosiderophages compared to the MEA horses. Bacterial culture results were not associated with the ultrasonographic appearance of the lung. This could be due to the fact that, in our cases, a lung infection was not present, but

the isolation of bacteria could only reflect a prolonged bacterial colonization of the airways.

The endoscopic scores for pharyngeal lymphoid hyperplasia were much lower in the SEA group compared to the MEA and the MEA + EIPH groups, while MEA + EIPH horses had greater scores compared to MEA horses. It has commonly been speculated that PLH may be associated with MEA (Auer *et al.* 1985, Burrell 1985, Christley *et al.* 2001b); however, no direct association has been proved and they just may coexist as a consequence to stabling and antigen exposure (Holcombe *et al.* 2001). It has been suggested that severe PLH and other upper airway disorders could contribute to the pathogenesis of EIPH by compromising upper airway airflow and increasing the negative pressure in the lower airway during inspiration (Rooney 1970); however, no association between PLH and EIPH has been demonstrated to date and a study reported that the severity of PLH did not have any effect on the incidence of EIPH (Burrell 1985). In our study, PLH was also negatively correlated with age. In fact, it is thought that PLH may regress as the horse matures and develops immunity, and an inverse relationship between age and the prevalence of PLH has been reported by numerous studies (Auer *et al.* 1985, Hobo *et al.* 1995, Christley *et al.* 2001b, Holcombe *et al.* 2006, Widmer *et al.* 2009, Koblinger *et al.* 2011).

The pathogenesis of PLH is unclear; however, it has been suggested that it may result as an immunological reaction to viral infections or secondary bacterial invasions or environmental antigens (Auer *et al.* 1985, Holcombe *et al.* 2001); in our study, no association between bacterial culture and PLH score has been observed. Some authors reported that pharyngeal microflora varies based on the inflammatory status and, in particular, the number of bacteria per gram in pharyngeal secretions of horses affected by grade 3-4 LPH was about 100-fold higher compared to normal horses (Hoquet *et al.* 1985). The divergence

between these findings and our results could be explained by the fact that, in our study, microbiological evaluation was per-formed on the TW and a degree of independence between upper and lower airway environments has been demonstrated by previous investigations (Koblinger *et al.* 2011).

In our study, PLH was correlated with increased percentages of BALf eosinophils and hemosiderophages, while an inverse correlation was observed with the neutrophils counts. A negative correlation between neutrophils and PLH scores has also been observed by Koblinger and colleagues (Koblinger *et al.* 2011), while no association between PLH and cytology of the BALf and the TW was detected in other studies (Holcombe *et al.* 2001, Holcombe *et al.* 2006). Our results can be easily explained. In fact, PLH decreases with age and is more common in horses affected by MEA and concomitant MEA and EIPH, eosinophils and hemosiderophages are more numerous in younger horses and horses affected by MEA and EIPH, while neutrophils increase with age and are abundant in SEA-affected horses. Moreover, PLH may represent a reaction to environmental antigens (Auer *et al.* 1985, Holcombe *et al.* 2001), and this would fit with BALf eosinophilia.

The endoscopic scores for tracheal mucus and tracheal bifurcation were significantly higher in the SEA group, compared to the MEA and the MEA + EIPH groups, and in-creased with age. Tracheal mucus grade and tracheal bifurcation score have been reported to be related to each other (Koblinger *et al.* 2011). The association between tracheal mucus score and age has been investigated by different authors with various results: in a study including only adult Warmbloods, no association has been observed (Widmer *et al.* 2009); in a study about young Thoroughbreds, mucus grade decreased with age (Holcombe *et al.* 2006); and in another study including horses of various age and breeds, mucus score increased with age (Koblinger *et al.* 2011). The variability of these results seems to be attributable to the different horse

populations enrolled in the studies. Our findings, based on a diverse population, agree with those of the latter study which included equally various patients. Contrasting results have also been obtained regarding the association of tracheal bifurcation score and age. In fact, some authors report that tracheal bifurcation grade increases in older horses (Gerber *et al.* 2004, Koch *et al.* 2007), while in another study no association with age has been detected; however, the same study reported greater tracheal bifurcation scores in the SEA-affected horses compared to the MEA-affected horses (Koblinger *et al.* 2011).

In our study, tracheal mucus and bifurcation scores were both positively correlated with BALf neutrophils counts and negatively correlated with hemosiderophages percentages. Moreover, tracheal mucus grade was also negatively correlated with mast cells counts. Mucus grade has been associated with increased neutrophils by different studies (Gerber *et al.* 2004, Holcombe *et al.* 2006, Koblinger *et al.* 2011) and to decreased mast cells (Koblinger *et al.* 2011); however, other authors report no correlation between tracheal mucus and BALf cytology (Holcombe *et al.* 2001, Richard *et al.* 2010b, Depecker *et al.* 2014). It has been hypothesized that neutrophils may play an important role in airway mucus accumulation by inducing an increase in the production of mucus and a decrease in the clearance, while the role of mast cells in airway inflammation remains unclear (Koblinger *et al.* 2011). The inverse correlation between tracheal mucus and hemosiderophages is in accordance with the findings of a previous study in which tracheal mucus was associated with a decreased likelihood of EIPH (Hinchcliff *et al.* 2010); however, other authors reported no association between EIPH and mucus (Hinchcliff *et al.* 2015, Couetil *et al.* 2016). Only one study investigated the association between tracheal bifurcation and BALf cytology, reporting a negative correlation with mast cells (Koblinger *et al.* 2011).

In our study, no correlation between bacterial culture and tracheal mucus and bifurcation has been observed; however, it has been reported that the isolation of specific bacterial species, such as *Streptococcus equi zooepidemicus* and *Pasteurellaceae*, may be associated with increased mucus grade in racehorses (Christley *et al.* 2001a, Wood *et al.* 2005b, Cardwell *et al.* 2014). It is not clear whether bacterial infections could contribute to mucus accumulation or bacterial colonization may be a consequence of impaired mucus clearance (Couetil *et al.* 2016). The lack of relationship between microbiology results and tracheal mucus in our study could be due to the fact that the horses having greater mucus grades were the SEA horses, in which less bacteria were isolated compared to the other groups.

In our study, some associations between ultrasonographic and endoscopic findings were observed. In particular, tracheal mucus grade was correlated with an increased number of lung focal lesions visible at ultrasound examination. Both these features have shown to be typical of a severe inflammation of the airways and have commonly been found in the SEA horses. Instead, an inverse correlation was detected between tracheal mucus and bifurcation grades and the number of comet-tail artifacts in the caudal lung fields. In fact, lesions in the caudal portions are more common in horses affected by EIPH, which was negatively associated with tracheal mucus and bifurcation scores.

3.2.5 Conclusions

In conclusion, ultrasonographic examination represents a useful and non-invasive diagnostic tool for horses affected by equine asthma and exercise-induced pulmonary hemorrhage, providing fast and easy information on the inflammatory status of the lung. Horses affected by SEA or concomitant MEA and EIPH have a significantly higher ultrasonographic score compared to

horses affected by MEA only. Moreover, ultrasonographic alterations proved to be associated with increased BALf neutrophils and hemosiderophages counts and to increased tracheal mucus accumulation. However, the diagnostic limit of thoracic ultrasonography is represented by its low specificity, not allowing to distinguish the nature of the lesions. Therefore, airway endoscopic and BALf collection remain essential in the diagnostic protocol of these respiratory conditions. Thoracic ultrasonography should be considered when selecting the bronchus from which to collect the BALf as it provides further information on the morphology of the lung, the localization and the extent of the sites of inflammation. Moreover, in young, moderately asthmatic racehorses, lung ultrasonography could represent a reliable indicator to distinguish between EIPH-affected and non-affected patients.

3.3 UPPER AND LOWER AIRWAYS EVALUATION AND ITS RELATIONSHIP WITH DYNAMIC UPPER AIRWAY OBSTRUCTION IN RACEHORSES

The present study has already been published in the following manuscript:

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Ethical statement: Ethical review and approval were waived for this study, as it consists of a retrospective study and all the procedures were performed on clinical horses for diagnostic purposes and included informed owner consent for the use of clinical data. Moreover, all the procedures were performed according to relevant guidelines as a part of standard diagnostic protocols.

3.3.1 Introduction

Horses are obligatory nasal breathers and cannot avoid the high pressures occurring at the level of the nasopharynx during exercise by switching to oral breathing like other species. Moreover, the nasopharyngeal region is not supported by osseous or cartilaginous structures and relies only on muscle activity to maintain its stability (Lane *et al.* 2006, Van Erck-Westergren *et al.* 2013): therefore, during exercise, when airflow turbulence and negative pressures occur at the floor of the nasopharynx and within the larynx, these structures may collapse, and horses may develop different forms of dynamic upper airway obstruction (DUAO) (Rakesh *et al.* 2018). As a consequence, respiratory function and gas exchanges at the alveolar-capillary level may be impaired, determining poor athletic performance, especially in racehorses working at supramaximal exercise (Martin *et al.* 2000, Lane *et al.* 2006, Franklin *et al.* 2012, Lo Feudo *et al.* 2022a).

Different types of DUAO have been described, including dorsal displacement of the soft palate (DDSP), medial deviation of aryepiglottic folds (MDAF), nasopharyngeal collapse (NPC), dynamic laryngeal collapse (DLC), epiglottis entrapment (EE) and epiglottis retroversion (ER) (Franklin and Allen 2017). Although these conditions have been studied for a long time, their exact pathogenetic mechanisms are still not completely clarified and are likely to be multifactorial (Courouce-Malblanc *et al.* 2010, Franklin and Allen 2017, Parente 2018). Most DUAOs are thought to result from neuromuscular dysfunction, fatigue, immaturity or conformational change of the structures controlling the patency of the nasopharyngeal region (Tan *et al.* 2005, Courouce-Malblanc *et al.* 2010, Franklin and Allen 2017). In particular, multiple researchers have hypothesized that epiglottic conformation may play an important role in the stability of upper airways (Allen and Franklin 2013): in fact, the loss of epiglottis convexity has been associated with the

development of DDSP (Haynes 1981, Allen and Franklin 2013) and MDAF (Strand and Skjerve 2012, Allen and Franklin 2013) and is frequently reported as a flaccid appearance of the epiglottis. Abnormal epiglottis conformation was associated with lower earnings in young racehorses (Garrett *et al.* 2010): this result could be attributable to the development of DUAO in horses with a dysplastic epiglottis. Another study reported improved performance in horses after epiglottic augmentation, which may overcome deficits in neuromuscular function (Parente *et al.* 2002). However, the findings of other studies do not support this hypothesis, as they failed to find an association between a flaccid appearance of epiglottis at rest and the detection of DDSP during exercise (Parente and Martin 1995). Moreover, some authors proposed that a short epiglottis, measured radiographically, may contribute to the pathogenesis of DDSP (Linord *et al.* 1983), but other studies reported normal epiglottis length in horses with DDSP (Rehder *et al.* 1995, Courouce-Malblanc *et al.* 2010). Therefore, it is still debated whether a functional epiglottis with normal conformation is required to maintain soft palate stability. Other nasopharyngeal abnormalities, visible at resting endoscopy, have been proposed as predictors of DUAO, including episodes of DDSP, which can occur spontaneously or be induced by swallowing, and the presence of ulceration on the free margin of the soft palate (Hobo *et al.* 1995, Kannegetier and Dore 1995, Beard 1996, Courouce-Malblanc *et al.* 2010). Spontaneous DDSP during resting endoscopy has been reported as a highly specific but extremely insensitive test for DDSP during exercise (Barazkai and Dixon 2011); however, the clinical significance of DDSP at rest remains uncertain, as it can also occur in horses with a normal function of the upper airway during exercise (Kannegetier and Dore 1995, Parente and Martin 1995). Palate ulceration may occur due to the friction between the ventral surface of the epiglottis and the free border of the soft palate (Parente *et al.* 2002); however,

in a study, only 16% of the horses with DDSP had palate ulceration (Gille and Lavoie 1996), and vice versa—many horses with palate ulceration do not show DDSP during exercise (Parente and Martin 1995). It has been hypothesized that horses displacing quickly do not have enough abrasion between the epiglottis and palate to induce ulceration (Parente *et al.* 2002).

Different authors reported that inflammation of the nasopharyngeal region, visible at endoscopy as pharyngeal lymphoid hyperplasia (PLH), may impair the function of pharyngeal mechanoceptors (Tessier 2006) and contribute to the neuromuscular dysfunction and instability of the upper airway, predisposing horses to the onset of DUAOs, such as DDSP, NPC and ER (Holcombe *et al.* 1998, Holcombe *et al.* 1999, Courouce-Malblanc *et al.* 2002, Courouce-Malblanc *et al.* 2010, Van Erck 2011, Franklin and Allen 2017, Tomaszewska *et al.* 2019). Recently, the "one airway, one disease" concept, long known in human medicine and describing a relationship between the health of the upper and lower airways, has also been proposed and investigated in equine medicine (Joo *et al.* 2021). Lower airway inflammation (LAI) has been associated with DUAO (Courouce-Malblanc *et al.* 2002, Courouce-Malblanc *et al.* 2010, Van Erck 2011, Trope 2013, Wysocka and Klucinski 2018, Joo *et al.* 2021), probably because of the increased negative pressure driven by lower respiratory tract obstruction and increased respiratory impedance and work of breathing, which may impair upper airway patency and accelerate the onset of neuromuscular fatigue (Couetil *et al.* 2001, Richard *et al.* 2009, Van Erck 2011, Wysocka and Klucinski 2018, Joo *et al.* 2021). Another proposed theory is that DUAO may predispose horses to lower airway disease via unknown mechanisms (Courouce-Malblanc *et al.* 2010, Wysocka and Klucinski 2018). Another lower airway disorder that has been associated with DUAO is exercise-induced pulmonary hemorrhage (EIPH) (Cook *et al.* 1988, Courouce-Malblanc *et al.* 2010, Trope 2013). This may

be due to the increase in the transmural pulmonary capillary pressure gradient resulting from upper airway obstruction and leading to the rupture of pulmonary capillaries and therefore EIPH (Cook *et al.* 1988, Ducharme *et al.* 2010, Van Erck-Westergren *et al.* 2013, Joo *et al.* 2021). In contrast, no association between DUAO and LAI inflammation or EIPH was detected in another study (Davidson *et al.* 2011).

As the role of upper and lower airways resting evaluation has not been fully under- stood yet, the present study aims to investigate, in a wide population of racehorses, the clinical significance of upper airway anatomical and functional resting abnormalities and the possible contribution of upper and lower airway inflammation to the development of DUAO.

3.3.2 Materials and Methods

3.3.2.1 Horses

The clinical records of Standardbred and Thoroughbred racehorses referred to the Equine Sports Medicine Unit of the Veterinary Teaching Hospital of the University of Milan (Italy) between 2000 and 2021, with a history of poor performance or abnormal respiratory noises during exercise, were retrospectively reviewed. All horses (n = 366) were in full training upon admission. Each horse underwent a complete clinical examination, laboratory analyses and upper airway endoscopy at rest and during exercise on a high-speed treadmill. In 158 horses, the length of epiglottis was measured radiographically. In 339 horses, tracheobronchoscopy was performed 30 min after maximal exercise on a treadmill for EIPH evaluation. In 297 horses, lower airway endoscopy was performed at rest, at least 24 h after exercise, and bronchoalveolar lavage fluid (BALf) was collected in 265 horses and subjected to cytological examination.

3.3.2.2 Upper Airway Endoscopy at Rest

Following a complete clinical examination, resting upper airway endoscopy was performed in all of the horses (n = 366). With this aim, the horses were contained in a stock; to prevent interferences with upper airway function, no other physical or pharmacological restraint techniques were applied. A flexible videoendoscope (EC-530WL-P, Fujifilm, Tokyo, Japan) was passed through the left nasal passage, and the upper tract of the respiratory system was visualized. A 0–4 score was assigned to pharyngeal lymphoid hyperplasia (PLH) (Holcombe *et al.* 2007) observed at the level of the nasopharyngeal mucosa and/or dorsal pharyngeal recess. The larynx was visualized, and its function was assessed during spontaneous breathing and after the stimulation of laryngeal movements by inducing swallowing, performing nasal occlusion maneuvers and during the "slap test" (thoraco-laryngeal reflex). Epiglottis conformation was evaluated, and its alterations were recorded, including the loss of rigidity and convexity (epiglottis flaccidity) or the presence of an entrapping mucosal fold. Whenever dorsal displacement of the soft palate was observed spontaneously or after the induction of swallowing, this finding was recorded. The observation of an ulceration on the free margin of the soft palate was registered. Based on arytenoids abductive function, a 1–4 score was assigned to recurrent laryngeal neuropathy (RLN) (Robinson 2004). Whenever an alteration suggestive of a previous surgical treatment of the upper airway was observed (i.e., laryngoplasty, ventriculectomy, cordectomy, staphylectomy, etc.), the horse was excluded from the study (n = 6).

3.3.2.3 High-Speed Treadmill Endoscopy

Before performing high-speed treadmill endoscopy (HSTE), the horses were conditioned to the high-speed treadmill (Sato I, Uppsala, Sweden) by at least

two training sessions. All the horses (*n* = 360) were tacked with the same equipment used for racing and wore a heart rate (HR) meter (Polar, Equine Inzone FT1, Steinhausen, Switzerland) during exercise. To perform HSTE, the horses were first warmed up by a 4-min walk (1.5 m/s) and a 5-minute trot (4.5 m/s for Thoroughbreds, 6 m/s for Standardbreds), with a 6° slope for Thoroughbreds and 3° slope for Standardbreds. After warm-up, the treadmill was temporarily stopped, and a videoendoscope (ETM PVG-325, Storz, Tuttlingen, Germany) was passed into the nasopharynx of the horse and held in position with Velcro® straps. Then, the treadmill was rapidly accelerated up to maximal speed (corresponding to HR ≥ 220 bpm) for a distance ranging from 1600 to 2100 m (based on the usual racing activity of each individual) or until the horse's fatigue (Ferrucci *et al.* 2003). The endoscopic images were visualized in real-time on a monitor and recorded on analogic (from 2000 to 2004) or digital supports (from 2005 to 2021) to allow for slow-motion analysis and storage. All the registered videos were later evaluated by the same operator, and the absence or presence of single or multiple DUAOs (DDSP, NPC, MDAF, EE, ER, DLC) was recorded. Based on the entity of airway obstruction, the DUAOs were classified as severe (DDSP, NPC, DLC and ER) or mild (MDAF and EE) (Lo Feudo *et al.* 2022a).

3.3.2.4 Radiographic Measurement of Epiglottis Length

To measure the length of the epiglottis radiographically, a lateral view of the pharyngo-laryngeal region was taken on standing horses (*n* = 158), with the head placed in a normal resting position. Radiodense markers of a known length were fixed on both sides of the neck and superimposed to the nasopharynx or the guttural pouches (Figure 5). The lengths of the markers were measured on the obtained radiographs and were used to determine the grade of radiographical magnification. Thyroepiglottic length was then

measured on the radiograph, and a correction factor for the previously assessed magnification was applied to obtain the actual epiglottis length (Linford *et al.* 1983). The reference values for epiglottis length were considered to be 8.76 ± 0.44 cm for Thoroughbreds (Linford *et al.* 1983, Davenport-Goodall and Parente 2003) and 8.74 ± 0.38 cm for Standardbreds (Hobo *et al.* 1995, Butler *et al.* 2000, Davenport-Goodall and Parente 2003).

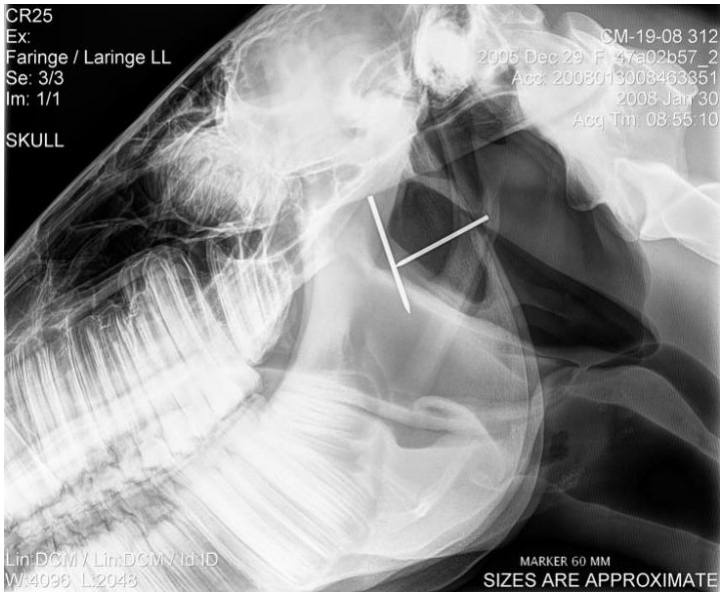

Figure 5. Example of how the radiographs were taken, with the markers of known length in place, to allow for epiglottis length measurements.

3.3.2.5 Post-Exercise Tracheobronchoscopy

Thirty minutes after the end of the HSTE, a tracheobronchoscopy was performed to verify whether exercise-induced pulmonary hemorrhage (EIPH) had occurred. The horses (n = 339) were contained in a stock and restrained with a twitch; endoscopy was performed as described above, and the lower tract of the respiratory system was examined. A 0–4 score was assigned to the presence of blood in the trachea and the mainstem bronchi (Hinchcliff *et al.* 2005).

3.3.2.6 Tracheobronchoscopy at Rest and BALf Collection

At least 24 h after HSTE, an endoscopy of the lower airways was performed as de-scribed above. With this aim, the horses (n = 297) were sedated with detomidine hy-drochloride (0.01 mg/kg IV) and restrained with a twitch. A 0–5 score was assigned to tracheal mucus accumulation (TM) (Gerber *et al.* 2004). The BALf was collected in 265 horses as follows: 60 mL of a 0.5% lidocaine hydrochloride solution was sprayed at the level of the carena to inhibit a coughing reflex, and the endoscope was passed into the bronchial tree until it was wedged firmly within a segmental bronchus; here, a 300 mL sterile saline 0.9% was in-stilled, and the fluid immediately aspirated (Lo Feudo *et al.* 2021b).

3.3.2.7 BALf Cytological Examination

The collected BALf was stored in sterile EDTA tubes and processed within 90 min. A few drops of pooled BALf were cytocentrifugated (Rotofix 32, Hettich Cyto System, Tuttlingen, Germany) at 500 rpm for 5 min. The slides were air dried, stained with May–Grünwald Giemsa and Perl's Prussian blue and observed under a light microscope at 400× and 1000× for 400-cell leukocyte differential count and the calculation of a simplified total hemosiderin score (THS) (Lo Feudo *et al.* 2022b).

3.3.2.8 Statistical Analysis

The data were collected on an electronic sheet (Microsoft Excel, Redmond, WA, USA), analyzed with descriptive statistics and evaluated for normality by the Shapiro–Wilk test. Ages were compared between males and females and between Standardbreds and Thoroughbreds using the Mann–Whitney test. The associations between age and endoscopic scores (PLH, EIPH, TM), epiglottis length, BALf leukocyte populations and THS were evaluated by

means of the Spearman correlation. The Mann–Whitney test was used to compare ages between horses with normal upper airways at rest and horses with any anatomical or functional alterations and between horses with and without DDSP at rest, epiglottis flaccidity and RLN. The same test was used to compare endoscopic scores, epiglottis lengths and BALf cytological findings between males and females and between Standardbreds and Thoroughbreds. The frequency of resting pharyngo-laryngeal alterations was compared between horses of different sexes and breeds using the Fisher's exact test. The horses were divided into four groups on the basis of the HSTE findings: no-DUAO, mild-DUAO, severe-DUAO or multiple-DUAOs. Ages was compared between the groups using the Kruskal–Wallis test and Dunn's multiple comparisons test; the distribution of sex and breed was compared between the groups by the Chi-square test. The Kruskal–Wallis test and the Dunn's multiple comparisons test were used to compare the endoscopic scores, epiglottis lengths, and BALf cytological results between the groups. The PLH score was also compared between the no-DUAO horses and the horses with dynamic DDSP, the horses with NPC and the horses with MDAF by the Mann–Whitney test; the same test was used to compare epiglottis lengths between the no-DUAO horses and the horses with dynamic DDSP. The frequency of pharyngo-laryngeal alterations at rest was compared between the groups by means of the Chi-square test and between the horses without DUAOs and the horses with dynamic DDSP, the horses with NPC and the horses with MDAF using the Fisher's exact test. The resting RLN grade was compared between the horses with and without DLC by means of the Mann–Whitney test. Moreover, the PLH score was compared between the horses with normal upper airway at rest and the horses with resting DDSP and between the horses with normal resting endoscopy and the horses with epiglottis flaccidity using the Mann–Whitney test. Finally, the frequency of

DDSP at rest was compared between the horses with normal epiglottis and the horses with epiglottis flaccidity by means of the Fisher's exact test. The data are presented as the mean ± standard deviation (SD) if normally distributed and as the median and interquartile ranges (IQRs) if not normally distributed. The statistical significance was set at $p < 0.05$. The data were analyzed using a commercially available statistical software package (GraphPad Prism 9.3.1 for MacOS; GraphPad Software, San Diego, CA, USA).

3.3.3 Results

3.3.3.1 Horses

A total of 360 horses (312 Standardbreds and 48 Thoroughbreds) met the inclusion criteria. The population consisted of 233 males (203 stallions, 30 geldings) and 127 females, aged from 2 to 8 years (median 3 years, IQR 3–4 years). Among the Standardbred population, 200 horses were males (64.1%) and 112 were females (35.9%), while, among the Thoroughbred population, 33 subjects were males (68.75%) and 15 were females (31.25%). The median age was 3 years in both the Standardbred and Thoroughbred populations, with different IQRs (Standardbreds: IQR 3–4 years; Thoroughbreds: IQR 3–3 years). The horses were divided into the no-DUAO group (169 horses), mild-DUAO group (23 horses), severe-DUAO group (111 horses) and multiple-DUAOs group (57 horses).

3.3.3.2 Upper Airway Endoscopy at Rest

The frequency and grade distribution of PLH among the study population are shown in Table 10; the median PLH score was 2 (IQR 1–2). The normal appearance and function of the pharyngo-laryngeal region were observed in 160 horses (44.44%), while alterations were detected in 200 horses (55.56%); the

distribution of upper airway alterations detected at resting endoscopy is displayed in Table 11.

PLH Grade	% of the Study Population
0	16.67%
1	17.22%
2	43.89%
3	20.83%
4	1.39%

Table 10. Frequency and distribution of pharyngeal lymphoid hyperplasia (PLH) grades among the study population.

Endoscopic Finding	% of the Study Population
Normal appearance and function	44.44%
Recurrent Laryngeal Neuropathy	30.27% 25.83% Grade II, 3.33% Grade III (0.28% III.a, 1.38% III.b, 1.67% III.c), 1.11% Grade IV
One or more episodes of dorsal displacement of the soft palate	27.22%
Epiglottis flaccidity	17.5%
Ulceration of the free margin of the soft palate	3.89%
Persistent epiglottis entrapment	1.11%

Table 11. Distribution of upper airways endoscopic findings at rest.

3.3.3.3 High-Speed Treadmill Endoscopy

During HSTE, no DUAO occurred in 169 horses (46.95%), one single DUAO was observed in 134 horses (37.22%) and multiple concomitant DUAOs were detected in 57 horses (15.83%). In order of frequency, the observed DUAOs included DDSP (107 horses, 29.72%), MDAF (63 horses, 17.5%), NPC (59 horses, 16.39%), DLC (19 horses, 5.28%), EE (7 horses, 1.94%) and ER (2 horses, 0.56%). The frequency of single or concomitant DUAOs is displayed in Table 12.

DUAOs	% of the Study Population
DDSP	20.83%
NPC	7.22%
MDAF	6.11%
DLC	2.5%
EE	0.28%
ER	0.28%
MDAF + NPC	5.28%
MDAF + DDSP	2.22%
NPC + DDSP	1.94%
DDSP + EE	1.67%
MDAF + DLC	1.39%
DDSP + DLC	0.83%
MDAF + ER	0.28%
MDAF + NPC + DDSP	1.67%
MDAF + DDSP + DLC	0.28%
MDAF + NPC + DDSP + DLC	0.28%

Table 12. Frequency of single or multiple dynamic upper airway obstructions (DUAOs) detected during high-speed treadmill endoscopy. DDSP = dorsal displacement of the soft palate; NPC = nasopharyngeal collapse; MDAF = medial deviation of aryepiglottic folds; DLC = dynamic laryngeal collapse; EE = epiglottis entrapment; ER = epiglottis retroversion.

3.3.3.4 Epiglottis Length

The epiglottis length was radiographically measured in 145 Standardbreds and 13 Thoroughbreds. The median epiglottis length in the investigated population was 8.2 cm (Standardbreds: IQR 7.88–8.7 cm; Thoroughbreds: IQR 7.5–8.8 cm). In the Standardbred population, the epiglottis length was within the reference ranges in 60 horses (41.67%), lower in 73 horses (50.69%) and higher in 11 horses (7.64%); in the Thoroughbreds population, the epiglottis length was within the normal limits in 2 horses (15.38%), lower in 8 horses (61.54%) and higher in 3 horses (23.08%).

3.3.3.5 Lower Airway Endoscopy and BALf Cytology

The tracheobronchoscopy performed 30 min after maximal exercise was negative for EIPH in 145 horses (42.77%) and positive in 194 horses (57.23%);

the frequency and grade distributions of EIPH among the study population are shown in Table 13. The median EIPH grade was 1 (IQR 0–2). On the lower airway endoscopy performed at least after 24 h, tracheal mucus accumulation was observed in 296 horses (86.6%); the grade distribution is displayed in Table 14. The median TM score was 1 (IQR 1–2). The cytological examination of BALf showed a median of 46% (40–50.5%) macrophages, a mean of 35.72% ± 11.85% lymphocytes, a median of 10% (5–18%) neutrophils, a median of 1% (0–3%) eosinophils and a median of 4% (3–6%) mast cells. The median THS was 36 (12–66).

EIPH Grade	Number of Horses	% of the Study Population
0	145	42.77%
1	75	22.12%
2	74	21.83%
3	34	10.03%
4	11	3.25%

Table 13. Frequency and grade distribution of exercise-induced pulmonary hemorrhage (EIPH) among the study population.

TM Grade	Number of Horses	% of the Study Population
0	45	13.2%
1	142	41.64%
2	117	34.31%
3	33	9.68%
4	4	1.17%

Table 14. Frequency and grade distribution of tracheal mucus accumulation (TM) among the study population.

3.3.3.6 Influence of Age, Breed and Sex

Among the study population, the Thoroughbreds were younger than the Standardbreds ($p = 0.0054$), and the females were younger than the males ($p < 0.0001$). Sex was equally distributed among the different breeds. Age was inversely correlated with PLH grade ($p < 0.0001$, $r = -0.45$) and TM score ($p =$

0.0083, r = −0.14) and was positively correlated with EIPH score (p = 0.0053, r = 0.15) and THS (p = 0.0165, r = 0.15); PLH score was significantly higher in females (median 2, IQR 2–3) than in males (median 2, IQR 1–2) (p < 0.0001). The horses showing pharyngo-laryngeal alterations at resting endoscopy were younger than the horses with normal appearance and function (p = 0.0213); moreover, pharyngo-laryngeal alterations were significantly more frequent in Thoroughbreds (83.33%) than in Standardbreds (51.28%) (p < 0.0001). In particular, DDSP at rest and grade > 1 RLN was more frequently observed in Thoroughbreds (DDSP 47.92%, RLN 63.83%) than in Standardbreds (DDSP 24.04%, RLN 25.32%) (DDSP p = 0.0014; RLN p < 0.0001). At BALf cytology, the neutrophils counts were higher in Thoroughbreds (median 17%, IQR 11–22.5%) than in Standardbreds (median 9%, IQR 4–17%) (p = 0.0016), while the lymphocyte counts were higher in Standardbreds (36.25% ± 11.78%) than in Thoroughbreds (30.92% ± 11.63%) (p = 0.0293). Among the different groups (no-DUAO, mild-DUAO, severe-DUAO and multiple-DUAOs), no differences were observed in age and sex distribution, while a significant difference in breed distribution was detected (p = 0.0064); in particular, in Thoroughbreds, DUAOs were significantly more frequent than they were in Standardbreds (Thoroughbreds 75%, Standardbreds 50.32%; p = 0.001).

3.3.3.7 Upper Airway Endoscopy and Epiglottis Length vs. DUAOs

The PLH grade was significantly lower in the multiple-DUAOs group (median 1, IQR 0–2) compared to the no-DUAO group (median 2, IQR 1–3) (p = 0.0066) and the severe-DUAO group (median 2, IQR 1–2). No differences were observed in PLH between the horses with and without DDSP, MDAF and NPC. The epiglottis length did not differ between the groups, nor did it differ between the horses with and without DDSP. The frequency of abnormalities of pharyngo-laryngeal appearance and function detected at resting endoscopy

differed between the groups ($p = 0.0003$) (Figure 6); in particular, alterations were more frequently observed in the severe-DUAO group (71.17%) compared to the no-DUAO group (44.97%) ($p < 0.0001$). While no differences were observed between the groups regarding the frequency of DDSP episodes observed during resting endoscopy, a trend was detected for different frequencies of epiglottis flaccidity among the different groups ($p = 0.0534$). In particular, a flaccid appearance of epiglottis was more frequently observed in the horses with DDSP diagnosed at HSTE (60.78%) compared to the horses without DDSP (39.22%) ($p = 0.0007$) (Figure 7). No differences were observed in the frequency of DDSP at rest between the horses with and without DDSP during HSTE, nor in the frequency of resting DDSP and epiglottis flaccidity between the horses with and without NPC and MDAF. The horses showing DDSP episodes at rest had a higher PLH grade (median 2, IQR 1–3) compared to the horses without resting DDSP (median 2, IQR 1–2) ($p = 0.0052$) and more frequently showed a flaccid appearance of the epiglottis (resting DDSP 31.63%, no resting DDSP 12.21%, $p < 0.0001$). Among the seven horses showing EE during exercise, three also presented persistent EE visible at resting endoscopy, two had a normal epiglottis and two had a flaccid appearance of the epiglottis; in four cases, an ulceration of the free margin of the soft palate was observed. Ulcerations of the margin of the soft palate were also observed in the four horses without DUAO, the two horses with MDAF + NPC, the two horses with DDSP and the two horses with NPC + DDSP. In the horses with DLC, the RLN grade was significantly higher (median 3, IQR 3–3) compared to the horses with normal dynamic arytenoids abduction (median 1, IQR 1–2) ($p < 0.0001$). In particular, all the horses with grade 1 (normal) RLN showed a normal laryngeal abduction during HSTE, and among the horses with grade 2 RLN, only two horses showed DLC (2.15%); in contrast, all the horses with grade 3 and grade 4 RLN showed DLC on HSTE.

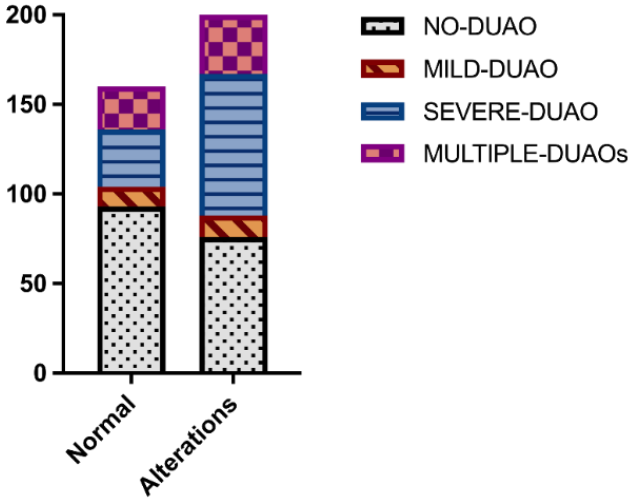

Figure 6. Stacked bars showing the frequency of alterations at upper airway resting endoscopy in the different groups (no-DUAO, mild-DUAO, severe-DUAO and multiple-DUAOs).

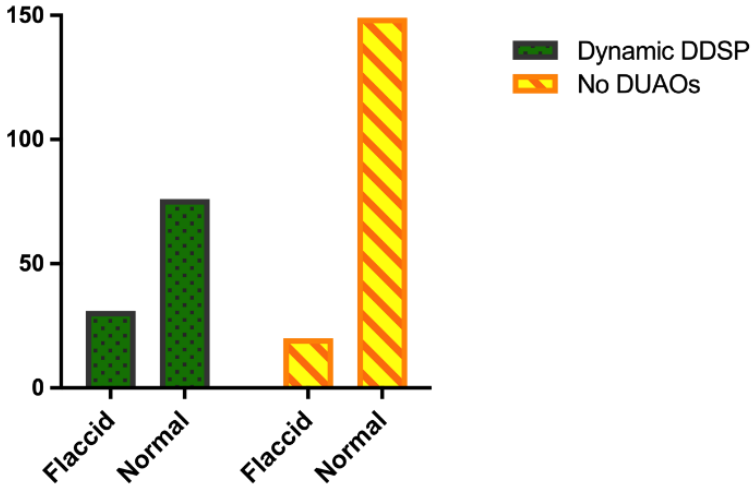

Figure 7. Bar graph showing the frequency of normal or flaccid appearance of epiglottis at upper airway resting endoscopy in the horses with no DUAOs and with DDSP diagnosed at HSTE.

3.3.3.8 Lower Airway Endoscopy and BALf Cytology vs. DUAOs

No differences between the groups were observed regarding the TM score, EIPH score, leukocyte differential count of BALf or THS.

3.3.4 Discussion

Although DUAOs are disorders that have long been known to cause poor performance in racehorses, their precise pathogenetic mechanisms have not been fully understood yet, and the possible role of structural or functional abnormalities of the nasopharynx has not been univocally clarified (Tan *et al.* 2005, Courouce-Malbland *et al.* 2010, Franklin and Allen 2017, Parente 2018). Moreover, some authors suggest that inflammation of the upper and lower respiratory tract may contribute to the development of DUAO, but contrasting results have been reported (Holcombe *et al.* 1998, Holcombe *et al.* 1999, Courouce-Malblanc *et al.* 2002, Van Erck 2011, Trope 2013, Wysocka and Klucinski 2018, Tomaszewska *et al.* 2019, Joo *et al.* 2021). Therefore, the present study aimed to investigate the diagnostic role of upper and lower airways resting evaluation for a better understanding of DUAO development.

The value of this study relies on a very large and homogeneous population of racehorses, including a total of 360 patients, and on a highly standardized protocol for airways evaluation in both resting and dynamic conditions. Among the study population, 53.05% of the horses showed DUAO on HSTE; the prevalence of DUAO reported in the literature ranges from 22.6% to 80.1% (Morris and Seeherman 1991, Davidson *et al.* 2017), based on the kind of population (i.e., racehorses or other performance horses), inclusion criteria (randomized or horses with a history of poor performance and respiratory noises) and diagnostic technique (HSTE or overground endoscopy). Among reports including both Standardbreds and Thoroughbreds diagnosed by HSTE, DUAO prevalence varies from 22.6% (Morris and Seeherman 1991) to 65% (Kannegieter and Dore 1995). Interestingly, the highest frequencies have been reported by studies only including Thoroughbred racehorses, with percentages ranging from 72.6% to 80.1% (Lane *et al.* 2006, Mirazo *et al.* 2014, Davidson *et al.* 2017, Oellers *et al.* 2018); similarly, in our study 75% of the

Thoroughbred patients showed DUAO during HSTE, which was significantly more frequent than in Standardbreds (50.32%), suggesting a breed predisposition. However, in our population, most Standardbreds were referred for poor performance, while Thoroughbreds were more often referred for abnormal respiratory noises during exercise, with a consequent higher probability of being diagnosed with DUAO; this could have biased the results of our study. An apparent predisposition of Thoroughbreds to develop DUAO, and especially DDSP, in comparison with Standardbreds was also observed by previous researchers, but the reason for this breed discrepancy is unclear (Durando *et al.* 2002, Tan *et al.* 2005, Durando *et al.* 2006). In our study, the Thoroughbreds more commonly showed upper respiratory tract alterations at rest; it must be noticed that the Thoroughbreds were younger than the Standardbreds, probably due to the shorter racing career, and that alterations at rest were more frequently observed in younger horses. Some authors suggest that young age may predispose horses to the development of DUAO (Lane *et al.* 2006, Courouce-Malblanc *et al.* 2010) and that affected horses could spontaneously resolve this condition over time (Parente 2018); in fact, it has been hypothesized that increased palatal musculature in older horses may improve muscular activity and provide a better resistance against collapsing forces (Lane *et al.* 2006). In contrast, other reports found no association between age and DUAO (Tan *et al.* 2005) or even a higher susceptibility in older horses (Ordidge 2001).

In the present study, the most frequently observed forms of DUAO were DDSP (29.72%), MDAF (17.5%) and NPC (16.39%); likewise, most studies about racehorses report DDSP as the most common DUAO, with the prevalence ranging from 14% to 45.2% (Morris and Seeherman 1991, Kannegetier and Dore 1995, Lane *et al.* 2006, Priest *et al.* 2012, Strand and Skjerve 2012, Mirazo *et al.* 2014, Davidson *et al.* 2017, Oellers *et al.* 2018). MDAF

was the most common DUAO in a previous study (Tan *et al.* 2005), but its prevalence in the literature is highly variable, ranging from 4% to 40.4% (Kannegetier and Dore 1995, Tan *et al.* 2005, Lane *et al.* 2006, Mirazo *et al.* 2014, Davidson *et al.* 2017, Oellers *et al.* 2018). Both in the present study and in the previous reports, MDAF is rarely diagnosed alone and is associated, in most cases, with other types of DUAO, especially NPC and DDSP; therefore, various authors have suggested that MDAF may either contribute or follow the development of associated DUAOs (Tan *et al.* 2005, Lane *et al.* 2006, Davidson *et al.* 2011, Allen and Franklin 2013, Franklin and Allen 2017). More generally, multiple concomitant DUAOs were detected relatively frequently in our population, with a prevalence of 15.83% of the total horses and of 29.84% of the horses with DUAO. Multiple DUAOs have also been commonly reported by previous studies, with a wide range of prevalence varying from 7% to 56.9% (Kannegetier and Dore 1995, Martin *et al.* 2000, Tan *et al.* 2005, Lane *et al.* 2006, Garret *et al.* 2010, Mirazo *et al.* 2014, Davidson *et al.* 2017). In the present report, NPC showed a prevalence of 16.39%, which was similar to those reported by some authors (Martin *et al.* 2000, Strand and Skjerve 2012, Mirazo *et al.* 2014, Oellers *et al.* 2018) but quite higher when compared to other studies (Kannegetier and Dore 1995, Tan *et al.* 2005, Lane *et a.* 2006, Barakzai and Dixon 2011, Davidson *et al.* 2017). The great variability of the prevalence of DUAOs, as reported by different studies, may be attributable to heterogeneous enrolled populations, diagnostic techniques and the lack of a consensus statement which could provide universal guidelines for the classification of different DUAOs (Barnett *et al.* 2015). The only upper respiratory tract for which a consensus statement exists is RLN detectable at rest (Robinson 2004); in the present study, all of the horses with grade 1 RLN and 97.85% of the horses with grade 2 RLN showed a normal laryngeal function during exercise. In contrast, some authors have reported that low

percentages (from 0.34% to 3.5%) of horses with grade 1 RLN and up to 11.9% of horses with grade 2 RLN may show DLC on HSTE (Lane *et al.* 2006a, Barakzai and Dixon 2011, Elliot and Cheetham 2019). Moreover, in our study, all horses with grade 3 and grade 4 RLN developed DLC on HSTE; conversely, previous studies reported a normal laryngeal function during exercise in 16% to 33% of the horses with grade 3 RLN (Barakzai and Dixon 2011, Elliot and Cheetham 2019), and it has been reported that 2% of horses with grade 4 RLN may still show a residual laryngeal activity during exercise without experiencing DLC (Elliot and Cheetham 2019). Therefore, independently from the RLN grade at rest, HSTE should be always encouraged to evaluate dynamic laryngeal function.

It has been hypothesized that epiglottic hypoplasia may predispose horses to DUAOs, such as DDSP, MDAF and EE (Haynes 1981, Linford *et al.* 1983, Garret *et al.* 2010, Strand and Skjerve 2012, Allen and Franklin 2013); in the present study, epiglottis length was assessed radiographically, and alterations of its conformation visible at endoscopic examination were recorded. Interestingly, only 39.49% of our patients had a normal epiglottis length falling within the reference ranges (Linford *et al.* 1983, Beard 1996, Butler *et al.* 2000, Davenport-Goodall and Parente 2003); a shorter epiglottis was observed in 51.59%, while the remaining 8.92% had a longer epiglottis. As the technique used for radiographic measurement was the same as that described by Linford (1983) (Linford *et al.* 1983), and our population of Standardbreds is by far the widest that underwent radiological assessment of epiglottis length, it is possible that the reference ranges may be reviewed, including more numerous populations of Standardbreds and Thoroughbreds. Unfortunately, only 158 horses in the present study underwent epiglottis length measurement, of which 145 were Standardbreds; the low number of Thoroughbreds subjected to this measurement represents a main limitation for the evaluation of the

possible relationship between epiglottis length and the development of DUAOs. In our study epiglottis length did not influence the development of DUAO or the development of any resting alterations; this finding is in contrast with a previous one reporting a shorter epiglottis as a predisposing factor to DDSP (Linford *et al.* 1983). However, similarly to our findings, other studies failed to detect any association between epiglottis length and DUAO (Rehder *et al.* 1995, Courouce-Malblanc *et al.* 2010). Therefore, some authors suggested that the epiglottis may play a role in DUAO development—not for its length but for its conformation. In particular, the loss of rigidity and of the normal convex appearance has been associated with DDSP and MDAF (Allen and Franklin 2013, Kelly *et al.* 2013). Our findings partially confirm this hypothesis: among our population, the horses showing a flaccid appearance of epiglottis at endoscopic examination were more prone to develop DDSP both at rest and during exercise, while no relationship was observed with MDAF. As alterations at resting endoscopy were more frequent in the severe-DUAO group, we also evaluated whether DDSP detected at rest could be a good predictor of DDSP on HSTE; however, no association between DDSP at rest and dynamic DDSP was observed. Conversely, previous studies reported a low sensitivity but a high specificity of DDSP detected at rest (Kannegetier and Dore 1995, Lane *et al.* 2006a, Tomaszewska *et al.* 2019). In any case, upper respiratory tract endoscopy at rest is advised in cases of suspected DUAO, but dynamic endoscopy is confirmed to always be essential for a certain diagnosis. It has been hypothesized that inflammation of the upper airways may predispose horses to DUAO (Holcombe *et al.* 1998, Holcombe *et al.* 1999, Tessier 2006, Courouce-Malblanc *et al.* 2002, Courouce-Malblanc *et al.* 2010, Van Erck 2011, Franklin and Allen 2017, Tomaszewska *et al.* 2019). In particular, PLH has been associated with DDSP (Courouce-Malblanc *et al.* 2002, Courouce-Malblanc *et al.* 2010, Tomaszewska *et al.* 2019), NPC

(Holcombe *et al.* 1999, Van Erck 2011) and ER (Franklin and Allen 2017). Surprisingly, in our study, PLH was lower in horses belonging to the multiple-DUAOs group; a possible explanation for this may be that the horses in the multiple-DUAOs group were older than those in the other groups, even if a statistical significance was not detected. As the PLH grade was lower in older horses, a lower PLH grade in the multiple-DUAOs group may ensue, without implying any causative relation between a low PLH grade and the concomitant occurrence of multiple DUAOs. When individually evaluating different DUAOs, no association was detected between PLH and DDSP, MDAF or NPC. Unfortunately, in our study, it was not possible to individually evaluate the other types of DUAOs due to the low number of affected cases. Interestingly, the PLH grade was higher in horses experiencing DDSP at rest but not during exercise. It could be hypothesized that upper airway inflammation may have an influence on pharyngo-laryngeal movements at rest; however, during exercise, more forces come into play, and the contribution of pharyngeal inflammation may be too low to have an impact on pharyngeal stability.

Some authors have also reported an association between DUAO and LAI (Courouce-Malblanc *et al.* 2002, Courouce-Malblanc *et al.* 2010, Van Erck 2011, Trope 2013, Wysocka and Klucinski 2018, Joo *et al.* 2021); in particular, a relation between BALf neutrophilia and DDSP was reported in two studies from the same research group (Courouce-Malblanc *et al.* 2002, Courouce-Malblanc *et al.* 2010), while other studies detected an association between mild-moderate equine asthma and NPC (Van Erck 2011, Wysocka and Klucinski 2018). However, multiple studies reported no association between upper and lower airways inflammation based on endoscopic and cytological findings (Holcombe *et al.* 2001, Holcombe *et al.* 2006, Koblinger *et al.* 2011, Lo Feudo *et al.* 2021b), and other authors found no relationship between LAI and

DUAO (Davidson *et al.* 2011). Similarly, in the present study, no differences between the groups were observed concerning tracheal mucus accumulation or the BALf cytological profile, suggesting that LAI is not associated with the onset of DUAO. Another lower airway disorder which has been associated with DUAO is EIPH (Cook *et al.* 1988, Ducharme *et al.* 2010, Van Erck-Westergren *et al.* 2013, Joo *et al.* 2021); however, analogously to a previous study (Davidson *et al.* 2011), we found no relationship between DUAO and EIPH based on both post-exercise endoscopy and total hemosiderin score. Therefore, the findings of the present study do not support the theory of a possible contribution of upper and lower airways inflammation to the onset of DUAO, nor the cause–effect relationship between DUAO and EIPH; however, given the contrasting results reported by different studies, these hypotheses cannot be ruled out.

3.3.5 Conclusions

In conclusion, DUAOs were more commonly observed in Thoroughbred racehorses than in Standardbred racehorses, confirming a possible breed predisposition; conversely, age did not seem to influence the onset of DUAO on HSTE, as pharyngo-laryngeal alterations were detected more frequently in younger horses only at resting upper airway endoscopy. The detection, at resting endoscopy, of the abnormal appearance and function of the pharyngo-laryngeal region was associated with the development of DUAO: in particular, a flaccid appearance of the epiglottis, with a loss of convexity and rigidity, was associated with the occurrence of DDSP both at rest and on HSTE. In contrast, the epiglottis length measured radiographically was not associated with DUAO, suggesting that the epiglottis may be involved in maintaining the stability of the upper respiratory tract, not based on its dimensions but on its conformation. Finally, in the present study, no association was observed

between DUAO and the inflammation of the upper and lower airways, nor between DUAO and EIPH.

3.4 IMPACT OF LOWER AIRWAY INFLAMMATION ON FITNESS PARAMETERS IN STANDARDBRED RACEHORSES

The present study has already been published in the following manuscript:

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Ethical statement: Ethical review and approval were waived for this study, as it consists of a retrospective study and all the procedures were performed on clinical horses for diagnostic purposes and included informed owner consent for the use of clinical data. Moreover, all the procedures were performed according to relevant guidelines as a part of standard diagnostic protocols.

3.4.1 Introduction

The racehorse industry is spread worldwide, with important economic, social, and employment implications in many countries. During the last few years, the attention to horse welfare and health protection has increased; therefore, research on disorders typically affecting racehorse health and performance is of special interest. There is evidence that the respiratory system represents the main performance-limiting factor in racehorses, undergoing maximal and supramaximal exercise (Poole and Erickson 2011); in particular, oxygen delivery during strenuous exercise primary limits the increase in maximal oxygen consumption (VO2 max) (Franklin *et al.* 2012), which reflects the aerobic capacity and, therefore, the athletic potential of the horse (Harkins *et al.* 1993). As a consequence, any disorder of the respiratory system may affect aerobic metabolism, finally leading to sport performance impairment (Franklin *et al.* 2012).

Among young racehorses, mild-moderate equine asthma (MEA) is a common inflammatory disease of the lower airway, characterized by mucus hypersecretion, airflow obstruction and airway hyperreactivity. Affected horses commonly show chronic, occasional cough, serous to mucopurulent nasal discharge, and poor performance (Couetil *et al.* 2016). However, the effects of MEA on performance can be difficult to define and quantify: some studies rely on trainers' or owners' expectations and impressions (Fogarty and Buckley 1991, Bedenice *et al.* 2008, Widmer *et al.* 2009), other studies on races placements (Holcombe *et al.* 2006, Saulez and Gummow 2009, Salz *et al.* 2016, Ivester *et al.* 2018), others instead on fitness parameters measured during standardized exercise tests (Couetil and Denicola 1999, Mazan and Hoffman 2001, Sanchez *et al.* 2005, Richard *et al.* 2010b, Nolen-Walston *et al.* 2013, Stucchi *et al.* 2020). Probably due to the different study designs, the literature includes contrasting results about the specific effects of lower airway inflammation -

either identified as tracheal mucus accumulation or increased inflammatory cells in tracheal wash (TW) or bronchoalveolar lavage (BAL) samples - on the athletic capacity of horses. A high degree of mucus accumulation in the trachea has been associated with lower race positions in both Thoroughbred (Holcombe *et al.* 2006) and Standardbred racehorses (MacNamara *et al.* 1990), and with performance below expectations in Standardbreds (Richard *et al.* 2010b); conversely, other studies found no association between tracheal mucus score and racing results in Thoroughbreds (Saulez *et al.* 2009, Salz *et al.* 2016). Neutrophilia of the BAL has been associated with exercise intolerance (Fogarty and Buckley 1991) and unsatisfactory racing results in Thoroughbreds (Ivester *et al.* 2018) and Standardbreds (Richard *et al.* 2010b); moreover, one study reported a strong negative association between BAL mastocytosis and racing placements (Ivester *et al.* 2018). Some other studies investigated the relationship between BAL cytological profile and fitness parameters measured during incremental exercise tests on treadmill; while BAL neutrophilia was associated with a lower aerobic-anaerobic threshold in one study on Standardbreds (Stucchi *et al.* 2020), no relationship between BAL differential cell count and several fitness parameters was observed by other authors nor in Standardbred nor in Thoroughbred racehorses (Mazan and Hoffman 2001, Nolen-Walston *et al.* 2013). Instead, when authors considered the cytological results of the TW, associations with racing results were lacking in Thoroughbreds (Holcombe *et al.* 2006, Salz *et al.* 2016), while one study reported a negative impact of TW neutrophilia on performance in Standardbreds (Richard *et al.* 2010b).

Overall, to date, no consistent evidence for relationships between performance and tracheal mucus accumulation, and between performance and BAL cytological profile, has been reached in racehorses (Kinnison *et al.* 2022). The present study aims to investigate the impact of lower airway inflammation on

athletic capacity in Standardbred racehorses, by evaluating the associations of the findings of both airway endoscopy and BAL cytology with a wide range of fitness parameters measured during a standardized incremental treadmill test.

3.4.2 Materials and Methods

3.4.2.1 Study population

The clinical records of the Standardbred racehorses referred for poor performance evaluation to the Equine Unit of the Veterinary Teaching Hospital of the University of Milan (Italy) between 2002 and 2021 were retrospectively reviewed. As all the procedures were performed on clinical patients for diagnostic purposes and included informed owner consent for the use of clinical data, ethical review and approval were waived.

All horses were in training upon admission and underwent a complete diagnostic protocol, including: collection of history, clinical examination and lameness evaluation, laboratory analyses, electrocardiogram at rest, upper airway endoscopy at rest, incremental treadmill exercise test, high-speed treadmill endoscopy, tracheobronchoscopy 30 minutes post-exercise for the evaluation of exercise-induced pulmonary hemorrhage, TW and BAL collection with cytological examination of the BAL and microbiological examination of the TW (Lo Feudo *et al.* 2022b).

Horses showing signs of systemic illness, lameness, clinically significant cardiac arrhythmias or valvular regurgitation, dynamic upper airway obstructions or rhabdomyolysis were excluded from the study, since these disorders may influence athletic performance.

3.4.2.2 Incremental Metabolic Test on Treadmill

After one day of being acclimated to a high-speed treadmill (Sato I, Uppsala, Sweden), after one or two training sessions, the horses underwent an incremental treadmill test. An inclination of 5% was applied to the belt: after a warmup phase (4 min walking at 1.5 m/s and 3 min trotting at 6 m/s), the protocol consisted of 1 min phases, increasing the speed by 1 m/s until the onset of fatigue. At the end of the test, horses were cooled down by a 30 min walk with 0% slope. Throughout the duration of the test, horses were wearing a heart rate monitor (Polar, Equine Inzone FT1, Steinhausen, Switzerland), and a continuous ECG was obtained before, during, and after exercise using a Holter recorder (Custo Flash 200, Sylco, Monza, Italy; Cardioline® Click Holter, Trento, Italy). Blood samples were collected through an extension tube connected to a 14 G Teflon catheter placed in the left jugular vein at different timepoints: at rest, at the end of the warmup phase and of each speed phase, and 1, 5, 15, and 30 min postexercise. Blood samples of 1 mL each were transferred into tubes containing 10 mg sodium fluoride and 2 mg potassium oxalate, centrifugated within 15 min, and refrigerated. Plasma lactate values were obtained using the enzymatic colorimetric method with a lactate dry-fast kit due to the automatic system and its proper reagents (Uni Fast System II Analyzer, Sclavo, Italy). In some horses, at each phase, an aliquot of blood was transferred into heparinized syringes, and blood pH (n = 69) and hematocrit (n = 46) were measured using a blood gas analyzer (IL 1630, Instrumentation Laboratory, Milan, Italy; Opti CCA, Opti Medical System, Roswell, USA) (Lo Feudo *et al.* 2022a).

The fitness parameters obtained during the incremental treadmill test included:

- V200: Speed at a heart rate of 200 bpm;
- VLa4: Speed at a plasma lactate concentration of 4 mmol/L;

- HRLa4: Heart rate at a plasma lactate concentration of 4 mmol/L;
- Lacmax: Maximum plasma concentration of lactate reached during the test or cool down;
- Vmax: Maximum speed reached during the test (until the horse became fatigued);
- Lac1, 5, 15, 30: Plasma lactate concentrations at 1, 5, 15, and 30 min postexercise, during the cool down;
- HR1, 5, 15, 30: Heart rate at 1, 5, 15, and 30 min post-exercise, during the cool down;
- pHmin: Minimum pH measured during or after the test;
- Htmax: Maximum hematocrit measured during or after the test.

A specific software (Lactate-E 1.0, Dr. David Higgins), which provides precise lactate threshold markers via inverse prediction (Newell *et al.* 2007), was used to calculate exact values of VLa4 and HRLa4.

3.4.2.3 Airway Endoscopy and Collection of TW and BAL

After one day of active rest (hand walking), horses underwent high-speed treadmill endoscopy and postexercise tracheobronchoscopy. At least twenty-four hours later, airway endoscopy with TW and BAL collection was performed. To this end, horses were contained in a stock and sedated with detomidine hydrochloride (0.01 mg/kg IV). A flexible videoendoscope (ETM PVG-325, Storz, Tuttlingen, Germany; EC-530WL-P, Fujifilm, Tokyo, Japan) was passed through the left nasal passage, and the upper and lower tracts of the respiratory system were visualized (Lo Feudo *et al.* 2022c).

Endoscopic scores were assigned to:

- Pharyngeal lymphoid hyperplasia (PLH), from 0 to 4 (Burrell 1985);
- Tracheal mucus accumulation (TM), from 0 to 5 (Gerber *et al.* 2004);
- Tracheal bifurcation blunting (TB), from 0 to 4 (Ferrucci *et al.* 2008).

A TW was performed by flushing a 60 mL prewarmed sterile saline (0.9%) into the intrathoracic portion of the tracheal lumen through a sterile single-lumen catheter advanced through the biopsy channel of the endoscope; at the level of the curvature of the distal trachea, the fluid deposit was re-aspirated and transferred into sterile plain tubes for microbiological evaluation. Immediately following the TW, a BAL sample was collected for cytological examination. The endoscope was advanced to the tracheal bifurcation, where 60 mL of a 0.5% lidocaine hydrochloride solution was sprayed through a sterile single-lumen catheter inserted into the biopsy channel of the endoscope; as the coughing reflex was inhibited, the endoscope was furtherly advanced into the bronchial tree. Once the endoscope was wedged within a segmental bronchus, a 300 mL sterile saline (0.9%) was instilled, and the fluid was immediately aspirated. The collected BAL was stored in sterile EDTA tubes and subjected to processing within 90 min (Lo Feudo *et al.* 2021b).

3.4.2.4 Cytological Examination of the BAL and Microbiological Examination of the TW

To perform cytological examination, 300 μL of pooled BAL was cytocentrifugated (Rotofix 32, Hettich Cyto System, Tuttlingen, Germany) at 26 g for 5 min. The slides were air dried, stained with May–Grünwald–Giemsa and Perl's Prussian blue, and observed under a light microscope at 400× and 1000× for a 400-cell leukocyte differential count. To perform the microbiological examination, 10 μL of TW were cultured on blood agar plates (5%) and incubated at 37 °C; after 48 h, bacterial species identification and CFU/mL count were performed (Lo Feudo *et al.* 2021b).

3.4.2.5 Group Allocation

Based on the results obtained with BAL cytology, horses were divided into groups following two different models.

In the first model, horses were divided into MEA and non-MEA groups: in particular, horses were considered affected by MEA when BAL cytology consisted of neutrophils > 10% and/or eosinophils > 5% and/or mast cells > 5%. In this case, we decided to adopt these wider cutoff values because 115/116 horses showed an increase in at least one population of inflammatory cells in the BAL compared with normal values (neutrophils ≤ 5%, eosinophils ≤ 1%, mast cells ≤ 2%). The BAL cutoff values used in this model are those considered unequivocally consistent with MEA regardless of the technique used for cytological examination (Couetil *et al.* 2016).

In the second model, horses were divided into neutrophilic MEA, eosinophilic–mastocytic MEA, and mixed MEA groups based on the following inclusion criteria (Nolen-Walston *et al.* 2013, Lo Feudo *et al.* 2021a):

- Neutrophilic MEA (n-MEA): neutrophils > 5%, eosinophils ≤ 1%, mast cells ≤ 2%;
- Eosinophilic–mastocytic MEA (e-MEA): neutrophils ≤ 5%, eosinophils > 1%, and/or mast cells > 2%;
- Mixed MEA (m-MEA): neutrophils > 5%, eosinophils > 1% and/or mast cells > 2%.

In this model, stricter cutoff values were adopted in order to distinguish the different MEA subtypes. Only one horse presented all BAL cell types within normal limits and was not included in this classification.

3.4.2.6 Statistical Analysis

The data obtained by the present study were analyzed using descriptive statistics and evaluated for normality through the Shapiro–Wilk test. The

associations between endoscopic scores (PLH, TM, and TB) and fitness parameters were evaluated with the Spearman correlation. Analogously, the relationships between BAL cell-type percentages and fitness parameters were investigated using Spearman correlation or simple linear regression according to data distribution. All fitness parameters and BAL cytology results were then compared between MEA and non-MEA groups by means of the Mann–Whitney test or the unpaired t-test and between the n-MEA, e-MEA, and m-MEA groups by means of the Kruskal–Wallis test and Dunn's multiple comparisons test or ordinary ANOVA and Fisher's LSD test based on data distribution. Moreover, fitness parameters were compared between horses with TW-positive and -negative bacterial cultures through the Mann–Whitney or unpaired t-tests. The associations between age and endoscopic scores, BAL cytology, and fitness parameters were evaluated by means of Spearman correlation. The possible influence of sex on fitness parameters was evaluated with the Mann–Whitney test. Finally, age and sex were compared between MEA and non-MEA groups using, respectively, the Mann–Whitney test and Fisher's exact test and between n-MEA, e-MEA, and m-MEA by means of the Kruskal–Wallis test, Dunn's multiple comparisons test, and the chi-square test.

Normally distributed data are displayed as mean ± standard deviation (SD), while non-normally distributed data are expressed as median and interquartile range (IQR). Statistical significance was set at $p < 0.05$. Data were analyzed using a commercially available statistical software package (GraphPad Prism 9.4.1 for MacOS; GraphPad Software, San Diego, CA, USA).

3.4.3 Results

3.4.3.1 Horses

Among the Standardbred racehorses that were subjected to the diagnostic protocol described above, between 2002 and 2021, 116 patients met the inclusion criteria of the present study. The study population included 76 males (63 stallions and 13 geldings) and 40 females, ranging in age from 2 to 8 years (median 3, IQR 3–4 years). The MEA group included 80 horses (10 geldings, 46 stallions, 24 mares), from 2 to 8 years old (median 3, IQR 3–5 years), while the non-MEA group included 36 horses (3 geldings, 17 stallions, 16 mares), from 2 to 6 years old (median 3, IQR 3–4 years). The two groups were age- and sex-matched. The n-MEA group included 14 horses (2 geldings, 6 stallions, 6 mares), ranging in age from 2 to 8 years (median 3, IQR 3–4 years); the e-MEA group included 36 horses (3 geldings, 25 stallions, 8 mares), from 2 to 7 years old (median 3.5, IQR 3–4.75); the m-MEA group included 66 horses (8 geldings, 32 stallions, 26 mares), from 2 to 7 years old (median 3, IQR 3–4). The three groups were age- and sex-matched.

3.4.3.2 Endoscopic Scores, BAL Cytology, and TW Microbiology

Among the study population, a median PLH score of 2 (IQR 1–2), a median TM score of 1 (IQR 1–2), and a median TB score of 1 (IQR 1–2) were observed. The results of the BAL cytological examination in the study population and in different groups are shown in Table 15. Lymphocyte percentages were lower ($p = 0.0001$) and neutrophil percentages were higher ($p < 0.0001$) in the MEA group compared with the non-MEA group. Lymphocyte percentages were higher and neutrophil percentages were lower in the e-MEA group compared with the n-MEA (lymphocytes, $p = 0.0002$; neutrophils, $p < 0.0001$) and the m-MEA (lymphocytes, $p < 0.0001$; neutrophils, $p < 0.0001$) groups. Eosinophil and

mast cell counts were lower in the n-MEA group compared with the e-MEA (eosinophils, $p = 0.0008$; mast cells, $p < 0.0001$) and the m-MEA (eosinophils, $p = 0.0098$; mast cells, $p < 0.0001$) groups.

The bacterial culture of TW was negative in 62 horses (53.45%) and positive in the remaining 54 horses (46.55%); the most commonly isolated bacteria were *Streptococcus* spp. (39 horses, 72.22%), *Klebsiella* spp. (6 horses, 11.11%), and *Pasteurell*a spp. (5 horses, 9.26%).

Cell Type (%)	Population	MEA	Non-MEA	n-MEA	e-MEA	m-MEA
Macrophages	44.79 ± 9.33	43.79 ± 9.25	47.03 ± 9.23	46.43 ± 6.35	41.86 ± 9.52	46.05 ± 9.49
Lymphocytes	36.1 ± 12.25	33.25 ± 12.14 [a]	42.44 ± 10.05 [b]	32.36 ± 10.29 [a]	45.19 ± 8.71 [b]	31.94 ± 11.71 [a]
Neutrophils	10 (5–17)	14 (7–20) [a]	5 (3.25–8) [b]	18.5 (13.75-–30.25)[a]	3 (3–4.75) [b]	13.5 (8.75–20) [a]
Eosinophils	1 (0–3)	1 (0–4.75)	1 (0–2)	0 (0–1) [a]	2 (0–5.75) [b]	1 (0–3) [b]
Mast cells	4 (3–6)	5 (3–7)	4 (3–5)	2 (1–2) [a]	5 (4–6.75) [b]	4 (3–6) [b]

Table 15. Results of the cytological examination of the BAL in the whole study population; the MEA and the non-MEA groups; and the n-MEA, e-MEA, and m-MEA groups. Data are expressed as mean ± standard deviation if normally distributed and as median (interquartile range) if not normally distributed. Data on lines with different superscript letters are significantly different from each other ($p < 0.05$).

3.4.3.3 Fitness Parameters

The results regarding the fitness parameters measured during the incremental treadmill test in the study population; the MEA and non-MEA groups; and the n-MEA, e-MEA, and m-MEA groups are displayed in Table 16.

Variable	Population	MEA	Non-MEA	n-MEA	e-MEA	m-MEA
V200 (m/s)	8 (7–8.5)	8 (7–9)	8 (7–8.38)	7 (6.38–8.25)	8 (7.5–8.5)	8 (6.37–8.63)
VLa4 (m/s)	8.45 (7.15–9.3)	8.4 (7.03–9.35)	8.75 (7.65–9.3)	8.05 (6.63–8.75)[a]	9.1 (8.03–9.7)[b]	8.2 (7–9.13)[a]
HRLa4 (bpm)	206 (198–214)	204.8 (197.6–211.2)	210.2 (198.9–216.2)	199.1 (193.8–215.8)	210.6 (202.1–216.7)[a]	204.1 (195.7–210.9)[b]
Lac max (mmol/L)	20.71 ± 6.89	21.36 ± 6.99	19.27 ± 6.49	23.12 ± 4.89 [a]	18.22 ± 5.79 [b]	21.56 ± 7.46 [a]
Lac 1 (mmol/L)	19.26 ± 6.07	19.62 ± 6.09	18.41 ± 6.03	21.42 ± 5.69 [a]	17.13 ± 5.07 [b]	20.01 ± 6.39 [a]
Lac 5 (mmol/L)	20.14 ± 6.94	20.77 ± 6.99	18.65 ± 6.69	21.79 ± 4.99	17.93 ± 6.08 [a]	21.04 ± 7.47 [b]
Lac 15 (mmol/L)	16.8 ± 7.85	17.58 ± 8.02	14.95 ± 7.22	17.94 ± 4.27	14.11 ± 6.62 [a]	18.06 ± 8.68 [b]
Lac 30 (mmol/L)	10.77 (6.36–15.27)	11.71 (7.54–15.97)	9.07 (4.9–14.49)	11.24 (8.73–14.39)	8.38 (4.29–13.82) [a]	11.83 (7.76–15.83) [b]
HR 1 (bpm)	152 ± 18.64	150.7 ± 19.19	154.9 ± 17.2	153.8 ± 18.55	150 ± 18.8	152.7 ± 18.78
HR 5 (bpm)	117.5 (107.3–127.8)	117 (108.3–127.5)	119 (106–127.8)	122 (111.5–130)	120 (110.8–128.5)	117 (105.8–125.3)
HR 15 (bpm)	93.41 ± 17.1	92.74 ± 15.64	95 ± 20.32	94.75 ± 12.9	98.5 ± 18.43 [a]	90.35 ± 16.57 [b]
HR 30 (bpm)	74.8 ± 18.14	75.74 ± 17.68	72.56 ± 19.31	78.75 ± 14.54	74.71 ± 19.34	74.09 ± 18.26
V max (m/s)	11 (11–11)	11 (11–12)	11 (11–11)	11 (10.75–11)	11 (11–11.75)	11 (11–12)
Ht max (%)	64.78 ± 4.03	64.86 ± 3.89	64.44 ± 4.8	65.11 ± 3.52	64.80 ± 3.11	64.69 ± 4.37
pH min	7.16 ± 0.1	7.16 ± 0.1	7.15 ± 0.1	7.15 ± 0.1	7.14 ± 0.08	7.16 ± 0.11

Table 16. Fitness parameters obtained through the incremental test on a treadmill in the whole study population; the MEA and the non-MEA groups; and the n-MEA, e-MEA, and m-MEA groups. Data are expressed as mean ± standard deviation if normally distributed and as median (interquartile range) if not normally distributed. Data on lines with different superscript letters are significantly different from each other ($p < 0.05$).

3.4.3.4 Statistical Results

The PLH score was inversely correlated with HR5 ($p = 0.0322$, $r = -0.21$), HR15 ($p = 0.0214$, $r = -0.22$), HR30 ($p = 0.0218$, $r = -0.22$), and Htmax ($p = 0.0009$, $r = -0.47$). The TM score was inversely correlated with Htmax ($p = 0.021$, $r = -0.34$). No association was observed between the TB score and any fitness parameter.

The percentage of macrophages in the BAL was inversely correlated with VLa4 ($p = 0.0259$, $r = -0.21$), HRLa4 ($p = 0.0014$, $r = -0.29$), HR15 ($p = 0.0329$, $r = -0.21$), and HR30 ($p = 0.0441$, $r = -0.19$).

The lymphocyte count was positively correlated with VLa4 ($p = 0.0003$, $r = 0.33$), HRLa4 ($p = 0.0008$, $r = 0.31$), and HR15 ($p = 0.0379$, $r = 0.20$), while inversely correlated with Lacmax ($p = 0.0290$, $r = -0.20$), Lac1 ($p = 0.0231$, $r = -0.22$), Lac5 ($p = 0.0146$, $r = -0.24$), Lac15 ($p = 0.0142$, $r = -0.24$), and Lac30 ($p = 0.0024$, $r = -0.29$).

The percentage of neutrophils was inversely correlated with V200 ($p = 0.0466$, $r = -0.19$), VLa4 ($p < 0.0001$, $r = -0.35$), HRLa4 ($p = 0.0083$, $r = -0.24$), and Vmax ($p = 0.0241$, $r = -0.21$) and positively correlated with Lacmax ($p = 0.0011$, $r = 0.30$), Lac1 ($p = 0.0017$, $r = 0.30$), Lac5 ($p = 0.0013$, $r = 0.31$), Lac15 ($p = 0.0032$, $r = 0.28$), and Lac30 ($p = 0.0007$, $r = 0.32$).

No association was observed between eosinophil and mast cell percentages and any fitness parameter. Similarly, the microbiological results of the TW were not related to any fitness parameter.

When dividing the population into the MEA and non-MEA groups, no differences were observed between groups for any fitness parameters. In contrast, when dividing the population into n-MEA, e-MEA, and m-MEA groups, several differences in fitness parameters were detected between groups. In particular, VLa4 was higher in the e-MEA group compared with the n-MEA ($p = 0.0112$) and the m-MEA ($p = 0.0050$) groups; the HRLa4 was also higher in the e-MEA group compared with the m-MEA group ($p = 0.0103$).

The values of Lacmax and Lac1 were lower in the e-MEA group compared with the n-MEA (Lacmax, p = 0.0223; Lac1, p = 0.0333) and the m-MEA (Lacmax, p = 0.0179; Lac1, p = 0.0249) groups; finally, Lac5 and Lac15 were statistically lower in the e-MEA group compared with the m-MEA group (Lac5, p = 0.0356; Lac15, p = 0.0180).

Age showed a positive correlation with BAL mast cells percentages (p = 0.0342, r = 0.20), VLa4 (p = 0.0197, r = 0.22), Vmax (p = 0.0001, r = 0.35), and Htmax (p = 0.0034, r = 0.42); conversely, an inverse correlation was observed between age and BAL eosinophil counts (p = 0.0287, r = −0.20), PLH score (p < 0.0001, r = −0.50) and the TM score (p = 0.0239, r = −0.21). Compared with females, males had higher V200 (p = 0.0081), VLa4 (p = 0.0047), HRLa4 (p = 0.0137), and Vmax (p = 0.0382) and lower Lacmax (p = 0.0204), Lac1 (p = 0.0132), and Lac5 (p = 0.0123). No differences in age or sex distribution were detected between the MEA and non-MEA groups, nor between the n-MEA, e-MEA, and m-MEA groups.

3.4.4 Discussion

Mild–moderate equine asthma is considered one of the most common causes of poor performance in young racehorses, second only to orthopedic disorders (Allen *et al.* 2006, Wilsher *et al.* 2006). Several studies have attempted to identify and measure the effects of lower airway inflammation on the athletic capacity and consequent sports performance of racehorses. However, to date, the evidence for a relationship between MEA and performance is not yet consistent (MacNamara *et al.* 1990). The present study aimed to evaluate associations between the findings of airway endoscopy, BAL cytology, and TW bacteriology with a series of fitness parameters measured during a standardized incremental treadmill test in a population of poorly performing Standardbred racehorses. Briefly, only neutrophilic inflammation in the lower

airway proved to negatively affect aerobic capacity, while tracheal mucus, BAL mastocytosis and eosinophilia, and bacterial tracheal colonization did not seem to be associated with decreased athletic performance.

In previous studies, the associations between lower airway inflammation and performance were investigated either by evaluating BAL or TW cytology and microbiology and airway endoscopic scores or by dividing horses into MEA-affected and not-MEA-affected groups; performance assessment relied either on trainers' or owners' impressions, racing results, or fitness parameters measured during standardized exercise testing. In the present study, we decided to take into consideration a complete assessment of airway inflammation, including the endoscopic scores of PLH, TM, and TB; a cytological examination of the BAL; and a microbiological examination of the TW.

Concerning endoscopic scores, in our study, horses with higher PLH scores showed lower heart rates during the recovery period; the clinical meaning of this association is unsure, as it may be the consequence of the less-demanding physical work undergone by young horses. In fact, it is known that PLH is more severe in younger horses and tends to de- crease naturally with age (Auer *et al.* 1985, Hobo *et al.* 1995, Holcombe *et al.* 2006, Koblinger *et al.* 2011, Lo Feudo *et al.* 2021); accordingly, in our study, younger horses had higher PLH scores and reached a lower maximal speed, and this could explain why horses with higher PLH scores recovered faster. Few studies had previously investigated the possible relationship between the severity of PLH and sports performance, and none of them reported any association in racehorses or in dressage and show-jumping horses (Auer *et al.* 1985, Hobo *et al.* 1995, Holcombe *et al.* 2006, Saulez and Gummow 2009, Widmer *et al.* 2009, Richard *et al.* 2010b), suggesting that PLH does not impact athletic capacity; only in one study on endurance horses was more severe PLH observed in poorly

performing horses compared with controls (Fraipont *et al.* 2011). In the present study, higher scores for both PLH and TM were associated with a lower maximum hematocrit. It is known that, during exercise, the oxygen-carrying capacity of the horse's blood increases proportionally with the rise in hematocrit; however, postexercise hematocrit failed to be associated with performance in a previous study (Evans *et al.* 1993). Therefore, the clinical meaning of this finding and its effects on performance are unclear, as the lower Ht observed in our study may be related to the younger age of horses showing higher PLH and TM scores. Interestingly, in our study, the TM score was not associated with any other fitness parameter, suggesting that no direct effect of mucus accumulation on decreased athletic capacity exists; nevertheless, the median and interquartile ranges of the TM score were low, indicating that most horses in the study population only showed a little or moderate accumulation of tracheal mucus, while a more marked accumulation was rarely observed. Therefore, the paucity of horses with high TM scores may have prevented us from highlighting any significant correlation between tracheal mucus accumulation and fitness parameters; as a matter of fact, some previous studies reported an impact from mucus accumulation on performance only when present in large amounts (Holcombe *et al.* 2006, Widmer *et al.* 2009). In general, an association between TM scores and poor performance was reported by numerous studies on Standardbred trotters (MacNamara *et al.* 1990, Richard *et al.* 2010b), Thoroughbred racehorses (Holcombe *et al.* 2006), endurance horses (Fraipont *et al.* 2011), and dressage and show-jumping horses (Widmer *et al.* 2009). However, in two studies on Thoroughbreds, no association between TM score and racing results was detected (Saulez and Gummow 2009, Salz *et al.* 2016), in accordance with our findings. Therefore, consistent evidence about the impact of TM accumulation on performance is still lacking (Kinnison *et al.* 2022). In the present study, the

blunting of the tracheal bifurcation was also considered, but no association between the TB score and any fitness parameters was found; to the best of the authors' knowledge, no studies have previously investigated its possible association with sports performance. In the case of TB, scores were generally low among our population, probably because thickening and edema of the tracheal bifurcation are more typical of severe equine asthma (Robinson 2003, Koch *et al.* 2007, Koblinger *et al.* 2011, Lo Feudo *et al.* 2021b).

When considering the cytology of the BAL, it is known that neutrophilia is a common finding among poorly performing racehorses (Allen *et al.* 2006). In the present study, we found significant effects caused by the percentage of neutrophils on several fitness parameters: in particular, as neutrophils increased, the values of V200 and VLa4 and the maximal exercise speed decreased, while the peak of plasma lactate concentration and plasma lactate concentrations during recovery time increased. These results suggest that neutrophilic inflammation in the lower airway impairs aerobic capacity, probably by interfering with gas exchanges at the alveolar–capillary level; as a consequence, hypoxemia may occur at lower speeds, with an earlier switch to anaerobic metabolism and prolonged lactate accumulation. Indeed, horses become fatigued at lower speeds, and high lactate concentrations remain longer after the end of the exercise. A number of previous studies reported a relationship between BAL neutrophilia and decreased performance: in particular, it was associated with exercise intolerance during strenuous exercise (Fogarty and Buckley 1991) and worse racing results in Thoroughbred racehorses (MacNamara *et al.* 1990) and with performance below expectations in Standardbred trotters (Richard *et al.* 2010b) and endurance horses (Fraipont *et al.* 2011). To the best of the authors' knowledge, no studies have denied the association between BAL neutrophilia and performance when defined on the basis of trainers' impressions or racing results. Conversely, contrasting results

have been reported when investigating the effects of BAL neutrophilia on fitness parameters measured during exercise tests on treadmills. In a study on Standardbreds, the cytological findings of the BAL were not associated with any indices of athletic capacity, including V200, VO2 max, maximal carbon dioxide production, respiratory quotient, and the number of steps completed during the treadmill test (Mazan and Hoffman 2001). Similarly, another study including Thoroughbred and Standardbred racehorses reported no relationship between BAL cytology and blood gas values, the peak of lactate concentration, and minimum pH measured during treadmill exercise (Nolen-Walston *et al.* 2013). Only a preliminary study by our research group observed an association between BAL neutrophilia and a lower aerobic–anaerobic thresh- old in Standardbreds (Stucchi *et al.* 2020). Therefore, the present study is the first one that highlights any measurable effects of BAL neutrophilia on physiological responses to treadmill exercise in horses. It is worth mentioning that macrophage and lymphocyte percentages were also correlated with some fitness parameters; however, it is hard to hypothesize a causative relationship, as the relative percentages of these cell populations may increase or decrease as a consequence of neutrophil variations. Finally, no association between eosinophil and mast cell percentages and any fitness parameters was observed; while no studies have reported a relationship between BAL eosinophils and performance, BAL mastocytosis has been shown to be strongly correlated with worse racing results and a decreased likelihood of winning in a study on Thoroughbred racehorses, impairing performance even to a greater degree compared with BAL neutrophilia (Ivester *et al.* 2018). No other studies have reported a similar association, which certainly deserves further investigation. Interestingly, some previous studies investigated the relationship between performance and the cytological profile of the TW. Two studies, based on racing results, detected no association in Thoroughbred

racehorses (Holcombe *et al.* 2006, Salz *et al.* 2016); in particular, in one of them, horses that actually raced showed higher TW neutrophil percentages compared with those that did not race, suggesting that the increase in tracheal neutrophils may be a consequence of intense exercise (McKane *et al.* 1993, Couetil and Denicola 1999, Holcombe *et al.* 2006). Probably for the same reason, in dressage and show-jumping horses, tracheal neutrophilia was associated with a strong will to perform and a better general impression (Widmer *et al.* 2009). Conversely, other authors reported a relationship between higher percentages of neutrophils in the TW and poor performance in Standardbreds (Richard *et al.* 2010b) and endurance horses (Fraipont *et al.* 2011). The discordance between these studies may be due to the fact that tracheal neutrophilia does not necessarily reflect neutrophilic inflammation within the lung, as the lack of correlation between TW and BAL cytology has been widely documented (Derksen *et al.* 1989, Malikides *et al.* 2003); in fact, for this reason, the cytology of the TW is considered inappropriate for the diagnosis of MEA, which should rely on BAL cytological examination (Couetil *et al.* 2016).

Besides the evaluation of the direct effects of BAL inflammatory cells on fitness parameters, we also decided to compare them between horses affected and not affected by MEA and between different MEA subtypes. Previous studies, which classified horses merely based on the diagnosis of MEA, reported variable results about its effects on fitness parameters. In particular, two studies reported a more pronounced exercise-induced hypoxemia in MEA horses (Couetil and Denicola 1999, Sanchez *et al.* 2005), while other authors observed no difference in PaO2 during a treadmill test between healthy horses and horses with lower airway inflammation (Courouce-Malblanc *et al.* 2002). Regarding lactate concentration, MEA was not associated with VLa4 (Couetil and Denicola 1999, Sanchez *et al.* 2005) or with lactate concentrations at

different steps of the treadmill test (Courouce-Malblanc *et al.* 2002, Sanchez *et al.* 2005) in previous studies; conversely, MEA horses reached higher peaks of lactate in one study (Couetil and Denicola 1999) but not in another one (Sanchez *et al.* 2005). In a study reporting an association with maximum lactate, lactate concentrations during recovery time were also higher in MEA horses, while blood pH and bicarbonate concentrations were lower; moreover, no associations with V200 and the maximal speed were observed (Couetil and Denicola 1999). In our study, no differences between MEA and non-MEA horses were detected for any fitness parameter; it must be underlined that we selected the suggested wide cutoff values (Couetil *et al.* 2016); this could have prevented mildly asthmatic horses from being recognized as MEA-affected. The possible allocation of these "borderline" subjects in the healthy group may not have allowed us to detect significant differences between MEA and non-MEA groups. Unfortunately, we could not have used stricter cutoff values, as almost all the horses in our population would have been allocated in the MEA group. In general, MEA affects up to 80% of racehorses in training (Christley and Rush 2007, Ivester *et al.* 2018), but its higher prevalence in our population was probably biased by the exclusive inclusion of poorly performing horses. Therefore, in our population, no healthy control horses could have been included, which represents the main limitation of the present study. Nevertheless, to overcome this limit, we divided the population based on MEA subtypes: neutrophilic, eosinophilic–mastocytic, and mixed (Couetil *et al.* 2016); indeed, different cytological profiles of MEA may reflect different etiopathogenetic mechanisms and implications for performance, as previously hypothesized by various authors (Nolen-Walston *et al.* 2013, Ivester *et al.* 2018). Our study revealed multiple differences in fitness parameters between horses with eosinophilic–mastocytic MEA and horses with either neutrophilic or mixed MEA, confirming this hypothesis; moreover, this finding once again

proved that neutrophilic inflammation was the main cause of decreased athletic capacity in our study population.

As the role of bacteria in MEA has long been discussed (Burrell *et al.* 1996, Christley *et al.* 2001a, Newton *et al.* 2003, Wood *et al.* 2005b), we also examined the possible relationship between the results of TW bacterial cultures and fitness parameters; however, we did not find any significant association. Only one previous study reported a negative impact of bacterial colonization on performance in racehorses, but in that study, the microbiological examination was performed on the BAL (Fogarty and Buckley 1991). In our study, bacteria were isolated from the TW: bacterial colonization in the trachea does not necessarily implicate lung neutrophilia (Lo Feudo *et al.* 2021b), and low bacterial counts could even be the result of sample contamination during endoscopy, which is not uncommon in MEA-affected horses (Wood *et al.* 2005b). Moreover, the isolation of bacteria is frequent in young racehorses due to the immaturity of their immunity (Wood *et al.* 2005b, Lo Feudo *et al.* 2021b). Therefore, results of microbiological examinations should be interpreted cautiously when considering a possible relationship with performance.

Finally, the effect of age and sex on fitness parameters was also noticed. In particular, age was positively correlated with VLa4 and the maximal speed reached during the treadmill test; an explanation for these results may be that adult horses are probably fitter and more physically mature, in agreement with previous studies (Thiruvenkadan *et al.* 2009, Lo Feudo *et al.* 2022b). Similarly, males showed better fitness parameters compared with females, in agreement with previous reports (Persson 1997, Tanner *et al.* 2012, Lo Feudo *et al.* 2022b). However, as all groups were age and sex-matched, it is likely that the contribution of age and sex to influencing fitness parameters was equal among different groups and that detected differences between MEA subtypes are exclusively attributable to lower airway inflammation.

The results of the present study open the door to the possibility of further investigating the possible relationship between airway inflammation and other innovative parameters related to exercise response. In fact, over the last few years, some novel biomarkers have been identified for monitoring physiological responses to training in horses. In particular, multiple studies have reported an increase in circulating levels of acute phase proteins, such as serum amyloid A, associated with exercise intensity and the level of fitness of the horse, which may be useful for the detection of subclinical pathologies affecting performance (Witkowska-Piłaszewicz *et al.* 2018, Arfuso *et al.* 2020). Moreover, oxidant and antioxidant parameter assessment may aid in the evaluation of fitness in racehorses (Rossi *et al.* 2021, Arfuso *et al.* 2022). Finally, a recent preliminary study reported a correlation between infrared thermography performed at the level of the *musculus trapezius pars thoracica* region and blood lactate values during race training, suggesting its possible applicability in future studies to obtain useful fitness measurement in a completely noninvasive way (Witkowska-Piłaszewicz *et al.* 2020).

3.4.5 Conclusions

The present study confirms that lower airway neutrophilia negatively affects athletic performance in Standardbred racehorses by impairing aerobic capacity and causing a prolonged accumulation of lactate, probably due to inefficient gas exchanges at the alveolar–capillary level. In particular, the affected fitness parameters include a lower speed at a heart rate of 200 bpm, a lower speed and heart rate at the aerobic–anaerobic threshold, higher peak lactate, and prolonged lactate accumulation during recovery. Conversely, eosinophilic and mastocytic lung inflammation does not seem to interfere with any fitness parameters, nor does the possible concomitant bacterial colonization in the trachea. Endoscopic signs of upper and lower airway

inflammation, including pharyngeal lymphoid hyperplasia, tracheal mucus accumulation, and edema of the tracheal bifurcation, do not represent risk factors for decreased athletic capacity. In conclusion, the results of the present study suggest that the association between mild–moderate equine asthma and poor performance in racehorses should be attributed to neutrophilic inflammation, while the role of other signs of airway inflammation should be interpreted cautiously and probably need further investigation.

CHAPTER 4.

RESEARCH:

PROSPECTIVE STUDIES

4.1 CYTOKINE MRNA EXPRESSION IN THE BRONCHOALVEOLAR LAVAGE CELLS FROM HORSES AFFECTED BY DIFFERENT EQUINE ASTHMA SUBTYPES

The results of the present study have been presented as an oral communication at the VAS Days meeting organized by the Veterinary and Animal Sciences PhD Course of the University of Milan on 29th September 2023. Moreover, the full study has been published in the following manuscript:

Lo Feudo CM, Stucchi L, Bazzocchi C, Lange Consiglio A, Comazzi S, Cozzi MC, Gusmara C, Gaspari G, Cialini C, Bizzotto D, Dellacà RL, Ferrucci F (2024) Cytokine mRNA expression in the bronchoalveolar lavage cells from horses affected by different equine asthma subtypes. J Equine Vet Sci, 27:135:105033.

The study team included researchers from the Department of Veterinary Medicine and Animal Sciences of the University of Milan, the Department of Veterinary Medicine of the University of Sassari, and the TechRes Lab, Department of Electronics, Information and Biomedical Engineering of the Polytechnic of Milan.

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Conflicts of Interest: The authors declare no conflict of interest.

Ethical statement: Ethical approval for the study was obtained from the Animal Welfare Organization of the University of Milan (Protocol Number OPBA_122_2021). Written informed consent for the inclusion of patients in the present study was obtained by all owners.

4.1.1 Introduction

Equine asthma (EA) is a non-infectious, inflammatory, recurrent, and chronic condition of horses, clinically characterized by airflow obstruction, mucus hypersecretion and airway hyperreactivity (Bullone and Lavoie 2020). Based on the severity and recurrence of the condition, it can be classified as mild-moderate (MEA) or severe equine asthma (SEA). While MEA can affect horses of any age and is associated with decreased athletic performance, tracheal mucus accumulation, occasional cough, and nasal discharge (Couetil *et al.* 2016), SEA affects adult horses over 7 years of age (Hotchkiss *et al.* 2007) and is characterized by recurrent episodes of dyspnea, cough, and bronchoconstriction (Leclere *et al.* 2011). Clinical EA severity is reflected by impaired respiratory function and, in particular, by increased pulmonary resistance and decreased pulmonary reactance, indicating structural obstruction and loss of tissue elasticity (Stucchi *et al.* 2022). Moreover, based on the relatively increased cell populations in the bronchoalveolar lavage fluid (BALf), different cytological EA subtypes have been described, including neutrophilic EA, mastocytic EA, eosinophilic EA, and mixed granulocytic EA (Bedenice *et al.* 2008, Lavoie *et al.* 2011). However, etiopathogenetic, clinical and prognostic differences between different EA severity and cytological subtypes are still loosely defined (Couetil *et al.* 2020).

In EA-affected horses, symptoms are exacerbated by exposure to environmental antigens, which determines a hypersensitivity reaction in genetically susceptible patients (Leclere *et al.* 2011, Bullone and Lavoie 2015, Bullone and Lavoie 2020). However, pathogenetic mechanisms of EA have not been fully understood, and previous studies reported inconsistent results regarding the type of immune response involved. Indeed, most studies reported a predominant Th2 immune response in asthmatic horses (Lavoie *et al.* 2001, Bowles *et al.* 2002, Cordeau *et al.* 2004, Dewachi *et al*. 2006, Beekman *et*

al. 2012), but other authors observed the involvement of other immune response types, such as innate (Laan *et al.* 2006a, Hughes 2011), Th1 (Ainsworth *et al.* 2003), Th17 (Debrue *et al.* 2005, Korn *et al*. 2015, Pacholweska *et al.* 2017, Gressler *et al.* 2022), or mixed responses (Giguere *et al.* 2002, Horohov *et al.* 2005, Lavoie-Lamoureux *et al.* 2010, Lavoie *et al.* 2011). The heterogeneity of these findings suggests that multiple molecular endotypes of EA may exist, characterized by different pathogenetic pathways, which may be associated with different EA subtypes or disease stages (Horohov *et al.* 2005, Padoan *et al.* 2013, Richard *et al.* 2014, Montgomery *et al.* 2018, Davis and Sheats 2021, Taylor *et al.* 2021). Recently, it has been hypothesized that, similarly to human asthma (Wenzel 2012, GINA Report 2023), EA may be distinguished into non-allergic, allergic, and late-onset phenotypes, which may differ in their pathogenetic mechanisms: differences may include the release of distinct cytokines and recruitment of specific inflammatory cell types, responsiveness or resistance to standard treatments, chronicity features, and reversibility of airway smooth muscle remodeling (Bond *et al.* 2018). The recognition of different immunological signatures may lead to a more accurate subcategorization of EA, allowing the development of novel therapeutic approaches based on new immunomodulatory agents (Klier *et al.* 2022) or precision medicine (Carnino *et al.* 2021).

To date, most studies investigating pathogenetic pathways of EA focused on one or two immune response types and evaluated their associations with EA clinical severity or cytological subtypes; however, to the authors' knowledge, no studies considered the contribution of each immune response type in a single population of EA-affected horses, nor associations with lung function have been reported. Therefore, in the present study, we decided to select at least one representative cytokine for every immune response type, including IL-1β (innate), IL-2 and IFN-γ (Th1), IL-4 (Th2), and IL-17 (Th17). We

hypothesized that different EA subtypes may be associated with the involvement of different cytokines, reflecting different immunological pathways. Hence, the aim of the present study was to evaluate the associations between the mRNA expression of the above-mentioned cytokines in the BALf of EA-affected horses and EA severity, cytological profile, and lung function measurements.

4.1.2 Materials and Methods

4.1.2.1 Ethics statement

Ethical approval for the study was obtained from the Animal Welfare Organization of the University of Milan (Protocol Number OPBA_122_2021). All procedures were collected as part of a routine diagnostic protocol for lower airway evaluation and written informed consent for the inclusion of patients in the present study was obtained by all owners.

4.1.2.2 Study population

Among a population of equine patients referred to the Equine Unit of the Veterinary Teaching Hospital (University of Milan, Italy) for lower airway evaluation or for a poor athletic performance protocol, fifteen client-owned horses were prospectively selected for inclusion in the present study. To be included in the study, horses had to undergo at least physical examination, airway endoscopy and collection of the BALF for cytological examination; exclusion criteria were represented by the presence of signs of systemic illness, a diagnosis of respiratory diseases other than EA, or having received steroidal or bronchodilator therapy during the previous 30 days. Based on the clinical and laboratory findings, horses were classified as SEA or MEA according to the following inclusion criteria:

- SEA: history of dyspnea, cough and/or exercise intolerance, and BALf neutrophils > 15% (Robinson 2001);
- MEA: history of poor performance, cough and/or nasal discharge, and BALf neutrophils > 10% and/or eosinophils > 1% and/or mast cells > 2% (Couetil *et al.* 2016, Woodrow *et al.* 2023).

Moreover, regardless of the clinical severity of EA, horses were classified as affected by neutrophilic EA subtype, when only increase in BALf neutrophils percentage was observed, or mixed EA subtype, when increase of more than one leukocyte population in the BALf was detected (Nolen-Walston *et al.* 2013). One horse was considered as healthy, as it did not have history of respiratory clinical signs, was normal at physical examination, and showed a normal BALF cytological profile.

4.1.2.3 Lung function testing

Since the severity of EA has been associated with different grades of lung function impairment, due to airflow obstruction, airway remodeling and progressive loss of tissue elasticity (Richard *et al.* 2009, Stucchi *et al.* 2022), we decided to test lung function in included horses. However, due to the unavailability of the necessary equipment during some periods of the study, only 8/15 horses underwent this procedure. The test was performed after clinical examination, and before airway endoscopy. During the test, horses were not sedated, and their head was maintained in a neutral position to avoid any interference with spontaneous breathing. A recently developed oscillometry portable device, based on the forced oscillation technique, was used (Bizzotto *et al.* 2022). The measurements were performed by applying at the horse airway opening small amplitude sinusoidal pressure waveforms at frequencies ranging from 2 to 6 Hz, with each frequency being applied for 30 seconds. Airflow and pressure were sampled and stored on a laptop for

subsequent analysis. After data recording, the respiratory input impedance was computed and reported as resistance (Rrs) and reactance (Xrs) by a least squares algorithm using Matlab software framework (Matlab, MathWorks, USA). Briefly, Rrs is defined as the resistance to flow in the airways, which depends on the caliber and architecture of the airways; Xrs, instead, represents their capacitive and inertive properties, reflecting the stiffness of the lungs and their distensibility (Oostven *et al.* 2003). From within-breath impedance tracings, the whole breath, inspiratory and expiratory Rrs and Xrs were calculated. Data were filtered following visual inspection and the values of the flow-shape index, excluding breaths showing the presence of artifacts (Bizzotto *et al.* 2022).

4.1.2.4 Airway endoscopy and BALf examination

Airway endoscopy and BALf collection were performed as described elsewhere (Lo Feudo *et al.* 2022). In brief, after horse sedation with detomidine hydrochloride (0.01 mg/kg IV; Domosedan; Vetoquinol, Italy) and restriction with a twitch, a flexible videoendoscope (EC-530WL-P, Fujifilm, Tokyo, Japan) was passed through the left nasal passages and the upper and lower tracts of the respiratory system were examined. Once the tracheal bifurcation was visualized, 60 mL of a 0.5% lidocaine hydrochloride solution was sprayed to inhibit coughing reflex; then, the endoscope was passed into the right bronchial tree until it was wedged firmly within a segmental bronchus. Here, a 300 mL pre-warmed sterile saline 0.9% was instilled through a sterile catheter inserted into the biopsy channel of the endoscope, and immediately re-aspirated. The BALf sample was then divided into aliquots: 10 mL were stored in sterile EDTA tubes for cytological examination, while the residual BALf was stored in one or more 50 mL sterile plain tubes for cytokine

expression analysis. For the cytological examination, within 90 min from the BALf collection, 300 μL of pooled BALf were cytocentrifugated (Rotofix 32, Hettich Cyto System, Germany) at 25 g for 5 min. The slides were air-dried, stained with May-Grünwald Giemsa and observed under a light microscope at 400x and 1000x for 400-cell leukocyte differential count (Lo Feudo *et al.* 2021).

4.1.2.5 Cells isolation, RNA extraction and cDNA synthesis

To allow cytokines expression analysis, cells were isolated from the residual BALF. With this aim, the residual BALF was centrifugated at 250 g for 10 min at 20 °C to obtain a cell pellet, and the supernatant was removed. Cells were re-suspended in 2 mL Dulbecco modified Eagle's medium (DMEM) high glucose supplemented with 2 mM L-glutamine, 0.1 mg/mL streptomycin, 100 IU/mL penicillin, and 5 μg/mL gentamicin, and counted using a Burker chamber with the Trypan Blue dye exclusion assay. A suspension of 2 million cells was then transferred into a sterile plain 2 mL tube, centrifugated at 250 g for 10 min at 20 °C to obtain a cell pellet, and the supernatant was removed. A 350 μL lysis buffer, composed by RLT buffer (a guanidine-thiocyanate–containing lysis buffer) supplemented with β--mercaptoethanol at 1:100 concentration, included in a commercial kit for RNA isolation (RNeasy Mini Kit, Qiagen, Venlo, The Netherlands), was added to the tubes, and the cells were resuspended. The obtained suspension was stored in a -80 °C freezer to allow later cytokines expression analysis. Total RNA was extracted from each sample using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and eluted in a final volume of 30 μl of RNase-free water. In order to remove any genomic DNA contamination, a DNase enzyme treatment was performed according to manufacturer instructions. Five hundred nanograms of RNA were retro-

transcribed to cDNA using the Quantitect Reverse Transcription Kit (Qiagen, Hilden, Germany) following manufacturer protocol, and cDNAs were stored at -80°C until use.

4.1.2.6 Target genes selection and gene expression profile

Genes involved in immune response (IL-1β, IL-4, IL-2, IFN-γ, and IL-17) were selected for subsequent molecular analyses. β-actin gene was used as reference as described by previous studies (Giguere *et al.* 2002, Perkins *et al.* 2008, Hughes *et al.* 2011, Joubert *et al.* 2011, Woodrow *et al.* 2023). Primer sequences and the amplification size of each fragment are described in Table 17.

Target gene	Sequence (5′→ 3′)	Amplification fragment size (bp)	Reference
IL-1β	F: TGGCAGAGGGGAATAGAAGGGTTTG R: ATAGGGAAGGCAGCTGGGCATTGA	86	Laan *et al.* 2006a
IL-4	F: TCGTGCATGGAGCTGACTGTA R: GCCCTGCAGATTTCCTTTCC	75	Gold *et al.* 2007
IL-2	F: TGCATCGCACTAACTCTTGC R: CAATTCTGTGGCCTTCTTGG	195	Sánchez-Matamoros *et al.* 2013
IFN-γ	F: TGGACACCATCAAGGAGGAC R: GGACCTTCAGATCATTTACCG	108	Sánchez-Matamoros *et al.* 2013
IL-17	F: GGCCTCAGATTACCACAACC R: ATCTCTCAGGGTCCTCGTTG	68	Padoan *et al.* 2013
β-actin	F: CAAGGCCAACCGCGAGAAGATGAC R: GCCAGAGGCGTACAGGGACAGCA	103	Laan *et al.* 2006a

Table 17. Primers sequences, amplification size and literature.

Genes expression was evaluated by quantitative PCR (qPCR) using an iQ5 Real-Time PCR instrument (Bio-Rad, California, USA) and Universal SYBR® Green Supermix (Bio-Rad, California, USA) as fluorescent molecule. The amplification conditions were: 150 nM (final concentration) of forward and reverse primers for β-actin gene fragment; 250 nM (final concentration) of forward and reverse primers for the other analysed genes. Annealing temperatures were 60°C for β-actin, IL-1β and IL-4 and 58°C for IL-2, IFN-γ and IL-17 fragment genes respectively. A melting profile was also included. Cycle threshold (Ct) values were determined for each sample and normalized

using β-actin gene as reference. The sample of the healthy horse was used as the reference sample to normalize cytokine expression data: in particular, the relative gene expressions for SEA and MEA samples were calculated using the ΔΔCt method and compared to the healthy sample considered as calibrator.

4.1.2.7 Statistical analysis

Data were collected on an electronic sheet (Microsoft Excel, Redmond, WA, USA) and analyzed using two commercial statistical software packages (GraphPad Prism 10.0.0 for MacOs, GraphPad Software, San Diego, CA; JASP 0.17.2.1 Intel, University of Amsterdam, Amsterdam, The Netherlands). Data normality was assessed by Shapiro-Wilk test, and descriptive statistics were performed. Normally distributed data are presented as mean ± standard deviation, while non-normally distributed data are reported as median and interquartile range (IQR). Cytokines mRNA expression was compared between horses affected by MEA and those affected by SEA, and between horses with neutrophilic EA or mixed EA, by unpaired t test or Mann-Whitney test, based on data distribution. Then, the associations between cytokines mRNA expression and BALF leukocyte populations were evaluated by Spearman's partial correlations, conditioned on the variable "age". The same test was used to assess the associations between cytokines mRNA expression and lung function measures, including total, inspiratory, and expiratory Rrs and Xrs at each frequency. To exclude the possible role of sex as confounding variable, cytokine expression was compared between horses of different sex by Kruskal-Wallis test. Statistical significance was set at $p < 0.05$. Correlation was considered as negligible if the Spearman r coefficient was 0.00 - 0.10, weak if 0.10-0.39, moderate if 0.40 - 0.69, strong if 0.70 - 0.89 and very strong if 0.90 - 1.00 (Schober *et al.* 2018).

4.1.3 Results

4.1.3.1 Horses

Fifteen horses were enrolled in the present study, including 2 mares, 7 geldings and 6 stallions, aged between 2 and 20 years old (median 5, IQR 2-12 years old). The population included 8 Thoroughbreds, 3 Warmbloods, 2 ponies, 1 Arabian, and 1 Quarter Horse. Based on inclusion criteria, 1 horse was healthy, 4 horses were classified as SEA, and 10 horses as MEA. Among asthmatic horses, 6 showed neutrophilic EA, and 8 showed mixed EA.

4.1.3.2 Lung function and BALf cytology

Lung function was tested by oscillometry in 8/15 horses, of those 1 healthy horse, 4 affected by mixed asthma, and 3 by neutrophilic asthma. Mean results and SDs are displayed in Table 18. Regarding BALf cytology, horses showed the following median (IQR) BALf differential count: macrophages 44 (40 – 51)%, lymphocytes 33 (25 – 35)%, neutrophils 19 (15 – 23)%, eosinophils 1 (0 – 1)%, and mast cells 2 (1 – 3)%.

	Whole breath	**Inspiratory**	**Expiratory**
Resistance ($cmH_2O \cdot s/L$)			
2 Hz	0.528 ± 0.229	0.445 ± 0.137	0.497 ± 0.255
3 Hz	0.613 ± 0.271	0.514 ± 0.204	0.680 ± 0.343
4 Hz	0.677 ± 0.248	0.546 ± 0.154	0.753 ± 0.359
5 Hz	0.747 ± 0.290	0.593 ± 0.154	0.866 ± 0.483
6 Hz	0.746 ± 0.255	0.599 ± 0.236	0.805 ± 0.349
Reactance ($cmH_2O \cdot s/L$)			
2 Hz	-0.048 ± 0.067	-0.050 ± 0.099	-0.074 ± 0.067
3 Hz	0.013 ± 0.055	-0.003 ± 0.053	0.005 ± 0.082
4 Hz	-0.006 ± 0.067	-0.013 ± 0.033	-0.057 ± 0.156
5 Hz	0.011 ± 0.073	0.009 ± 0.040	-0.011 ± 0.061
6 Hz	-0.033 ± 0.079	0.019 ± 0.030	-0.045 ± 0.040

Table 18. Oscillometry results in 8 asthmatic horses from the study population. Data are presented as mean ± standard deviation.

4.1.3.3 BALf cells cytokine mRNA expression

The median (IQR) BALf relative mRNA expression of IL-1β in EA-affected horses was 4.65 (1.29 – 11.19), IL-2 was 2.82 (0.59 – 6.90), IFN-γ was 2.70 (1.35 – 10.27), IL-4 was 20.41 (10.55 – 78.96), and IL-17 was 3.57 (2.27 – 25.71). Cytokines mRNA expression did not differ between horses of different sex. Results in the MEA and SEA groups, and in horses affected by neutrophilic or mixed asthma phenotypes are displayed, respectively, in Figure 8 and Figure 9. In SEA horses, IL-1β mRNA expression was significantly higher compared to MEA horses ($p = 0.041$); in horses with mixed asthma phenotype, the mRNA expression of IL-4 was significantly higher compared to horses with neutrophilic asthma phenotype ($p = 0.008$). The expression of the other cytokines did not differ between EA subtypes.

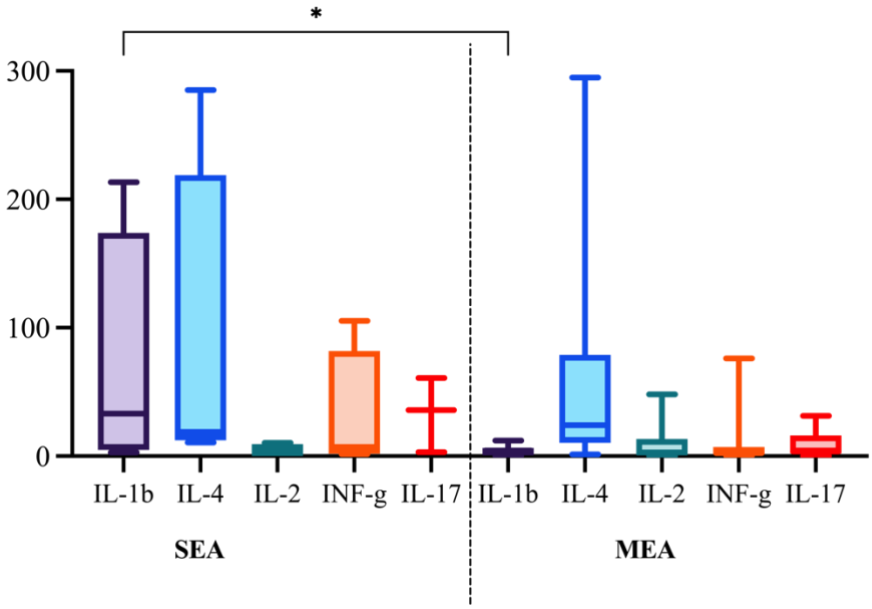

Figure 8. Scatter plots showing the medians (bars) and IQRs (fine lines with serifs) of the mRNA expression of IL-1β, IL-2, IFN-γ, IL-4, and IL-17 in horses affected by severe (SEA) or moderate equine asthma (MEA). Statistical significance is shown as * ($p < 0.05$).

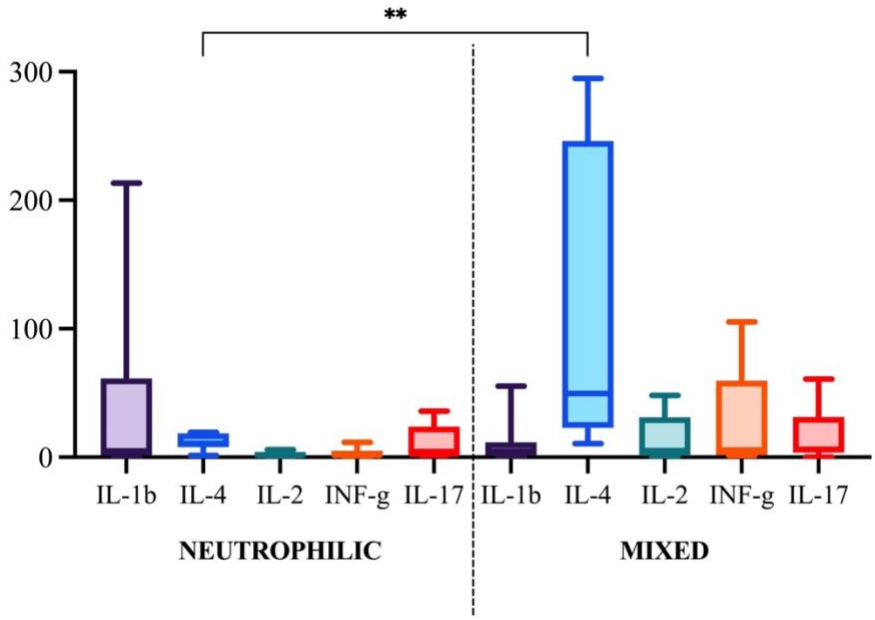

Figure 9. Scatter plots showing the medians (bars) and IQRs (fine lines with serifs) of the mRNA expression of IL-1β, IL-2, IFN-γ, IL-4, and IL-17 in horses affected by neutrophilic or mixed granulocytic equine asthma. Statistical significance is shown as ** ($p < 0.01$).

A positive moderate correlation was observed between the percentage of neutrophils in the BALf and the mRNA expression of IL-1β ($p = 0.038$, $r = 0.56$). The percentage of mast cells in the BALf was positively moderately correlated with the mRNA expression of IL-4 ($p = 0.028$, $r = 0.59$) and IFN-γ ($p = 0.029$, $r = 0.58$). A trend of positive moderate correlation was also observed between the BALf eosinophils percentage and the mRNA expression of IL-17 ($p = 0.058$, $r = 0.54$).

Concerning lung function results, the mRNA expression of IL-1β was strongly correlated with increased expiratory Rrs at 5 Hz ($p = 0.047$, $r = 0.76$). The mRNA expression of IL-4 was strongly inversely correlated with the whole-breath Xrs at 5 Hz ($p = 0.046$, $r = -0.76$), and very strongly with the expiratory Xrs at 5 Hz ($p = 0.003$, $r = -0.93$). Finally, the mRNA expression of IL-17 was very strongly inversely correlated with the expiratory Xrs at 5 Hz ($p = 0.009$, r

= -0.92). The mRNA expressions of IL-2 and IFN-γ were not associated with lung function.

4.1.4 Discussion

Multiple immunologic pathways have been implicated in equine asthma, and it has been hypothesized that different immune response types reflect different EA stages or subtypes. In the present study, the increased expression of cytokines reflecting innate, Th2 and Th17 immune response types (IL-1β, IL-4, IL-17) seemed to contribute to distinctive pathogenetic processes of EA, supporting the hypothesis that a mixed inflammatory/immune response is involved in this disease. Conversely, the involvement of Th1 response seemed questionable.

In the literature, the immune response type most commonly implicated in the pathogenesis of EA has been Th2 response; indeed, many authors reported its pivotal role in SEA (Lavoie *et al.* 2001, Cordeau *et al.* 2004, Bullone and Lavoie 2015) or MEA (Beekman *et al.* 2012), while others did not observe any increase of Th2 cytokines expression in asthmatic horses (Ainsworth *et al.* 2003, Kleiber *et al.* 2005, Hughes *et al*. 2011). Therefore, it has been hypothesized that Th2 response may play a role in EA only in specific disease stages, and some studies were conducted to test this hypothesis, leading to discordant findings (Horohov *et al.* 2005, Cordeau *et al.* 2004). In our study, we evaluated the expression of the Th2-related cytokine IL-4. Previous studies reported an increase of IL-4 expression in horses affected by SEA (Lavoie *et al.* 2001, Beadle *et al.* 2002, Bowles *et al.* 2002, Giguere *et al.* 2002, Cordeau *et al.* 2004, Horohov *et al.* 2005) and MEA (Lavoie *et al*. 2011); however, others did not detect any differences between healthy and asthmatic horses (Ainsworth *et al.* 2003, Hughes *et al.* 2011, Richard *et al*. 2014). In the present study, although IL-4 was over-expressed in EA-affected horses compared to the reference control horse,

it did not seem to increase with the severity of the disease. Conversely, its expression was higher in horses with a mixed granulocytic EA subtype compared to the neutrophilic subtype, and correlated, although only moderately, with increased BALF mast cells percentage, similarly to the findings of previous studies (Lavoie *et al.* 2011, Beekman *et al.* 2012). Moreover, IL-4 expression was strongly correlated with decreased pulmonary reactance, especially in the expiratory phase of the breath; this finding suggests that this cytokine may play a role in expiratory flow limitation due to bronchospasm (Dellacà *et al.* 2004), or in the elasticity reduction of the lung tissues, typically associated with progressive airway remodeling (Bullone and Lavoie 2020). Therefore, our results suggest that Th2 response may be involved in the airway hyperresponsiveness associated with BALF mastocytosis and, probably, in structural remodeling, analogously to what reported in human asthma (Ramakrishnan *et al.* 2019).

Pro-inflammatory Th1 response has also been widely investigated in the pathogenesis of EA. Some authors reported its implication in MEA (Richard *et al.* 2014), in the acute and chronic phases of SEA, and in airway remodeling (Horohov *et al.* 2005), while others did not find any role of Th1 response in EA pathogenesis (Kleiber *et al*. 2005, Hughes *et al.* 2011, Beekman *et al.* 2012). In the present study, the selected cytokines reflecting Th1 response were IL-2 and IFN-γ. Their expressions did not differ between horses affected by different severity nor cytological subtypes of EA, nor were associated with lung function. Only a moderate correlation was observed between IFN-γ and mast cells percentages, analogously to a previous report (Lavoie *et al.* 2011). In previous studies, IL-2 was not increased in horses with MEA compared to healthy horses (Hughes *et al.* 2011), or was correlated with BALF neutrophils count (Woodrow *et al.* 2023). Similarly, contrasting findings were observed concerning IFN-γ expression in EA; some studies reported an increase in SEA

(Beadle *et al.* 2002, Giguere *et al.* 2002, Ainsworth *et al.* 2003, Horohov *et al.* 2005, Woodrow *et al.* 2023) and in MEA (Lavoie *et al.* 2011, Richard *et al.* 2014), while others detected no associations with EA (Cordeau *et al.* 2004, Hughes *et al.* 2011) or even a decrease in SEA (Lavoie *et al.* 2001). Finally, some other studies reported its higher expression in horses with neutrophilic EA (Horohov *et al.* 2005, Beekman *et al.* 2012, Richard *et al.* 2014, Woodrow *et al.* 2023). The results of our study suggest that Th1 response does not seem to play a primary role in lower airway inflammation, nor in the pathogenesis of lung dysfunction. Indeed, the only correlation observed was not strong, and its significance may be considered cautiously due to the small size of the study population.

Airway hyperreactivity has also been associated with Th17 response (Debrue *et al.* 2005, Taylor *et al.* 2021). In the present study, the expression of IL-17 did not differ between horses affected by different severity and cytological subtypes of EA; only a trend of positive moderate correlation was observed with the BALF eosinophils percentage. This finding is supported by previous literature, reporting its involvement in abnormal immune responses and airway hyperresponsiveness (Debrue *et al.* 2005), typically associated with BALF eosinophilia (Richard *et al.* 2014). However, previous studies reported an association between IL-17 increased expression and neutrophilic lower airway inflammation (Beekman *et al.* 2012), both in horses with SEA (Debrue *et al.* 2005, Korn *et al.* 2015, Pacholewska *et al.* 2017, Boivin *et al.* 2018b, Gressler *et al.* 2022) and MEA (Bond *et al.* 2019a); conversely, other authors did not find IL-17 increase in MEA-affected horses (Taylor *et al.* 2021). Some studies also supported a role of Th17 in EA chronicization (Bullone and Lavoie 2015) and, in humans and mice, it is known to lead to mucous cell metaplasia and airway remodeling (Wang *et al.* 2010, Newcomb and Peebles 2013). According to the results of the present study, this phenomenon may also occur in EA: as a

matter of facts, IL-17 was very strongly correlated with decreased reactance, suggesting that it may contribute substantially to lung tissue elasticity loss.

Beside adaptive immune responses, pulmonary innate immunity also seems to be involved in the pathogenic pathway of EA (Franchini *et al.* 1998, Ainsworth *et al*. 2003, Laan *et al.* 2005). The increased expression of pro-inflammatory cytokines was reported in the BALF of SEA-affected horses by several studies (Franchini *et al.* 1998, Giguere *et al.* 2002, Ainsworth *et al.* 2003, Laan *et al.* 2005, Laan *et al.* 2006a, Lavoie *et al.* 2011, Padoan *et al.* 2013, Hansen *et al.* 2020), while discordant results were reported in MEA-affected horses (Hughes *et al.* 2011, Lavoie *et al.* 2011, Beekman *et al.* 2012, Richard *et al.* 2014, Davis and Sheats 2021, Taylor *et al.* 2021). In the present study, the expression of IL-1β was higher in horses with SEA than in horses with MEA, and was positively correlated with the percentage of neutrophils in the BALF, confirming the findings of previous reports (Giguere *et al.* 2002, Joubert *et al.* 2011, Lavoie *et al.* 2011, Beekman *et al.* 2012, Padoan *et al.* 2013, Hansen *et al.* 2020, Davis and Sheats 2021, Taylor *et al.* 2021). Interestingly, IL-1β was the only cytokine correlated with increased expiratory resistance: as resistance depends on airway caliber and architecture, its increase may be associated with bronchoconstriction and/or structural changes. Moreover, it is known that EA affects mainly the expiratory phase of the breath (Vandenput *et al.* 1998), which can further explain the correlations detected by this study.

Overall, the results of the present study agree with some previous reports and in contrast with others; the inconsistency of the literature on EA immunopathology is probably due to the subcategorization of EA mainly based on its severity rather than on different disease endotypes, which has not allowed to develop a proper clinically relevant classification yet (Woodrow *et al.* 2023). Therefore, the scientific community is still far from identifying potential biomarkers or therapeutic targets, which are presumably different

among distinct EA subtypes. The present study represents an attempt to identify different immunological pathways that may be related with different severity, cytological phenotypes, and degrees of lung dysfunction. However, several limitations were encountered, and many of them are intrinsic in the complexity of this disease. Indeed, horses sharing the same diagnosis may differ in their underlying pathogenetic mechanisms: for example, clinical signs may arise at a young age (early-onset) or during the adultness/elderly (late-onset), may differ in the allergic or non-allergic phenotype, and may be associated or not with other atopic disorders (Bond *et al.* 2018, David and Sheats 2021). Moreover, it has been demonstrated that cytokines expression in the BALF varies with time and is influenced by avoidance or exposure to allergens (Giguere *et al.* 2002, Cordeau *et al.* 2004, Horohov *et al.* 2005, Joubert *et al.* 2008), making it difficult to standardize and compare different studies. For this reason, to allow EA immunophenotyping, studies should be performed at several time points and be based on more sensitive and complete approaches, such as genome-wide transcriptomics. Another important factor influencing cytokines expression can be the wide range of age (Adams *et al.* 2008, Bullone and Lavoie 2017b) of EA-affected horses; in the present study, horses between 2 and 20 years old were included, which may have represented a bias for the conducted analyses. However, to overcome this limitation, we have considered age as a confounding variable in the correlation analyses between cytokines expression, BAL cytology and lung function. More numerous and better age-grouped populations are needed to confirm our findings. Some other limitations deserve to be discussed in the present study. First of all, normalization of mRNA cytokines expression was based on one single reference gene, and one single healthy control horse. The reference gene used in this study is β-actin, which was traditionally utilized in similar equine studies (Giguere *et al.* 2002, Laan *et al.* 2006a, Perkins *et al.* 2008, Hughes *et al.*

2011, Joubert *et al.* 2011, Woodrow *et al*. 2023); however, in one study, a low stability of β-actin in MEA-affected horses treated by corticosteroids was reported, suggesting more suitable reference genes (Beekman *et al.* 2012). Nevertheless, in the present study, recent pharmacological treatment represented an exclusion criterion, eluding possible biases. The lack of a control group of healthy horses remains the main limitation of the study: unfortunately, due to ethics issues, it was not possible to collect BALF from horses without the clinical indication for performing it, as it represents a relatively invasive procedure. In fact, the only healthy horse, used as the reference patient, underwent BALF collection as part of a poor athletic performance protocol to exclude pulmonary disorders; due to the absence of history of respiratory symptoms throughout the horse's life, the normal findings at clinical examination, and the within-ranges BALF cytology, we can be sure that the selected horse was really healthy. However, due to the lack of a control group, the present study could not compare EA-affected and healthy horses, but was rather focused on the evaluation of cytokines expression differences between different EA subtypes, and their associations with different cytological types of inflammation and lung function impairment. Unfortunately, lung function was evaluated only in 8 horses, due to the lack of availability of the oscillometry device during some parts of the study period, and reported correlations should be interpreted cautiously; however, despite the limited population, the novelty of these preliminary results deserves to be highlighted. Indeed, this is the first study reporting the associations between cytokines expression and lung function in horses; in order to understand whether the detected correlations are due to bronchoconstriction or chronic pulmonary remodeling, further studies should be conducted including endobronchial ultrasound and histomorphometric analysis of endobronchial biopsies.

4.1.5 Conclusion

The results of the present study support the hypothesis that multiple immune response types contribute to EA pathogenesis. While innate immune response seems to be associated with airway neutrophilic inflammation and disease severity, Th2 type response seems to be involved in the increase of airway mast cells. Moreover, lung function impairment may be correlated with different immune responses: the involvement of innate response seems to be associated with an increase of airway resistance, reflecting a reduction of airway caliber, while Th2 and Th17 type responses seem to contribute to reactance decrease, either due to acute bronchospasm or progressive loss of lung tissues elasticity. Due to the several limitations of the present study, further research is needed to confirm or confute our findings.

4.2 DIFFERENCES IN LUNG FUNCTION BETWEEN HORSES AFFECTED BY MILD-MODERATE EQUINE ASTHMA AND HEALTHY CONTROLS MEASURED BY A NOVEL PORTABLE OSCILLOMETRY DEVICE

The results of the present study are original. The research project was conducted by the Equine Sports Medicine Laboratory "Franco Tradati", Department of Veterinary Medicine and Animal Sciences of the University of Milan, with the technical and scientific support of the TechRes Lab, Department of Electronics, Information and Biomedical Engineering of the Polytechnic of Milan, and of the Equine Asthma Research Laboratory, Faculty of Veterinary Sciences of the University of Montreal.

The study is currently under review in Equine Veterinary Journal.

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Conflicts of Interest: The authors declare no conflict of interest.

Ethical statement: Ethical approval for the study was obtained from the Animal Welfare Organization of the University of Milan (Protocol Number OPBA_136_2022). Written informed consent for the inclusion of patients in the present study was obtained by all owners or delegated holders.

4.2.1 Introduction

Mild-moderate equine asthma (MEA) is a chronic disorder affecting the lower airways of horses, characterized by mild inflammation, subclinical airflow obstruction, excessive tracheobronchial mucus accumulation, and airway hyperreactivity. Clinical manifestations of MEA are typically subtle, including reduced athletic performance and chronic occasional or intermittent coughing. Therefore, ancillary procedures are essential for its diagnosis. This involves detecting tracheal mucus accumulation at airway endoscopy and observing mild increases in neutrophils, eosinophils, and/or mast cells percentages in the bronchoalveolar lavage fluid (BAL) (Couetil *et al.* 2016). However, these methods are invasive, requiring physical and pharmacological restraint. Moreover, they indicate lower airway inflammation, but only indirectly suggest airway obstruction. Thus, the development of non-invasive techniques capable of identifying airway obstruction represents a pivotal theme in respiratory research (Couetil *et al.* 2020).

In human asthma, the detection of alterations in pulmonary mechanics is considered the diagnostic gold standard (GINA Report 2023). In equine medicine, different lung function testing techniques have been reported, demonstrating a good sensitivity for the detection of severe airway obstructions (Young *et al.* 1997, Hoffman and Mazan 1999, Couetil *et al.* 2000, Couetil *et al.* 2001, Herzholz *et al.* 2003a, Herzholz *et al.* 2003b, Mazan *et al.* 2004, Van Erck *et al.* 2004a). However, contrasting results have been reported by different authors on their efficacy in identifying milder obstructive forms. Conventional lung mechanics showed higher respiratory resistance in horses with MEA compared to healthy controls in one study (Couetil *et al.* 2001), but not in another (Pirrone *et al.* 2007); similarly, forced expiration detected differences between healthy and MEA-affected horses4. However, these techniques are relatively invasive and not easily applicable in clinical

conditions. Electrical impedance tomography, though non-invasive and portable, lacks the sensitivity to detect mild obstructions (Herteman *et al.* 2021). Finally, oscillometry has been applied to horses with MEA, with two studies reporting higher inspiratory and expiratory resistance compared to healthy horses (Hoffman and Mazan 1999, Richard *et al.* 2009). While this method is non-invasive, sensitive, and does not need active collaboration of the patient (Klein *et al.* 2006), its application has, to date, been limited to research settings due to its complexity and limited commercial availability (Couetil *et al.* 2020).

Recently, a novel oscillometry device based on the forced oscillation technique (FOT) has been developed and preliminarily validated (Bizzotto *et al.* 2022). This device is wireless, portable, compact and, therefore, potentially applicable in clinical and field settings. The aim of the present study was to evaluate whether the new device can detect lung function differences between healthy horses and those affected by MEA.

4.2.2 Materials and Methods

4.2.2.1 Study population

Thirty-seven horses were enrolled in the present study. These horses were selected among a population of clinical patients examined at the Equine Unit of the Veterinary Teaching Hospital and/or referred to the Equine Sports Medicine Laboratory of the University of Milan between January and August 2023. The horses varied in age, sex, and breed, and were referred either for various purposes, including clinical check-up examinations, athletic fitness assessment, or respiratory tract evaluation. Horses without a history of respiratory symptoms and normal upon respiratory clinical examination were included as healthy controls (n = 15). Among these, horses with a recent

history of reduced athletic performance underwent additional assessments, including airway endoscopy and BAL cytology, to rule out lower airway inflammation. Horses were confirmed as healthy if presenting a tracheal mucus accumulation score < 2/5 and BAL cytology results showing neutrophils ≤ 10%, mast cells ≤ 5%, and eosinophils ≤ 5% (Couetil *et al.* 2016). Similarly, horses with a history and/or detection at physical examination of respiratory clinical signs were subjected to airway endoscopy and BAL cytology. Horses with a tracheal mucus score ≥ 2/5 and a BAL cytology consisting of neutrophils > 10%, and/or mast cells > 5%, and/or eosinophils > 5% (Couetil *et al.* 2016) were classified as MEA-affected horses (n = 22). Exclusion criteria included recent administration of corticosteroids or bronchodilators within the fourteen days prior to examination, presence of systemic illness, and current or historical presentation of dyspnea.

4.2.2.2 Airway endoscopy, BAL collection and cytology

Airway endoscopy and BAL collection were performed in all 22 MEA-affected horses, and in 5 out of 15 healthy horses. During endoscopy, performed as described elsewhere (Lo Feudo *et al.* 2022d), a 0 to 5 score was assigned to tracheal mucus accumulation (Gerber *et al.* 2004). For BAL collection, 60 mL of 0.5% lidocaine hydrochloride were sprayed at the level of the carena to suppress coughing reflex, and the endoscope was advanced into the bronchial tree, until firmly wedged. Here, 300 mL of sterile saline 0.9% were instilled and re-aspirated. The obtained BAL sample was preserved in sterile EDTA tubes and, within 90 minutes, 300 μL were cytocentrifugated at 25 g for 5 minutes. The resulting slides were air-dried, stained with May-Grünwald Giemsa, and 400-cell leukocyte differential count was performed (Lo Feudo *et al.* 2022d).

4.2.2.3 Oscillometry lung function testing

Lung function was tested in all enrolled horses using a recently validated oscillometry device, based on the forced oscillation technique (Bizzotto *et al.* 2022). During the tests, horses were either secured in stocks or kept in their boxes, with their heads loosely contained with halter and lead rope to maintain a neutral position and prevent any interference with spontaneous breathing. Measurements were taken at the horse's airway opening by applying small-amplitude sinusoidal pressure waveforms with frequencies ranging from 2 to 6 Hz, through a custom-designed horse mask. Each frequency was applied for 30 seconds, during which airflow and pressure data were recorded and stored on a laptop for subsequent analysis. The respiratory input impedance was calculated using a least squares algorithm in the Matlab software framework (Matlab; MathWorks) and expressed in terms of resistance (Rrs) and reactance (Xrs). Data were filtered based on visual inspection and the values of the flow-shape index, and breaths exhibiting artifacts were excluded. From within-breath impedance tracings, Rrs and Xrs values were obtained for the whole-breath, inspiratory and expiratory phases; moreover, ΔRrs and ΔXrs were calculated by the difference between the inspiratory and expiratory values. Frequency dependence of Rrs and Xrs, indicative of distal obstruction (Komarow *et al.* 2011), was calculated by the difference between whole-breath values obtained at 2 Hz and at 6 Hz (respectively, Rrs2 – Rrs6 and Xrs2 – Xrs6).

4.2.2.4 Data analysis

Data were gathered on an electronic spreadsheet (Excel; Microsoft Corp), and analyzed using two statistical software packages (Prism 10.0.3; GraphPad Software and JASP 0.17.3; JASP Team). The normality of data distribution was

assessed by the Shapiro-Wilk test, and descriptive statistics were performed. Normally distributed data are expressed as mean ± standard error (SE), while non-normally distributed data are presented as median and interquartile ranges (IQR). Age was compared between healthy and MEA groups by the Mann-Whitney test, whereas sex and breed distributions were compared between groups by the Chi-square test. To assess whether oscillometry parameters varied significantly with frequency, RM one-way ANOVA or Friedman test were performed within groups, based on data distribution. Oscillometry parameters and the values of frequency dependence of Xrs and Rrs were compared between groups using either the t-test or Mann-Whitney test, adjusted on the age variable. Statistical significance was set at $p < 0.05$.

4.2.3 Results

4.2.3.1 Horses

Among the study population (n = 37), 15 horses were classified as healthy (40.5%) and 22 horses were categorized as MEA (59.5%). Healthy horses were aged between 2 and 14 years (median 6, IQR 4 – 11 years), and MEA horses between 2 and 12 years (median 4, IQR 3 – 7 years). No significant difference in age was observed between the two groups. The healthy group included 9 mares (60%), 4 geldings (26.7%), and 2 stallions (13.3%), while the MEA group comprised 4 mares (18.2%), 8 geldings (36.4%), and 10 stallions (45.4%). Sex distribution was significantly different between groups ($p = 0.023$). A diverse array of breeds was represented in both groups. The healthy group was composed by 5 Thoroughbred (33.3%) and 4 Standardbred (26.7%) racehorses, 4 Warmbloods (26.7%), and 2 Quarter Horses (13.3%). The MEA group included 11 Thoroughbred (50%) and 7 Standardbred racehorses (31.8%), 2 Arabians (9.1%), 1 Warmblood (4.55%), and 1 Appaloosa (4.55%). No

significant disparities in breed distribution were discerned between the groups.

4.2.3.2 Airway endoscopy and BAL cytology

At airway endoscopy, the 5 examined healthy horses showed a median tracheal mucus score of 1 (0 – 1), while the 22 MEA horses showed a median score of 3 (2 – 4). Within the healthy group, the median percentages of BAL leukocytes were: 50 (49 – 50)% macrophages, 39 (39 – 41)% lymphocytes, 9 (8 – 9)% neutrophils, 0 (0 – 0)% eosinophils, and 2 (2 – 3)% mast cells. Conversely, in the MEA group, the median percentages were: 47 (41 – 51)% macrophages, 26 (24 – 33)% lymphocytes, 19 (16 – 23)% neutrophils, 0 (0 – 2)% eosinophils, and 3 (2 – 5)% mast cells.

4.2.3.3 Oscillometry

Whole-breath, inspiratory, expiratory, and Δ Xrs and Rrs obtained at each frequency in the healthy and MEA groups are displayed in Figure 1. Horses with MEA showed lower whole-breath ($p = 0.012$), inspiratory ($p = 0.018$), and expiratory ($p = 0.007$) Xrs at 2 Hz compared to their healthy counterparts. Moreover, horses with MEA had a higher Δ Xrs at 5 Hz in comparison to healthy horses ($p = 0.041$), with a similar trend observed at 4 Hz ($p = 0.064$). No other significant differences were detected between the groups for Xrs parameters at the remaining frequencies, nor for any of the Rrs measures.

In healthy horses, whole-breath, inspiratory, expiratory, and Δ Rrs significantly varied with oscillations frequency ($p < 0.001$); specifically, a positive frequency dependence was observed for the first three parameters, while Δ Rrs showed a negative frequency dependence. Conversely, all Xrs parameters did not change with frequency; only a trend was observed for positive frequency-dependence of inspiratory Xrs ($p = 0.069$). In MEA horses,

whole-breath, inspiratory, and expiratory Xrs and Rrs varied significantly with frequency ($p < 0.001$), showing a positive frequency dependence. Instead, Δ Xrs and Δ Rs were not frequency dependent. Finally, Xrs2 – Xrs6 was more negative in the MEA group (-0.110 ± 0.028 cmH20/L/s) compared to the healthy group (-0.031 ± 0.025 cmH20/L/s) ($p = 0.049$), confirming a stronger frequency dependence of Xrs in MEA-affected horses. Conversely, no significant differences were observed for Rrs2 – Rrs6 (MEA, -0.247 ± 0.040 cmH20/L/s; healthy, -0.199 ± 0.039 cmH20/L/s).

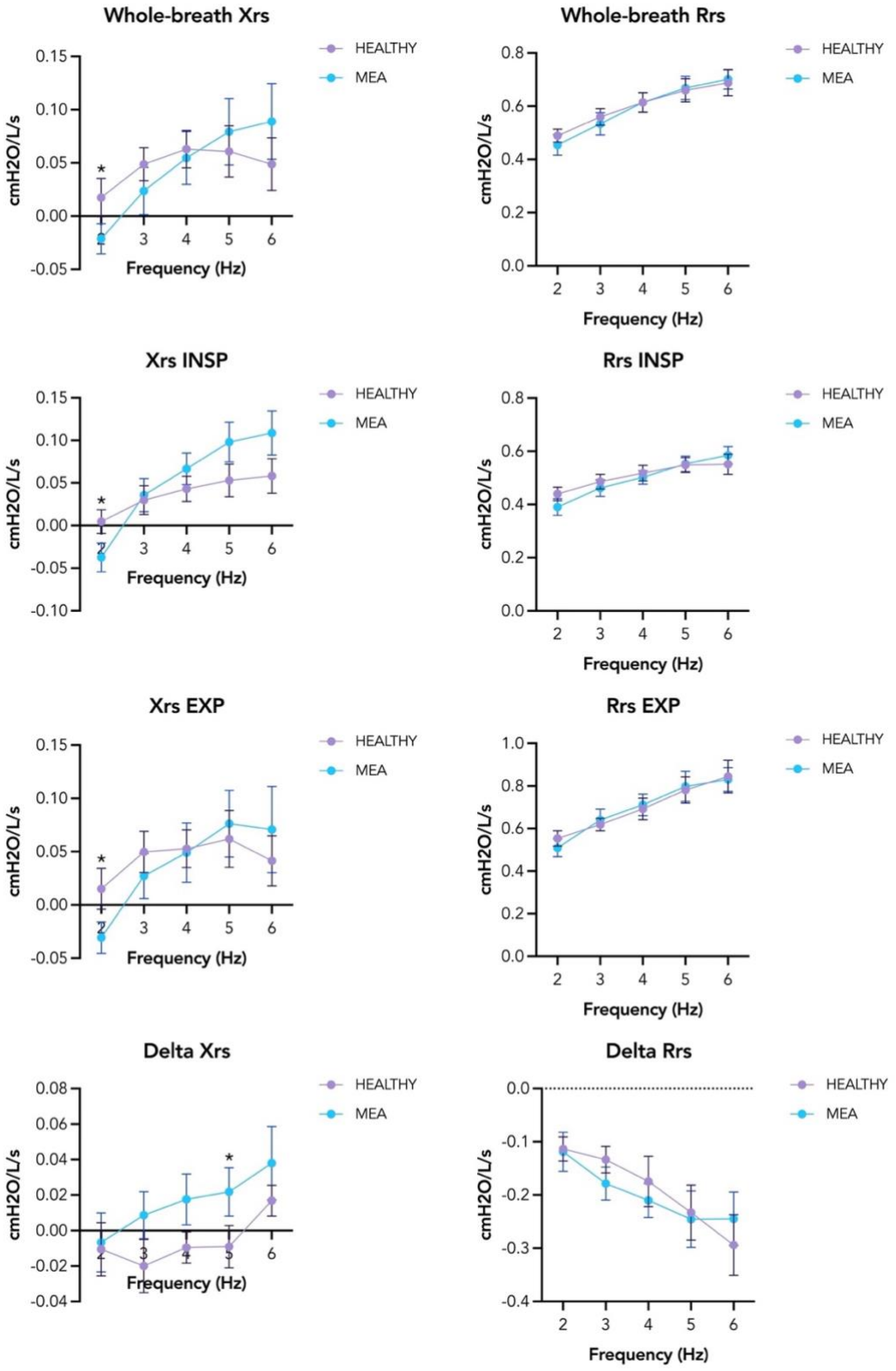

Figure 1. Oscillometry parameters, expressed as mean and SE, obtained in 15 healthy and 22 MEA horses at frequencies ranging from 2 to 6 Hz. Statistically significant differences between healthy and MEA horses are shown as * ($p < 0.05$).

4.2.4 Discussion

The diagnosis of MEA currently relies on quite invasive procedures, including airway endoscopy and BAL collection for cytological examination1. Therefore, the development of non-invasive systems capable of identifying lung dysfunction, even in cases of mild airway obstruction, is pivotal in equine respiratory medicine (Couetil *et al.* 2020). With this purpose, oscillometry has shown promise in previous studies; however, to date, its application has been limited to research settings. The present study aimed to evaluate whether a novel portable and wireless oscillometry device, designed for easy use in clinical and field settings (Bizzotto *et al.* 2022), could effectively detect lung function differences between healthy and MEA-affected horses. The results showed reduced Xrs at 2 Hz in MEA-affected horses, higher Δ Xrs at 5 Hz, and a more pronounced positive frequency dependance of Xrs. Conversely, parameters of Rrs did not exhibit significant differences between healthy ad MEA-affected horses.

Various techniques have been studied over the past decades for assessing lung function, including conventional pulmonary mechanics (Couetil *et al.* 2001, Pirrone *et al.* 2007), forced expiration (Couetil *et al.* 2000a, Couetil *et al.* 2000b), flowmetric plethysmography (Hoffman *et al.* 2007, Dixon *et al.* 2021), electrical impedance tomography (Herteman *et al.* 2021, Secombe *et al.* 2021), and oscillometry (Young *et al.* 1997, Hoffman and Mazan 1999, Van Erck *et al.* 2004a, Klein *et al.* 2006, Richard *et al.* 2009). While the first two methods are invasive, the latter three are not (Van Erck *et al.* 2004a, Hoffman *et al.* 2007, Herteman *et al.* 2021) and represent promising diagnostic tools for MEA. However, electrical impedance tomography did not detect any differences between healthy and MEA-affected horses in one study (Herteman *et al.* 2021), while flowmetric plethysmography has only been applied to MEA-affected horses after bronchoprovocation tests (Wichtel *et al.* 2016, Secombe *et al.* 2019b,

Dixon *et al.* 2021). Conversely, oscillometry studies have reported increased Rrs in MEA-affected horses under spontaneous-breathing conditions (Hoffman and Mazan 1999, Richard *et al.* 2009), suggesting its potential for clinical application. First described in 1956 by Dubois and colleagues (Dubois *et al.* 1956), oscillometry assesses the respiratory system's response to external superimposed forces, filtered from the signals obtained from spontaneous beathing (Hoffman 2002), by measuring resistance and reactance at different frequencies. Resistance reflects the resistive properties of the respiratory system, which depend on the caliber and architecture of the airways, while reactance describes its elastic and inertial properties (Dubois *et al.* 1956, Hoffman and Mazan 1999, Van Erck *et al.* 2004a). Oscillometry has demonstrated good sensitivity in horses affected by severe forms of equine asthma, showing increased Rrs and decreased Xrs compared to healthy horses (Hoffman and Mazan 1999, Van Erck *et al.* 2004a, Stucchi *et al.* 2022). Promising results have been reported also in the diagnosis of MEA, but its use has been confined to research conditions due to its complexity and lack of commercial availability (Couetil *et al.* 2020). A newly developed FOT device, specifically assembled and preliminarily validated for the equine species, may overcome this limitation. Indeed, this device is based on cost-effective technology and employs predominantly automated data processing algorithms (Bizzotto *et al.* 2022). In the present study, it enabled us to discern variations in Xrs between healthy horses and those affected by MEA: specifically, MEA-affected horses showed lower Xrs at 2 Hz across all phases of the breath, with the discrepancy being particularly enhanced during expiration. Conversely, no differences in Rrs parameters were observed between the two groups in our sample, which is in contrast with the findings of the two prior oscillometry studies on MEA. Specifically, one study reported elevated Rrs within the 1 to 3 Hz frequency range in MEA horses compared to controls, and no differences in Xrs

(Hoffman and Mazan 1999). In the other study, increased Rrs was observed at frequencies within 1 and 10 Hz, along with decreased Xrs at higher frequencies from 5 to 20 Hz; within-breath analysis confirmed these outcomes during both inspiratory and expiratory phases (Richard *et al.* 2009). To properly interpret these results, it is important to note that the resonance frequency in horses typically falls between 2 and 4 Hz (Young and Hall 1989, Onmaz *et al.* 2013). This suggests that lower frequencies likely pertain to the distal small airways, while higher frequencies are indicative of a more proximal respiratory tract, including central and upper airways. Therefore, while Rrs was significantly higher at lower frequencies, distinctions in Xrs were solely detectable at higher frequencies: this makes it improbable that these differences are indicative of distal airway obstruction. In our study, we observed lower Xrs only at 2 Hz: since frequencies ranging from 1 to 3 Hz are considered the most sensitive for the lower airways (Young *et al.* 1997, Hoffman 2002), and that the lungs' ability to store capacitive energy is mainly evident in the small airways (Bickel *et al.* 2014), our findings are likely representative of the smaller distal airways. Interestingly, differences were observed during each phase of the respiratory cycle, but the statistical significance was more pronounced expiration. This is consistent with the expiratory phase being the most impacted in equine asthma, characterized by increased expiratory effort and reduced expiratory flow (Couetil *et al.* 2001, Pirie 2014). To assess disparities between inspiratory and expiratory components, we calculated the Δ values. The Δ Xrs was higher in MEA-affected horses compared to their healthy counterparts at all frequencies, but statistical significance was only reached at 5 Hz. This observation further confirms the aforementioned explanations and is consistent with a previous study on horses with severe equine asthma during the remission phase (Stucchi *et al.* 2022), as well as in human patients with chronic obstructive pulmonary disease. This may be attributed to tidal

expiratory flow limitation, a consequence of the narrowing of some airway segments, known as choke points, resulting from dynamic compression of the peripheral airways. Essentially, when choke points develop, expiratory reactance decreases, leading to an increase in Δ Xrs (Pedersen and Butler 2011). The absence of observed differences in Rrs in our study population is not entirely clear. Similar to our results, a previous study found that horses with severe asthma during the remission phase did not exhibit variations in Rrs compared to healthy horses, but only in inspiratory Xrs (Stucchi *et al.* 2022). Our hypothesis is that Rrs is primarily linked to airway caliber and increases as it narrows (Kaminsky *et al.* 2022), and bronchoconstriction in MEA horses may be subclinical and so mild that it may not be easily detected (Couetil *et al.* 2020). Conversely, Xrs mainly reflects lung elasticity, which may decrease due to the progressive chronic remodeling of the lower airways, resulting in increased stiffness (Kaminsky *et al.* 2022). Indeed, in horses with MEA, airways alterations have been identified, including epithelial hyperplasia, smooth muscle fibrosis, and thickening of the lamina propria. These features are believed to contribute to airway obstruction in affected horses (Bessonat *et al.* 2022, Dupuis-Dowd and Lavoie 2022), potentially leading to reduced lung elasticity and, consequently, decreased Xrs. A recent study on human asthma found a correlation between increased bronchial wall thickness and both reactance and resistance (Chan *et al.* 2023), offering partial support for our hypothesis. While studies correlating oscillometry parameters and airway remodeling in horses are lacking, one study based on conventional lung mechanics reported an association between the deposition of collagen and elastic fibers and worsened pulmonary resistance and elastance (a component of reactance) values (Setlakwe *et al.* 2014). However, the relationship between lung function and airway remodeling remains unclear (Bedenice *et al.* 2008).

Therefore, the interpretation of our results should be considered as a hypothesis, which requires confirmation through future studies.

In the present study, in addition to assessing oscillometry parameters, we decided to analyze also the frequency dependence and compare it between healthy and MEA-affected horses. In particular, the frequency dependence of Rrs was positive in both groups, meaning that Rrs increased with increasing frequency, and no significant differences were observed between the groups. Interestingly, Xrs showed a positive frequency dependence exclusively in MEA-affected horses. Conversely, previous studies reported that Rrs did not physiologically change as a function of frequency, whereas horses with severe airway obstruction showed a negative frequency dependence of Rrs (Young *et al.* 1997, Hoffman and Mazan 1999, Van Erck *et al.* 2006). In another study including only MEA-affected horses, those with coughing exhibited a more negative frequency dependence of Rrs compared to non-coughing horses (Bedenice *et al.* 2008). A positive frequency dependence of Rrs was reported at low frequencies, consistent with our findings, in both a population of healthy horses (Van Erck *et al.* 2004a) and in horses with severe equine asthma during remission (Onmaz *et al.* 2013). Regarding the frequency dependence of Xrs, a positive frequency dependence has been reported in both healthy horses and those with lower airway obstruction (Van Erck *et al.* 2006, Richard *et al.* 2009, Onmaz *et al.* 2013). However, to the best of the authors' knowledge, no previous studies have investigated differences in the frequency dependence of Xrs between healthy and MEA-affected horses. In our study, the frequency dependence of Xrs appeared positive in both groups from a graphical perspective, but statistical significance was only reached in MEA-affected horses. Therefore, our study suggests that evaluating frequency dependence may be of interest in the diagnosis of MEA and deserves further investigation.

Overall, the results of the present study are encouraging and suggest that oscillometry testing could contribute to the diagnosis of MEA in clinical practice. However, several limitations still exist, and further studies are needed to establish it as a standalone diagnostic tool. Firstly, the sample size in our population was too small to determine possible cut-off values distinguishing between healthy and MEA-affected horses. Therefore, future studies should involve a larger number of patients to attempt to define normal ranges, with a specific focus on Xrs values, especially at lower frequencies (i.e., 2 Hz), as indicated by our results. Nevertheless, it is possible that universally applicable cut-off values may not be attainable, given that various variables beyond health status may influence lung function, including age, sex, and breed. Indeed, a previous study showed a correlation between age and both Rrs and Xrs in horses (Van Erck *et al.* 2004a); similarly, in humans, lung aging is characterized by lung function impairment, extracellular matrix remodeling, and airspace enlargement (Gremlich *et al.* 2021). For this reason, in the present study, we adjusted the statistical analyses for age, although it did not significantly differ between healthy and MEA groups. Lung function did not differ between horses of different genders in one study (Van Erck *et al.* 2004a), whereas a recent preliminary research suggested an influence of sex hormones on lung function in mares with severe equine asthma (Mainguy-Seers *et al.* 2022). In our study, groups were not sex-matched, because our study population consisted of referred clinical patients rather than a standardized research herd. For the same reason, horses of different breeds and uses were included and, although breed distribution did not differ between groups, the percentage of racehorses was higher in the MEA group. To the authors' knowledge, the influence of breed on lung function has never been investigated and cannot be ruled out. By exclusively including clinical patients, we were unable to perform a BAL on all healthy horses, but only in

those showing clinical signs possibly attributable to respiratory disorders. Indeed, performing a relatively invasive technique such as endoscopy and BAL for research purposes would have represented an experimental procedure. Therefore, in the remaining patients, the diagnosis of MEA was excluded based on clinical history and physical examination, albeit without cytological confirmation. Additional studies should be conducted to confirm or refute our findings, based on a larger, better-defined and more homogeneous population of horses.

In conclusion, the present study demonstrated a consistent reduction of Xrs at 2 Hz throughout the whole respiratory cycle in horses affected by MEA, which was highlighted during expiration. Furthermore, a notable increase in Δ Xrs at 5 Hz was observed in the MEA group, indicating potential expiratory flow limitation. Conversely, Rrs parameters did not show any significant variation based on the presence or absence of the disease. While the frequency dependence of Rrs was positive in all horses and did not differ between groups, a significant positive frequency dependence of Xrs was only evident in MEA-affected horses. These findings collectively highlight the potential utility of the novel oscillometry device in the assessment of MEA, with particular emphasis on the relevance of Xrs parameters.

4.3 RESPIRATORY OSCILLOMETRY TESTING IN RELATION TO EXERCISE IN HEALTHY AND ASTHMATIC THOROUGHBREDS

Some preliminary results of the present study, including only a small population of six horses, have been presented as a poster at the 28th SIVE (Italian Society of Equine Veterinarians) International Congress, held in Venice on 3rd-4th February 2023 (**Lo Feudo CM, Stucchi L, Bizzotto D, Dellacà R, Lavoie JP, Ferrucci F. Lung function testing by oscillometry and its relation to exercise in Thoroughbred racehorses: a preliminary study**).

The full study has been published in the following manuscript:

Lo Feudo CM, Stucchi L, Bizzotto D, Dellacà R, Lavoie JP, Ferrucci F (2024) Respiratory oscillometry testing in relation to exercise in healthy and asthmatic Thoroughbreds. Equine Vet J, doi: 0.1111/evj.14065.

The research project was conducted by the Equine Sports Medicine Laboratory "Franco Tradati", Department of Veterinary Medicine and Animal Sciences of the University of Milan, with the technical and scientific support of the TechRes Lab, Department of Electronics, Information and Biomedical Engineering of the Polytechnic of Milan, and of the Equine Asthma Research Laboratory, Faculty of Veterinary Sciences of the University of Montreal.

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Conflicts of Interest: The authors declare no conflict of interest.

Ethical statement: Ethical approval for the study was obtained from the Animal Welfare Organization of the University of Milan (Protocol Numbers OPBA_44_2022 and OPBA_37_2023). Written informed consent for the inclusion of patients in the present study was obtained by all owners or delegated holders.

4.3.1 Introduction

Thoroughbred racehorses exercise at maximal and supramaximal intensities, demanding an optimal aerobic capacity While the cardiocirculatory and musculoskeletal systems are highly efficient in horses, the respiratory system is considered the primary limiting factor to oxygen delivery, even in healthy subjects (Franklin *et al.* 2012). Hence, understanding the physiological changes in lung function associated with intense exercise becomes essential. While data on horses remain scarce and mostly date back to the 1980s (Art and Lekeux 1988, Art *et al.* 1988, Art *et al.* 1990, Davis *et al.* 2006), numerous studies have investigated the effects of exercise on lower airway tone in humans and, in recent years, researchers have attempted to clarify the heterogeneously reported findings (Pichon *et al.* 2005, Caggiano *et al.* 2017, Aggarwal *et al.* 2018, Engan *et al.* 2020, Gavrielatos *et al.* 2022). Indeed, exercise has been associated with two opposite phenomena: exercise-induced bronchodilation and exercise-induced bronchoconstriction. Bronchodilation appears to be the normal response to exercise (Lodrup Carlsen *et al.* 2006), due to parasympathetic activity withdrawal (Beck 1993, Pichon *et al.* 2005, Engan *et al.* 2020), increase in circulating cathecolamines (Kagawa and Kerr 1970, Pichon *et al.* 2005, Engan *et al.* 2020), release of prostaglandins by mast cells and airway epithelial cells (Mansfield *et al.* 1979, Beck 1993, Pichon *et al.* 2005, Engan *et al.* 2020), and recruitment of non-ventilated lung areas (Engan *et al.* 2020). However, exercise-induced bronchoconstriction is frequently observed in the human population, particularly among athletes (Weiler *et al.* 2016). Although the presence of asthma predisposes to airway obstruction during and after exercise, even healthy subjects can experience exertional dyspnoea (Caggiano *et al.* 2017, Aggarwal *et al.* 2018), especially when exercising intensely in a cold and dry environment, leading to airway epithelial damage (Sue-Chu *et al.* 1999, Krafczyk and Asplund 2011, Gavrielatos *et al.* 2022). The

diagnostic gold standard for this condition is based on lung function tests performed within 30 minutes after the cessation of challenge exercise (Anderson 2012, Anderson and Kippelen 2012, Del Giacco *et al.* 2015, Weiler *et al.* 2016) by spirometry or oscillometry techniques (Gavrielatos *et al.* 2022).

In healthy horses, traditional respiratory mechanics measurements showed an increase in total airway resistance during moderate and intense exercise, followed by a decrease during recovery. This is probably due to passive physical factors, such as the narrowing of the airway lumen due to compressing transmural pressure, which predominate during exercise, and active physiological adjusting factors, overcoming the first ones during recovery (Art *et al.* 1988, Art *et al.* 1990). Notably, the inspiratory resistance of the lower airways decreased with increasing exercise intensity, lending support to the hypothesis of exercise-induced bronchodilation also in equine athletes (Art *et al.* 1988). Recently, electrical impedance tomography allowed to identify impaired airflow indices, suggesting bronchoconstriction, induced by moderate lunging exercise in horses affected by moderate to severe equine asthma, but not in healthy horses (Herteman *et al.* 2021). Nevertheless, no studies have been conducted on post-exercise lung function changes in racehorses undergoing maximal exercise on the track, following an adapted diagnostic protocol based on the one used in human sports medicine.

We hypothesized that, similarly to human athletes, intense exercise induces changes in lung function detectable by oscillometry in racehorses, and that these changes may differ between healthy horses and those affected by mild equine asthma (MEA). Moreover, as repeated airway damage seems to facilitate the onset of exercise-induced bronchoconstriction in humans (Gavrielatos *et al.* 2022), we hypothesized that horses suffering from exercise-induced pulmonary hemorrhage (EIPH) may also be predisposed to exertional airway obstruction. Therefore, the objectives of the present study were to

compare lung function, assessed by oscillometry, in racehorses at rest and after intense racing exercise on the track (breeze), and to compare exercise-induced lung function variations between healthy horses and those with lower airway disorders.

4.3.2 Materials and Methods

4.3.2.1 Horses

Fourteen client-owned Thoroughbred racehorses in training were prospectively enrolled in the study from three distinct training centers. The study was performed at the facilities where horses normally lived and trained, between August 2022 and August 2023. Horses were stabled in box stalls bedded with straw and fed a diet of hay and concentrate tailored to their nutritional requirements. Horses were trained every morning from Monday to Saturday, following a standardized protocol, consisting of 20-minute walk in an automatic walker and 5- to 10-minute warm-up at trot, followed by a 1400- to 1600-meter canter at 35-40 km/h five days a week, or a breeze at 50-55 km/h once a week, and concluded with a 35-minute cool-down (5-minute trot and 30-minute walk). Horses referred to the Equine Sports Medicine Laboratory of the University of Milan (Italy) for racing fitness evaluation and/or a history of poor performance were selected; based on clinical history, a respiratory score was assigned to each horse according to the HOARSI scoring system (Ramseyer *et al.* 2007). Horses with a score ≤ 2 were included and underwent a complete physical examination to rule out signs of systemic diseases, lameness, and potential causes of decreased athletic capacity apart from lower respiratory disorders.

Ethical approval for the study was obtained from the Animal Welfare Organization of the University of Milan (Protocol Numbers OPBA_44_2022

and OPBA_37_2023) and written informed consent for the inclusion of patients in the present study was obtained by all horses' owners or caretakers.

4.3.2.2 Study protocol

The study protocol included evaluations before, during, and after exercise. Horses' lung function was first assessed at rest, before exercise. Then, horses underwent a standardized breeze exercise, monitored through a training tracker device. After the end of exercise, lung function was re-evaluated at 15 minutes and 45 minutes post-exercise. Finally, upper and lower airways endoscopy was performed and bronchoalveolar lavage fluid (BALf) was collected and subjected to cytological examination. Based on the cytological profile of the BALf, horses were divided into two groups: mild equine asthma (MEA) (neutrophils > 10%, and/or eosinophils > 5%, and/or mast cells > 5%) and healthy (neutrophils ≤ 10%, and/or eosinophils ≤ 5%, and/or mast cells ≤ 5%) (Couetil *et al.* 2016).

4.3.2.3 Lung function testing

Lung function was assessed in all horses using a recently validated wireless oscillometry device, based on the forced oscillation technique (Bizzotto *et al.* 2022). Tests were conducted at rest and repeated at 15 and 45 minutes after the end of the breeze exercise; indeed, as lung function changes are expected to occur within 30 minutes post-exercise and to return to resting values thereafter (Parsons *et al.* 2013), lung function testing was performed at two distinct post-exercise time points. During the tests, horses were kept in their box stalls, loosely contained with halter and lead rope; their heads were kept in a neutral position to prevent any interference with spontaneous breathing. The measurements were taken at the horse's airway opening by applying small amplitude sinusoidal pressure waveforms at frequencies ranging from 2 to 6

Hz, through a customized horse mask (Figure 10). Each frequency was applied for 30 seconds, and airflow and pressure data were recorded and stored on a laptop for subsequent analysis. The respiratory input impedance was then computed using a least squares algorithm in the Matlab software framework (Matlab, MathWorks, USA), and expressed as resistance (Rrs) and reactance (Xrs). From within-breath impedance tracings, the Rrs and Xrs were calculated for the whole breath and during inspiration and expiration. Data were filtered based on visual inspection and the values of the flow-shape index, and breaths showing artifacts were excluded (Bizzotto *et al.* 2022). Then, for each parameter, the average of the values across the different frequencies was calculated and utilized for data analysis and presentation.

Figure 10. A horse from the study population wearing the oscillometry device customized horse mask during a lung function testing session.

4.3.2.4 Exercise

Following the preliminary evaluation at rest, horses underwent standardized exercise. Environmental temperature and relative humidity during exercise were recorded in real-time by consulting the online platform www.meteonetwork.it. After a warm-up phase at walk (20 minutes) and trot (5 minutes), horses galloped for 1400-1600 meters (based on the racetrack length). Horses were ridden by their respective professional riders, who were instructed to push them to their maximum speed for the racetrack conditions (breeze). Horses were then trotted for 5 minutes, walked for 5 minutes to cool down and led to their boxes for lung function testing at 15 minutes post-exercise. After this evaluation, horses were walked for 25 minutes, before being led back to their boxes for lung function testing at 45 minutes post-exercise. Throughout the exercise session, horses were tacked with their daily equipment and wore a validated training tracker device (Equimetre, Arioneo, France) (Ter Woort *et al.* 2020), providing data on mean and maximum speed, mean and maximum heart rate (HR), and a continuous electrocardiogram (ECG). The speed at which a heart rate of 200 bpm was reached (V200) was obtained as a measure of aerobic capacity. Furthermore, as horses exercised at varying mean and maximum speeds, we selected a fixed speed of 40 km/h and recorded the heart rate of all horses at this speed (HR40). Finally, the ECG data were imported into a heart rate variability software (Kubios HRV), and the segment of strenuous exercise was analyzed, defined as the period from the onset of the gallop plateau (following the steep heart rate increase), until the point where the heart rate sharply decreased (Hammond *et al.* 2023). The R-wave detection was manually adjusted and the ECG findings were evaluated by two operators (CMLF and LS); recordings with sub-optimal quality or loss of connectivity were excluded from analysis. The presence of dynamic arrhythmias was recorded, and the heart rate variability was assessed using two time-domain measures calculated by the software: standard deviation of

R-R intervals (SDNN) and root mean square of successive differences (RMSSD)

4.3.2.5 Bronchoalveolar lavage

Following the third lung function measurement, horses were sedated with 0.01 mg/kg detomidine hydrochloride, restrained with a twitch, and airway endoscopy with BALf collection was performed as described elsewhere (Lo Feudo *et al.* 2022d). The collected BALf was processed for cytological examination within 2h; May-Grünwald Giemsa and Perl's Prussian blue stainings were used, respectively, for 400-leukocyte differential count and calculation of total hemosiderin score (THS) (Lo Feudo *et al.* 2022b), performed by the same operator (CMLF).

4.3.2.6 Data analysis

All data were collected on an electronic spreadsheet (Microsoft Excel, Redmond, WA, USA) and analyzed using two commercial statistical software packages (GraphPad Prism 10.0 for MacOS, GraphPad Software, San Diego, CA, USA; JASP 0.17.2.1 Intel, University of Amsterdam, Amsterdam, Netherlands). Data normality was assessed by the Shapiro-Wilk test and descriptive statistics were performed. Data are presented as mean ± standard deviation (SD) if normally distributed, and as median and interquartile range (IQR) if not normally distributed. Changes in average lung function at the three time points (at rest, at 15 minutes and 45 minutes post-exercise) within and between the healthy and MEA groups were evaluated using repeated-measures two-way ANOVA with the Geisser-Greenhouse correction. In case of significant time effect, group effect, or group*time effect, a post-hoc test was performed, correcting for multiple comparisons using the two-stage step-up method. To evaluate whether air temperature and humidity influenced post-

exercise changes in lung function, Pearson's correlations of environmental temperature and relative humidity with oscillometry parameter differences at rest and at 15 minutes post-exercise and were assessed. To evaluate the possible associations between EIPH and lung function, linear regression was used between oscillometry results and THS, calculated from the BALf. Finally, to assess associations between average oscillometry measurements at rest and exercise fitness data (mean and maximum speed, V200, mean and maximum HR, HR40, SDNN, RMSSD), Pearson's partial correlations were used, conditioned on the variable "age". Statistical significance was set at $p < 0.05$. Correlation was categorized as negligible if the r coefficient was 0.00 - 0.10, weak if 0.10-0.39, moderate if 0.40 - 0.69, strong if 0.70 - 0.89 and very strong if 0.90 - 1.00 (Schober *et al.* 2018).

4.3.3 Results

4.3.3.1 Study population

The study population included 14 Thoroughbred racehorses, aged from 2 to 7 years (median 3, IQR 2 – 5 years), consisting of 8 stallions (57.14%), 3 mares (21.43%), and 3 geldings (21.43%). Among them, 8 horses were referred for fitness assessment and had a HOARSI score of 1 (57.14%), while 6 horses presented with poor racing performance and had a HOARSI score of 2 (42.86%). Based on BALF cytology, 5 horses were classified as healthy, and 9 horses were classified as MEA.

4.3.3.2 Within- and between- groups oscillometry variations

All horses tolerated the oscillometry device and lung function testing procedures well. At rest and at 45 minutes post-exercise, all values were successfully obtained. Conversely, the 15 min post-exercise evaluation failed

in one horse due to a technical issue of the device, preventing timely lung function measurement; this horse, belonging to the MEA group, was excluded from the analysis of exercise-related lung function changes. The oscillometry measurements at the different time points in the healthy and MEA groups are displayed in Figure 11. In the healthy group, a significant time effect was detected for whole-breath ($p = 0.013$) and inspiratory Rrs ($p = 0.016$), which decreased at 15 minutes post-exercise and returned to normal at 45 minutes post-exercise. Specifically, whole-breath Rrs was significantly lower at 15 minutes post-exercise compared to values at rest ($p = 0.043$) and at 45 minutes post-exercise ($p = 0.004$). Inspiratory Rrs was significantly lower at 15 minutes post-exercise compared to pre-exercise measurements ($p = 0.049$), with a similar trend compared to the 45 minutes post-exercise values ($p = 0.066$). Expiratory Rrs and all Xrs parameters did not change significantly at different timepoints. In the MEA group, oscillometry parameters did not vary significantly with time. Concerning the between groups comparisons, no significant differences were detected at rest. At 15 minutes post-exercise, MEA-affected horses exhibited increased whole-breath ($p = 0.006$), inspiratory ($p = 0.016$), and expiratory Rrs ($p = 0.019$) compared to healthy horses. The whole-breath, inspiratory and expiratory Xrs did not differ between groups. Only trends were observed at rest, with MEA-affected horses showing lower whole-breath ($p = 0.097$) and expiratory Xrs ($p = 0.070$) compared to healthy horses. Environmental temperature and humidity did not demonstrate any association with lung function variations.

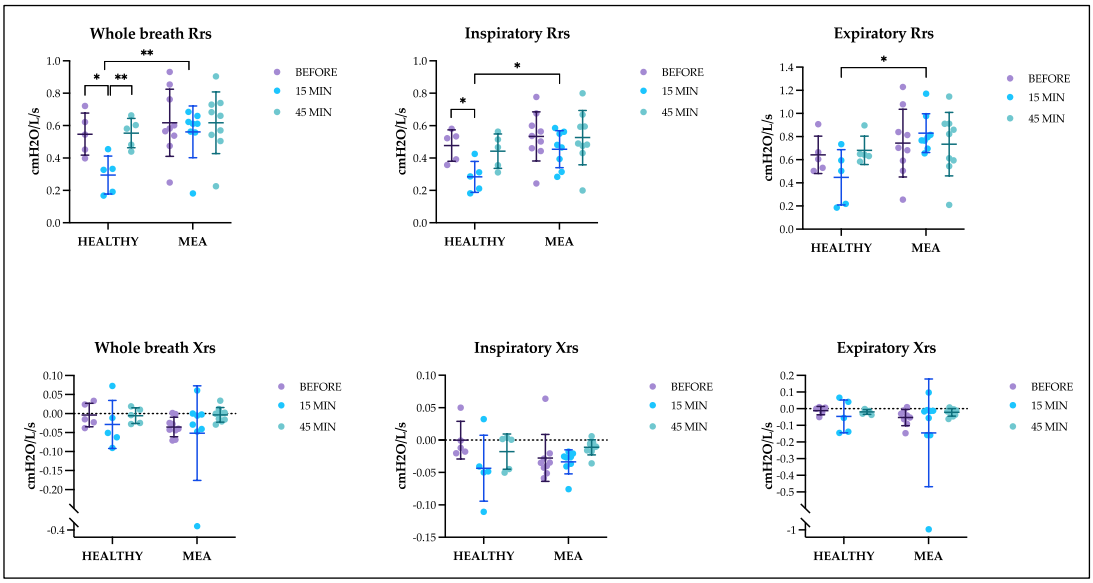

Figure 11. Interleaved scatter plots showing the means and standard deviations of oscillometry parameters at rest, at 15 minutes post exercise, and at 45 minutes post-exercise, in the healthy and MEA groups. Significant differences between different time points and between groups are expressed as * ($p < 0.05$) and ** ($p < 0.01$).

4.3.3.3 Associations between oscillometry and BALf cytology

The cytological findings in the whole study population, the healthy group, and the MEA group are presented in Table 18.

BALf cytology	Whole population	Healthy group	MEA group
Macrophages %	50.50 ± 7.49	50.00 ± 3.94	50.78 ± 9.12
Lymphocytes %	31.79 ± 8.47	38.60 ± 3.91	28.00 ± 7.98
Neutrophils %	13.86 ± 4.96	8.60 ± 1.14	16.78 ± 3.53
Eosinophils %	0.93 ± 2.13	0.40 ± 0.89	1.22 ± 2.59
Mast cells %	3.00 ± 1.84	2.40 ± 1.14	3.33 ± 2.12
THS	39.14 ± 27.90	33.60 ± 22.51	42.22 ± 31.32

Table 18. Results of the cytological analyses of the BALf, including leukocyte differential cell count, and total hemosiderin score (THS), in the whole population, in the healthy group, and in the MEA group. Data are expressed as mean ± standard deviation.

The THS was associated with decreasing whole-breath (p = 0.006, r2 = 0.51) and expiratory Xrs (p = 0.006, r2 = 0.51) at 15 minutes post-exercise (Figure 12).

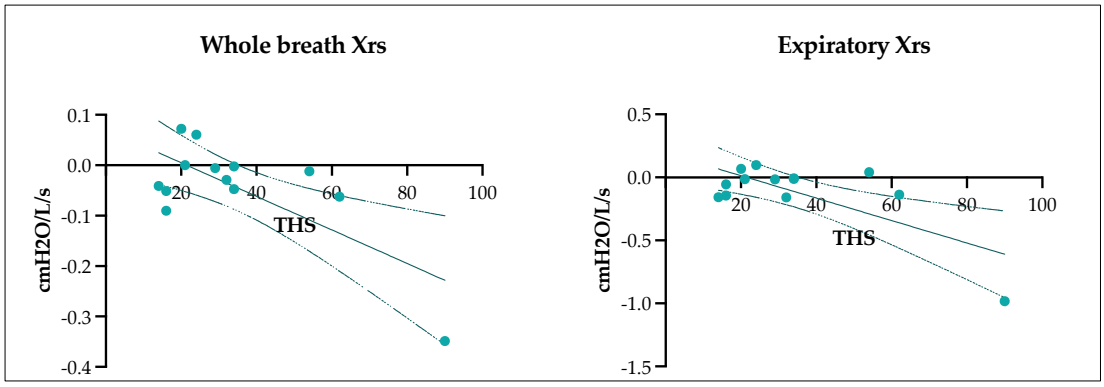

Figure 12. Linear regression graphs showing the associations between the total hemosiderin score (THS) and the whole-breath and expiratory Xrs measured by oscillometry at 15 minutes post-exercise.

4.3.3.4 Associations between oscillometry and fitness parameters

The fitness parameters obtained during the breeze exercise in the whole study population, the healthy group, and the MEA group are displayed in Table 3; clinically significant cardiac arrhythmias were not observed in any horse. Oscillometry measurements, both at rest and at 15 minutes after exercise, were associated with some of the fitness parameters. Among the oscillometry parameters at rest, whole-breath and expiratory Rrs were moderately inversely correlated with V200 (p = 0.027, r = -0.61 and p = 0.029, r = -0.60, respectively), and expiratory Xrs was moderately inversely correlated with maximum HR (p = 0.024, r = -0.62). Regarding oscillometry results at 15 minutes post-exercise, whole-breath Rrs was moderately inversely correlated with mean (p = 0.038, r = -0.60) and maximum speed (p = 0.039, r = -0.60), while expiratory Rrs was strongly inversely correlated with RMSSD (p = 0.009, r = -0.74).

Fitness parameter	Whole population	Healthy group	MEA group
Mean speed	41.99 ± 4.68	44.46 ± 3.30	40.62 ± 4.93
Maximum speed	53.03 ± 5.92	55.38 ± 4.09	51.72 ± 6.57
V200	44.75 ± 8.32	49.32 ± 8.75	42.21 ± 7.35
Mean HR	199 ± 13	193 ± 15	202 ± 12
Maximum HR	212 ± 10	208 ± 9	214 ± 10
HR_{40}	191 ± 20	179 ± 23	197 ± 16
SDNN	7.97 ± 5.62	10.35 ± 9.92	6.92 ± 2.51
RMSSD	7.39 ± 8.36	13.03 ± 14.66	4.88 ± 1.08

Table 19. Results of the fitness parameters obtained during exercise in the whole population, in the healthy group, and in the MEA group. Data are expressed as mean ± standard deviation.

4.3.4 Discussion

While the associations between lower airway inflammation and athletic performance in racehorses have been widely investigated (Richard *et al.* 2010b, Salz *et al.* 2016, Ivester *et al.* 2018, Lo Feudo *et al.* 2022d), studies on the relationships between lung function and exercise are still lacking. The present study addresses three main questions in order to overcome the existing literature gap: what are the physiological effects of intense exercise on lung function, do they differ between healthy horses and horses with lower airway disorders, and can lung function testing be predictive of fitness? The results of this study suggest that, in healthy horses, exercise induces a reduction in Rrs, presumably due to bronchodilation. However, this phenomenon does not seem to occur in horses with asthma. Moreover, healthy horses showed higher Xrs at rest, and lower post-exercise Rrs, compared to MEA horses. Post-exercise Xrs was inversely associated with THS, suggesting that also EIPH severity may impair the elasticity of lung tissues. Finally, pre- and post-

exercise lung function measurements showed potential as predictors of some fitness variables reflecting the level of aerobic and cardiovascular capacities.

The effects of exercise on pulmonary function have been widely discussed, without reaching a comprehensive understanding of the involved physiological processes, even in humans. The control of airway caliber is a dynamic process, resulting from the interaction between neural and humoral factors.8 Based on airway tone variations, two different and opposing phenomena have been observed: exercise-induced bronchodilation and exercise-induced bronchoconstriction. Generally, people with asthma are more susceptible to developing exercise-induced bronchoconstriction, reaching a prevalence of 90-100% (Weiler *et al.* 2010, Caggiano *et al.* 2017, Aggarwal *et al.* 2018). However, although the physiologic response to exercise is bronchodilation (Lodrup Carlsen *et al.* 2006), even healthy individuals can experience episodes of bronchoconstriction, with a prevalence of 5-20% (Weiler *et al.* 2016, Caggiano *et al.* 2017), which becomes even higher in elite athletes reporting a feeling of reduced fitness (Krafczyk and Asplund 2011). In horses, there are limited available data on lung function changes associated with exercise, mainly relying on traditional lung mechanics, which support the hypothesis of bronchodilation, as evidenced by a decreased resistance of the distal airways during the inspiratory phase (Art and Lekeux 1988, Art *et al.* 1988, Art *et al.* 1990, David *et al.* 2006). In our study, healthy horses showed a significant reduction of the whole-breath and inspiratory Rrs at 15 minutes post-exercise compared to their resting values, returning to baseline levels at 45 minute post-exercise. This suggests that intense breeze exercise physiologically induces a temporary bronchodilation in Thoroughbred racehorses, which may persist for a duration between 15 and 45 minutes post-exercise. Conversely, in horses with MEA, neither exercise-induced bronchodilation nor bronchoconstriction were observed. In these horses, it is

possible that the natural bronchodilator effect of exercise is counteracted by a bronchoconstrictor response associated with the airway hyperresponsiveness typical of MEA. The balance between these opposing effects may result in non-significant variations in lung function after intense exercise. This finding partly differs from a recent study using electrical impedance tomography, which showed a different response to exercise in healthy and asthmatic horses, with the former not experiencing any changes in lung function, and the latter exhibiting post-exercise bronchoconstriction (Herteman *et al.* 2021). To explain this discrepancy, differences in the study protocol must be considered, including the lung function testing technique (electrical impedance tomography vs. oscillometry), horse population (Warmbloods and other saddle horses vs. Thoroughbred racehorses), disease severity (mild-moderate and severe asthma vs. mild-moderate asthma exclusively), and exercise type and intensity (lunging at walk, trot, canter vs. ridden breeze). It may be hypothesized that the light exercise regimen employed in Herteman and colleagues' study was not intense enough to induce parasympathetic withdrawal, elevation of catecholamines and prostaglandins, and peripheral lung areas recruitment, all of which lead to bronchodilation. As a consequence, in asthmatic horses, the bronchoconstrictor effect associated with airway hyperreactivity predominates, determining an increase in peak expiratory flow (Herteman *et al.* 2021). However, despite the differences in results, both studies have observed a difference in airway tone between healthy and MEA horses emphasized by exercise. Therefore, post-exercise lung function testing may be useful for the non-invasive assessment of the lower airway health status in sport horses, overcoming the limitation of testing lung function solely at rest, which exhibits low sensitivity in mild cases. While various lung function techniques have demonstrated sensitivity for severe forms of equine asthma (Young *et al.* 1997, Hoffman and Mazan 1999, Couetil *et al.* 2001, Van

Erck *et al.* 2004a, Stucchi *et al.* 2022), results in MEA horses have shown less consistency. For instance, conventional lung mechanics and electrical impedance tomography failed to discern differences at rest between healthy and MEA horses in eupneic resting conditions (Pirrone *et al.* 2007, Herteman *et al.* 2021). Only during hyperventilation induced by the rebreathing method, standard lung mechanics allowed to identify MEA-affected horses (Pirrone *et al.* 2007). Conversely, studies based on oscillometry have reported either increased inspiratory and expiratory Rrs in MEA horses (Hoffman and Mazan 1999, Richard *et al.* 2009), or lower expiratory Xrs (Stucchi *et al.* 2021). In our study, no significant differences in oscillometry parameters were observed at rest. Only trends were observed, with MEA horses showing a lower whole-breath and expiratory Xrs compared to their healthy counterparts; significance may have not been reached due to the limited sample size. As reactance reflects the distensibility and elasticity of lung tissues, it is possible that these trends are a consequence of the progressive pulmonary remodeling resulting from chronic lower airway inflammation. Indeed, MEA horses exhibit alterations of the central airways, including epithelial hyperplasia, smooth muscle fibrosis and lamina propria thickening (Bessonat *et al.* 2022). Beyond the comparisons between healthy and MEA horses, our study explored also the associations between lung function and THS, reflecting EIPH severity. Interestingly, while THS was not associated with lung function parameters at rest, significant associations emerged in post-exercise measurements. Specifically, an inverse relationship between post-exercise Xrs and THS was observed: indeed, as EIPH severity increased, the whole-breath and the expiratory Xrs decreased. Similarly to the hypothesis proposed for MEA, these associations may be explained by the elasticity loss resulting from repeated EIPH episodes, which can lead to lung alterations (Hinchcliff *et al.* 2015), including the parenchyma becoming firm, pleural and septal fibrosis and

angiogenesis, and small pulmonary veins remodeling, characterized by collagen and smooth muscle hyperplasia (O'Callaghan *et al.* 1987a, Williams *et al.* 2008, Williams *et al.* 2013).

Our results confirm the promising role of post-exercise lung function testing in the non-invasive diagnosis of lower airway disorders. Indeed, the increased mechanical work of breathing may exacerbate any respiratory dysfunction associated with mild or subclinical lower airway disorders (Pirrone *et al.* 2007). Another interesting finding in our study is the identification of relationships between pre- and post-exercise lung function and fitness parameters. While many studies in the literature have explored the associations between lower airway inflammation and racing performance, to the authors' knowledge, none have investigated the correlations between respiratory resistance, reactance and athletic capacity indices (Richard *et al.* 2010b, Salz *et al.* 2016, Ivester *et al.* 2018, Lo Feudo *et al.* 2022d). In the present study, we detected a decrease of V200 with increasing whole-breath and expiratory Rrs at resting evaluation; since V200 has been associated with athletic ability and racing performance (Davie *et al.* 2002, Leleu *et al.* 2005), our results suggest that horses with higher respiratory Rrs, particularly during the expiratory phase, may exhibit poor performance. Moreover, horses with a more negative expiratory Xrs reached a higher maximum HR, suggesting a potentially less efficient cardiovascular condition. After exercise, higher respiratory Rrs was associated with lower mean and maximum speeds, confirming that horses with compromised lung function, indicative of bronchoconstriction, may be worse performing. Finally, horses with higher post-exercise expiratory Rrs showed a lower RMSSD. This metric is a heart rate variability index, traditionally considered to decrease in cases of chronic pain or diseases (Catai *et al.* 2020, Hammond *et al.* 2023). Interestingly, children with asthma seem to have a lower heart rate variability (Kazuma *et al.* 1997), which might elucidate the

association we observed between a decreasing RMSSD and bronchoconstriction. In summary, the assessment of lung function, both before and after exercise, may be predictive of racehorses' fitness and could represent an innovative tool for early detection of potential subclinical causes of poor performance, as well as for the monitoring of training, cardiovascular condition, and athletic capacity.

4.3.4.1 Limitations

Although the results of the present study are innovative and demonstrate potentially significant implications for performing pre- and post-exercise lung function testing, several limitations should be acknowledged. The small sample size restricts the generalizability of the results, which should be validated by larger-scale studies. As we observed associations between lung function and THS, it would have been interesting to divide the population into groups, not only based on MEA diagnosis but also considering EIPH. Unfortunately, the sample size was insufficient to support such a specific classification. Another limitation arises from the lack of a preliminary repeatability study on the used oscillometry device; however, each measurement lasted approximately 3 minutes, and non-sedated horses would become restless during a longer period. However, the encouraging observation was that lung function parameters tended to return to baseline values after 45 minutes. Moreover, the present study did not include dynamic upper airway endoscopy during exercise, thus we cannot rule out the interference of upper airway obstruction on oscillometry parameters; however, none of these horses made abnormal upper airway sounds during exercise, they had no known history of dynamic upper airway disorders, and their upper airway endoscopies were normal at rest. Future studies should be undertaken to assess the extent to which dynamic upper airway obstructions may affect lung function parameters obtained at frequencies reflecting the

central and lower respiratory tract; indeed, in our study we selected frequencies between 2 and 6 Hz. In contrast to prior oscillometry studies, we did not stratify the data by frequency for statistical analysis, but rather calculated mean values for each oscillometry parameter. This decision was made because there is no consensus on the frequencies most useful for detecting lower airway mechanics alterations in horses. Additionally, considering each individual frequency separately could have led to data dispersion in such a small population, potentially amplifying the impact of any artifacts on the final values. Finally, despite our efforts to standardize the exercise tests, numerous variables could have influenced the results. Horses were all in full training and managed similarly, but varied in age, sex, and genetic potential. Also, their riders had variable experience, weight, and sex. On different days, track conditions and weather may have differed; while we did not identify correlations between environmental temperature and humidity and lung function variations, we cannot exclude the potential influence of environmental conditions on horses' performance indices. Ideally, all of these factors should have been considered as confounding variables, but it was not feasible in our study due to the small sample size. Future studies, based on a larger and more standardized population of racehorses, should be conducted to either confirm or refute our findings.

4.3.5 Conclusions

In conclusion, under physiological conditions, breeze exercise appeared to induce bronchodilation in Thoroughbred racehorses, as evidenced by reduced respiratory resistance in post-exercise oscillometry. However, this response was not observed in horses with MEA. Oscillometry revealed higher resistance in MEA horses at 15 minutes post-exercise. Furthermore, post-exercise decreased reactance was linked to greater EIPH severity. Finally, oscillometry

demonstrated potential in predicting athletic capacity and cardiovascular condition in racehorses, both at rest and after exercise.

CHAPTER 5.
GENERAL DISCUSSION
AND FUTURE PERSPECTIVES

5.1GENERAL DISCUSSION

Over the past decades, knowledge and comprehension of equine asthma have rapidly advanced, with veterinary researchers and clinicians worldwide working to address gaps in the scientific literature. However, there is still much to be understood to enable a more effective and targeted diagnostic and therapeutic approach for affected patients. On one hand, advancements in human asthma research have paved the way for insights into equine asthma, as these syndromes exhibit several similarities. On the other hand, the horse serves as an ideal animal model for human asthma, and results obtained in equine patients may be translated to humans. In 2019, the Equine Asthma Group identified key areas in equine asthma research that require attention through novel studies. Key limitations in our understanding of equine asthma include its underlying etiopathogenesis, diagnostic approach, and therapeutic strategies.

In this context, the present PhD project attempted to answer some of the lingering questions in the literature and contribute to the panorama of equine asthma. As a referral center for equine respiratory and sports medicine, the Veterinary Teaching Hospital of the University of Milan has accumulated extensive clinical data from EA patients over the past twenty years. With this wealth of existing data, while planning novel prospective studies, we chose to analyze them and conduct retrospective observational studies. These studies were primarily addressed to assess the usefulness of ancillary procedures in the diagnosis of EA and to evaluate potential pathogenetic mechanisms from a clinical perspective. Simultaneously, we started working on prospective research projects, one focused on the immunological pathways involved in EA, and two on the potential use of lung function testing by oscillometry for the non-invasive diagnosis of MEA.

The etiopathogenetic mechanisms of EA were investigated in the studies presented in the paragraphs 3.1, 3.2, 3.3, and 4.1. The first study, presented in section 3.1, provided insight into the type of hypersensitivity reactions and the main allergens involved in the etiopathogenesis of both SEA and MEA. Specifically, the study revealed a predominance of late-phase type-I hypersensitivity response in horses affected by SEA, associated with IgE-mediated mast cells degranulation, and a predominance of delayed type-IV reaction in horses with MEA. While both horses affected by MEA and SEA exhibited a predisposition to develop hypersensitivity responses to numerous antigens, the most allergenic substances were insects. This finding supports the hypothesis of an "atopic march" and the association between EA and insect bite hypersensitivity (IBH). Other notable allergens causing hypersensitivity reactions were dog epithelium, mites, and pollens.

The prospective study, presented in section 4.1, also investigated the immunological mechanisms involved in EA pathogenesis, with a specific emphasis on varying severity and cytological subtypes of the disease. This study corroborated the hypothesis that the pathogenesis of EA entails a mixed inflammatory and immune response, differing between distinct subtypes. Notably, the Th2 response appeared to play a central role in cases of mixed granulocytic EA, especially when characterized by elevated mast cell percentages in the BALf. Innate immunity was associated with disease progression and severity, particularly in relation to neutrophilic inflammation of the lower airways. Conversely, the Th1 response did not seem to hold a primary role in the pathogenesis of EA. Furthermore, the study preliminarily explored the impact of specific cytokines on lung function impairment. The findings revealed the involvement of IL-1β in increased expiratory resistance and an association of IL-4 and IL-17 with decreased respiratory reactance. These results indicate their potential role not only in bronchoconstriction but

also in the process of progressive airway remodeling, leading to reduced airway caliber and loss of lung tissue elasticity.

Another study, detailed in section 3.3, provided a more clinically oriented perspective on the pathogenesis of EA by examining the potential link between lower airway inflammation and dynamic obstructions of the upper airways. Prior research had hypothesized a relationship between different segments of the respiratory tract, aligning with the theory in human medicine known as "*one airway, one disease*". However, our study countered this hypothesis, as the presence and type of upper airway obstructions were not associated with BALf cytology nor with tracheal mucus score. In the study presented in paragraph 3.2, the presence of pharyngeal lymphoid hyperplasia, indicative of upper respiratory tract inflammation, was solely linked to increased eosinophils in the BALf. However, it is important to note that this finding may be a consequence of both features being associated with young age and not necessarily a direct physiological correlation.

Additionally, this study examined the potential role of co-morbidities such as EIPH and lower airway bacterial colonization in the pathogenesis of EA. While microbiological findings did not show an association with BALf cytology, horses with MEA experienced concurrent bacterial colonization more frequently than horses affected by SEA. This phenomenon may be attributed to the younger age of affected horses, indicating a potentially immature immune system, or it may represent a contribution of bacteria to the development of mild to moderate lower airway inflammation in young horses. Regarding the associations between EIPH and MEA, horses with concomitant pulmonary hemorrhage did not exhibit a significant difference in in BALf leukocyte differential count from those exclusively affected by MEA. However, in another study conducted by our research group on a population of Standardbred trotters (Lo Feudo *et al.* 2022b) – though not included in the

present thesis – mast cell percentages were found to be increased in horses with higher endoscopic tracheal blood scores and total hemosiderin scores in the BALf. This suggests a potential contribution of concurrent EIPH to mastocytic inflammation of the lower airways in racehorses. These results confirm that multiple factors may contribute to the onset of MEA.

In our examination of racehorses, we also assessed the effects of MEA on athletic capacity, investigated by measuring a series of fitness parameters during a standardized incremental exercise test on high-speed treadmill. Although MEA has been recognized as a common cause of poor performance, earlier studies aiming to quantify this influence have provided disparate findings. Our study suggested that the diminished athletic capacity observed in association with MEA may be attributed to neutrophilic inflammation. Conversely, our findings indicated that other subtypes of MEA, as well as the quantity of mucus accumulation in the trachea, did not appear to exert a significant impact on the overall fitness of Standardbred trotters.

In addition to shedding light on the pathogenesis of EA, the present PhD project also explored the potential utility of various diagnostic procedure that could either assist or replace BALf collection and cytology, a relatively invasive procedure requiring sedation. In particular, the potential of intradermal testing (IDT), thoracic ultrasonography, and oscillometry were explored. The first study, presented in section 3.1, suggested that a comprehensive IDT, including evaluations of hypersensitivity reactions at 30 minutes, 4 hours, and 24 hours, could offer valuable insights into horses affected by EA. This approach could allow to identify hypersensitivities to specific allergens, potentially leading to more precise allergen avoidance strategies and paving the way for the use of allergen-specific immunotherapy (ASIT) as an alternative, steroid-free treatment. However, further research is

needed to validate the clinical significance of positive reactions and the efficacy of immunotherapy in treating EA.

Thoracic ultrasonography, a relatively underexplored yet rapid, easy, and completely non-invasive procedure, represented another focus of our research. Indeed, we wanted to assess its effectiveness in diagnosis EA. Our study, presented in section 3.2, demonstrated that horses with SEA presented more ultrasonographic alterations, including comet-tail artifacts and focal lesions, compared to horses with MEA. Similarly, horses with concurrent EIPH showed a higher frequency of alterations compared to horses exclusively affected by MEA, especially in the cranial and caudal regions. Indeed, the quantity and severity of these alterations correlated with BALf neutrophil and hemosiderophage percentages, as well as with higher tracheal mucus accumulation scores. Therefore, thoracic ultrasonography emerged as a valuable diagnostic tool, but with a low specificity, thus it cannot replace airway endoscopy and BALf collection. Nevertheless, compared to endoscopy, thoracic ultrasonography can provide information on the localization and extension of inflammatory lesions in the lung, warranting its inclusion in a comprehensive diagnostic protocol for EA. Additionally, this same study confirmed the importance of airway endoscopy, reporting more substantial tracheal mucus accumulation and greater blunting of the tracheal bifurcation in horses affected by SEA compared to those with MEA.

Finally, we conducted two prospective studies on the application of oscillometry for assessing lung function in horses with MEA. These studies represent the most innovative part of this doctoral thesis. Indeed, despite oscillometry has been used in equine research since 1989, its clinical and field applications have been limited by the lack of sensitive, portable, and easy-to-use devices. A collaborative effort involving the Equine Sports Medicine Laboratory of the University of Milan, the Equine Asthma Laboratory of the

University of Montreal, and the Techres Lab of the Polytechnic University of Milan, resulted in the development and preliminary validation of a novel oscillometry device based on the forced oscillation technique (Bizzotto *et al.* 2022). This device, distinguished by its low-cost technology and being user-friendly, wireless, compact, and portable, holds promise for practical and feasible application in clinical and field conditions. While previous literature already supported the sensitivity of oscillometry in diagnosing SEA, concerns have arisen about its sensitivity in detecting MEA. To address this, our research focused on evaluating the applicability of the new device for the diagnosis of MEA. The first study, presented in section 4.2, involved a diverse population of healthy and MEA-affected horses, with various ages and breeds. The second study, presented in section 4.3, specifically included a group of young Thoroughbred racehorses, assessing lung function both before and after intense exercise. While the first study aimed at identifying differences in lung function parameters between healthy and MEA-affected horses, the second one expanded its objectives to evaluate variations in lung function related to intense exercise and to assess associations between lung function and fitness parameters.

Results from the first study revealed a reduced reactance at 2 Hz in horses with MEA, throughout the whole respiratory cycle, but particularly enhanced during expiration. Additionally, a worsened expiratory phase, compared to the inspiratory phase, was observed at 5 Hz. Conversely, no differences in resistance were detected between groups. Another interesting result of this study was the distinct frequency dependance of reactance between healthy and MEA-affected horses. Indeed, while both groups showed a positive frequency dependence of resistance, only MEA-affected horses also showed a significant positive frequency dependence of reactance. These findings suggest that measuring reactance could be a valuable component of a

comprehensive diagnostic protocol for MEA, offering a non-invasive and straightforward indicator of subclinical lower airway obstruction. However, further studies with larger and more standardized populations should be conducted to establish cut-off values and determine sensitivity and specificity for diagnosing MEA.

The second study focusing on Thoroughbred racehorses provided several interesting results. Firstly, it elucidated that intense racing exercise induces bronchodilation, detectable 15 minutes post-exercise, in healthy horses but not in those affected by MEA. This underscores the distinct respiratory responses in these two groups, emphasizing the potential of post-exercise assessments for revealing underlying respiratory conditions. In addition to these insights, the study revealed noteworthy distinctions in lung function between healthy and MEA-affected horses when evaluated post-exercise. While oscillometry at rest primarily exhibited variations in respiratory reactance, post-exercise testing unveiled significant differences in resistance, specifically elevated in asthmatic horses. Furthermore, horses concurrently affected EIPH exhibited a decreased post-exercise reactance, illustrating the intricate interplay of respiratory factors in athletic performance. Beyond its diagnostic utility for identifying MEA in racehorses, this study highlighted the potential of oscillometry in predicting athletic performance. In fact, oscillometry parameters demonstrated associations with key fitness measures, suggesting a broader role in assessing the overall athletic capacity of Thoroughbred racehorses. Therefore, the novel oscillometry device demonstrated versatility, proving to be beneficial not only in diagnosing MEA in subclinically affected patients, but also in monitoring and predicting the athletic capacity of racehorses. The combination of its diagnostic capacity, feasibility in diverse conditions, and applicability across various fields emphasized the promising role of oscillometry as a valuable tool in equine respiratory health assessment.

5.2 FUTURE PERSPECTIVES

While the current PhD project has yielded intriguing results, some limitations have been acknowledged within each study: therefore, future investigations are needed to confirm our findings. Moreover, the promising outcomes of our studies not only underscore their significance, but also pave the way for further developments in both pathogenesis insights and diagnostic tools. Presented below are some potential future research perspectives, which may validate and extend the results reported in the studies included in the present project.

5.2.1 Clinical application of intradermal testing and novel related-opportunities in EA

In the present PhD thesis, IDT successfully identified the allergens most frequently associated with hyperresponsiveness in horses affected by MEA or SEA. However, further studies are necessary to confirm the involvement of these identified allergens in triggering clinical signs. To address this, an antigen inhalation challenge could be performed, using a nebulized formulation composed of the allergens identified by IDT. If this test induced a worsening in the clinical respiratory score, lung function, and/or BALf cytology, it would affirm the efficacy of IDT in pinpointing the allergens responsible for EA clinical signs. The reliable and accurate identification of the specific allergens would serve as the foundation for the development of an allergen specific immunotherapy, similarly to human medicine, which could allow to reduce the use of medications, directly targeting the hyperresponsiveness pathway (Erekosima *et al.* 2014).

5.2.2 Determination of sensitivity and specificity of thoracic ultrasonography for the diagnosis of EA

The differences observed in thoracic ultrasonographic scores and number of lung focal lesions between horses affected by MEA and SEA suggest that thoracic ultrasound may represent a valuable non-invasive diagnostic tool for EA. Therefore, future studies should be conducted to assess its sensitivity and specificity, aiming to establish potential cut-off values. These values could be used as a preliminary evaluation of lower airway inflammation, especially in cases where a clinician lacks access to an endoscope or the ability to perform a BALf. Moreover, thoracic ultrasound may be used daily to evaluate the effectiveness of a treatment or to monitor the progression of the disease over time. This underscores its potential utility not only in diagnosis but also in the ongoing assessment and management of EA.

5.2.3 Evaluation of the role of dynamic upper airway obstruction in the pathogenesis of EA in non-racing sport and leisure horses

In this project, we have shown that MEA is not associated with DUAOs in Standardbred trotter horses. However, existing literature suggests associations between EA and DUAOs in other horse populations, such as non-racing sport horses or leisure horses (Wysocka *et al.* 2018, Joo *et al.* 2021). Various potential explanations have been proposed. The heightened negative pressure resulting from obstruction in the lower respiratory tract and an increased respiratory effort might compromise the patency of the upper airway, leading to the onset of neuromuscular fatigue and, subsequently, DUAOs. On the other hand, it is plausible that DUAOs could predispose horses to lower airway disease through mechanisms not yet understood. This potential association could be crucial for the management of EA, and *vice versa*, as anecdotical reports suggest that DUAOs may be solved by treating lower

airway inflammation. Therefore, we suggest that this topic deserves further investigations, exploring large, unbiased horse populations according to standardized diagnostic protocols.

5.2.4 Comprehensive approach of EA subtypes categorization based on multi-omics techniques

Equine asthma is a syndrome including a spectrum of respiratory diseases, potentially differing in clinical presentation and severity, pathogenetic pathways, and recurrence rates (Bond *et al.* 2018). While the present project identified specific cytokines associated with different EA subtypes, our study was confined to the evaluation of expression of five cytokines reflecting four different immunological pathways. However, the involved mechanisms are very complex, and the evaluation of only one or two cytokines is overly simplistic. Moreover, cytokine expression may vary over time and depend on the stage of the disease. Therefore, it must be recognized that our approach, though based on previous literature, was relatively basic. Future investigations employing a multi-omics approach holds promise for a more comprehensive understanding of EA pathogenesis, allowing a finer subcategorization of this complex disease. Currently, our research group is working on the evaluation of the metabolome and miRNome of BAL-derived exosomes in horses affected by MEA and SEA. The identification of differentially expressed metabolites and miRNAs opens avenues for their potential application as biomarkers for early detection, prognosis, and personalized therapy delivery. To date, multi-omics techniques have been extensively applied to human asthma (Tyler and Bunyavanich 2019, Abdel-Aziz *et al.* 2020, Gautam *et al.* 2022, Radzikowska *et al,* 2022) and, in the last years, are gaining increasing popularity also in veterinary medicine. Multiple studies have been conducted on EA with various omic approaches, evaluating

the microbiome, transcriptome, miRNome, proteome, lipidome, and metabolome of respiratory secretions (BAL and TW), and exhaled breath condensate. Most of these studies investigated the role of microbiome in EA. The lower airways, once considered as a sterile environment, demonstrated to have their own microbiome, different from the one of the upper airways (Bond *et al.* 2019b, Fillion-Bertrand *et al.* 2019). Significant differences in tracheal and lung microbiome were reported both between healthy and SEA horses (Fillion-Bertrand *et al.* 2019) and between healthy and MEA horses (Bond *et al.* 2017). Moreover, steroidal treatment showed to influence the lower airway microbiome of MEA-affected horses in one study (Bond *et al.* 2017), while in another one it affected only the upper respiratory tract (Bond *et al.* 2019b). Moreover, some authors investigated a possible relationship between intestinal microbiome and EA, with contrasting results (Keiser-Thom *et al.* 2020, Leclere and Costa 2020). Transcriptomics studies showed differences in lung tissues genes expression between horses with SEA and healthy controls, but also between the remission and the exacerbation phases of EA (Hulliger *et al.* 2020). Similarly, differentially expressed miRNAs were identified in the serum of SEA-affected horses in another study based on a miRNomics approach (Pacholewska *et al.* 2017). Also, the proteomic profile of the BAL was different between horses with EPA in exacerbation of the symptoms and their healthy counterparts, with over-representation of proteins involved in neutrophils migration, chemotaxis and infiltration (Bright *et al.* 2019). A first study on the lipidome of BAL surfactant showed no differences between healthy and SEA-affected horses in remission, but only an alteration of the lipidomic profile in SEA-affected horses after exposure to hay, leading to disease exacerbation (Christmann *et al.* 2018). Conversely, a second study performed by the same research group reported significant alterations in the surfactant lipidome of horses with SEA, and mild changes in horses with

neutrophilic MEA. Additionally, the plasma lipidomic profile was significantly altered in all types of EA, including SEA, neutrophilic and eosinophilic MEA, compared to controls, and differed between distinct EA subtypes (Christmann *et al.* 2022). Finally, metabolomic analysis allowed to identify differences in the concentration of some metabolites in the TW of horses with EA compared to controls in one study, while no differences were observed in exhaled breath condensate samples (Bazzano *et al.* 2018). Conversely, another study of the same research group, restricted to horses with SEA, showed differences in the metabolomic profile of both BAL and exhaled breath condensate, in comparison with healthy horses (Bazzano *et al.* 2020). In summary, studies on EA based on omics approaches are still preliminary and may require standardization to obtain more homogeneous results. In particular, it has been suggested that the TW may be the best sample type for omic analyses, representing the right balance between procedure invasiveness and obtainable results (Karagianni *et al.* 2021). Multi-omics techniques represent an encouraging approach to EA diagnosis and may pave the way to novel targeted therapeutic approaches.

5.2.5 Identification of oscillometry parameters' cut-off values for the diagnosis of EA

The oscillometry studies presented in this PhD thesis revealed differences in lung function parameters, obtained by the novel FOT device, between healthy and MEA-affected horses. However, for a more definitive interpretation of oscillometry results and to ensure their diagnostic applicability, it is crucial to establish cut-off values for Xrs and Rrs and calculate their sensitivity and specificity. Nevertheless, it should be noticed that results varied between our two studies, likely because of the different populations of horses involved. Indeed, in the first study, a mixed population of horses of various ages, breeds,

and uses was included, while the second study focused on a more homogeneous group of young Thoroughbred racehorses in training. Therefore, the identification of universally applicable cut-off values may be challenging, given the potential variations between types of horses. These uncertainties underscore the need for future studies to address these questions and provide more insights into the application of oscillometry in EA.

5.2.6 Clinical application of oscillometry in equine medicine, including sports performance monitoring and therapeutic efficacy evaluation

Independently from the feasibility of establishing diagnostic cut-off values for oscillometry parameters, their measurement may find applications in various facets of equine respiratory and sports medicine. As previously mentioned, oscillometry parameters ranges may vary among different horse populations, and focusing on their assessment within individual subjects could yield additional benefits. For instance, continuous monitoring of lung function in racehorses, both before and after exertion, may enable the early detection of deviations from their baseline values obtained during healthy periods, thus highlighting potential airway disorders. This timely identification could facilitate prompt therapeutic interventions or suggest the need for adjustments in management practices, including stabling, training routines, and race scheduling. Moreover, it could prevent horses from competing in suboptimal respiratory conditions, thereby safeguarding both their physical and psychological welfare. These measures also aim to minimize and prevent lung damage resulting from chronic cycles of inflammation and repair leading to tissue remodeling, safeguarding the horse's long-term health status. Another potential application of longitudinal oscillometry measurements involves the objective evaluation of therapeutic interventions. This non-invasive approach could track progressive improvements in lung function

over time, offering guidance for determining therapeutic dosages and identifying the opportune moment to interrupt medical treatment.

5.3 CONCLUSIONS

In conclusion, this doctoral research has produced significant contributions to the understanding of equine asthma, offering valuable insights into its pathogenesis and proposing novel diagnostic approaches. Briefly, we discerned distinctive late-phase type I responses in SEA horses and type IV delayed hypersensitivity reactions in MEA horses; the identification of the most prevalent allergens in both groups further enhances our comprehension of this complex condition. Furthermore, the research delineated the involvement of various immunological pathways, such as Th2, Th17, and innate immune responses, in different EA severity and cytological subtypes. The investigation into Standardbred racehorses revealed that EA, contrary to expectations, showed no clear association with upper airway disorders. Instead, it was implicated in decreased fitness and athletic capacity, confirming the impact of this condition on racing performance. In terms of diagnostics, our research supports the inclusion of intradermal testing and thoracic ultrasonography into a comprehensive diagnostic protocol for EA. Additionally, oscillometry represents a promising non-invasive diagnostic tool, suggesting potential for further investigation and diverse applications.

for its non-invasive diagnosis and may find additional interesting applications. While acknowledging that our findings are still preliminary, we anticipate and encourage further research in this field, paving the way for enhanced diagnostic strategies and more targeted interventions in the future.

CHAPTER 6.
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