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PULMONARY ARTERY REMODELING IN SEVERE EQUINE ASTHMA

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SUMMARY

ABSTRACT

Recurrent episodes of airway obstruction, hypoxemia and pulmonary hypertension (PH) are present during exacerbations of severe equine asthma (SEA). Pulmonary hypoxic vasoconstriction is known to contribute to the development of PH, which may lead to *cor* pulmonale. However, as PH is only partially reversible by oxygen administration, other etiological factors are likely to be involved. In human chronic obstructive pulmonary disease, pulmonary artery (PA) remodeling contributes to the development of PH. Furthermore, allergic airway inflammation results in remodeling of pulmonary vasculature in mouse models, suggesting that similar findings may be present in asthma. We therefore postulated that PA remodeling is present in SEA and contributes to PH. The project aimed to investigate 1) the presence of PA remodeling in severe equine asthma and its distribution throughout the lungs, 2) the involvement of vascular smooth muscle (VSM) alterations, and 3) their reversibility following long-term antigen avoidance strategies or inhaled corticosteroids administration. Using histomorphometry and tissue bank (ERTB) lung samples, the PA wall was measured on sections stained with hematoxylin-eosin saffron, collected post-mortem from different lung regions of 12 asthmatic horses and 6 age-matched controls. Pulmonary vascular smooth muscle (VSM) mass was also measured on sections stained for α-smooth muscle actin collected with in vivo thoracoscopy or post-mortem peripheral lung biopsy from 5 controls, 6 asthmatic horses in remission, and 11 asthmatic horses while exacerbation and after 1 year of antigen avoidance alone (5 horses) or treatments with fluticasone (6 horses). Data were compared using one tailed unpaired t tests with Welch correction or paired t tests ($p<0.05$). Increased PA wall surface was detected in apical ($p=0.002$) and caudodorsal ($p=0.03$) lung regions of asthmatic horses in both exacerbation and remission, when compared to controls. The VSM mass was similarly increased ($p=0.03$) when compared to controls. A tendency for a normalization of the VSM mass was observed after treatment with antigen avoidance ($p=0.05$), but not with fluticasone ($p=0.27$). Remodeling of the PA develops in SEA and is associated with an increase in VSM. The resulting narrowing of the lumen of the PA

could enhance hypoxic vasoconstriction, contributing to PH during exacerbation of SEA. VSM mass normalization is better achieved by antigen avoidance than by corticosteroids.

PART 1: LITERATURE REVIEW

CHAPTER 1: SEVERE EQUINE ASTHMA

This research project aims to assess presence of pulmonary artery remodeling in severe equine asthma and to evaluate whether vascular changes are reversible after long-term asthma treatment. An overview about severe equine asthma pathogenesis and clinical consequences, as well as a revision of available therapeutic approaches and their efficacy are essential to understand hypothesis and results of the experimental study.

DEFINITION AND EPIDEMIOLOGY

Severe equine asthma is a chronic non-infectious obstructive and inflammatory disorder of the equine lower respiratory tract, affecting about 10-14% of horses living in the boreal hemisphere (1). Reversible bronchospasm due to bronchial hyperresponsiveness, airway neutrophilia, mucus increased production, systemic inflammation and pulmonary remodeling are typical features of the disease (2). Several terms were used in the past to define this condition including heaves, pulmonary disease, broken-wind, emphysema, chronic bronchiolitis, hay sickness, small airway disease, allergic airway disease, chronic obstructive pulmonary disease (COPD) and more recently recurrent airway obstruction (RAO) (3). Because latest evidences suggest lots of pathophysiological and clinical similarities with human chronic asthma, the definition “severe equine asthma” has been officially adopted since 2016 (4, 5). Severe equine asthma and human chronic asthma do not totally overlap because both are heterogeneous disorders, showing variable clinical presentation and involving

complex pathogenetic pathways (4). However, both conditions are reversible and recurrent, with alternation between episodes of clinical exacerbation, triggered by exposure to specific environmental antigens and periods of disease remission, after removal of the offending antigen (2, 6, 7). During exacerbation, the combination of bronchospasm, mucus accumulation and airway remodeling results in clinically remarkable airflow obstruction and dyspnea. During remission of the disease, airway neutrophilic inflammation regresses and severe asthmatic horses do not clinically differ from healthy horses (7). Nevertheless, systemic inflammation as well as structural remodeling of peripheral airways are only partially reversible, being responsible for persistence of subclinical airway obstruction and for the progressive degenerative nature of the disease (8-11).

ETIOPATHOGENESIS

ETIOLOGY

Severe equine asthma is a multifactorial disorder that involves combination of individual predisposition and exposure to environmental exacerbating allergens (2). Severe asthmatic horses are likely to have a genetically defective or altered immune response, particularly involving the IL-4/Th2 mediated pathway. Interestingly, severe equine asthma resulted associated with increased natural resistance to gastrointestinal helminths as well as predisposition to allergic skin diseases, that are both typical Th2-mediated responses (12). In some families of severe asthmatic Warmbloods, genetic studies have identified an association between the sequence of the IL-4 receptor gene and its increased expression (13). Although further investigation is required, the immune response of severe asthmatic horses is likely to be excessive and/or abnormally persistent towards antigens that are usually tolerated by the immune system of healthy horses. Both severe asthmatic and healthy horses develop pulmonary neutrophilia after exposure to environmental allergens. Regression of neutrophilic

response is observed in healthy horses but not in asthmatics after persistence of the antigenic challenge, supporting the hypothesis of individual susceptibility for an abnormal immune response (7) Furthermore, other non-genetic factors are associated with increased horse susceptibility such as advancing age (≥4 years), breed (Thoroughbreds), pollution, previous respiratory infections, winter and spring seasons, early exposure to hay and straw (14, 15).

In addition to a genetic and individual susceptibility, clinical manifestation of the disease requires exposure to specific exacerbating factors that usually consists of inhalation of environmental allergens. The pivotal role in inducing exacerbation is played by mold spores particularly belonging to *Aspergillus fumigatus*, *Faenia rectivirgula* and *Thermoactinomyces vulgaris* species (16-19). Other organic dust components such as bacterial endotoxin as well as inorganic particulate and noxious gases are not determinant but contribute to the inflammation degree and clinical syndrome severity (20). Because most involved allergens are found in moldy hay, straw and stable dust, severe equine asthma has been considered as a "disease of domestication and stabling". However, less frequently, horses may present severe asthma exacerbation following exposure to grass pollen at pasture. This syndrome corresponds to what was previously known as Summer-Pasture Associated-RAO (SPA-RAO) and reflect the variability of antigens that are implicated in the equine asthma-like response, similarly to human asthma (4, 21).

PATHOGENESIS

It is widely accepted that severe equine asthma consists in a hypersensitivity reaction to inhaled allergens, however specific immunological pathways are far to be completely clarified. Conversely to the human disease, an early phase immune response is not implicated in severe equine asthma. Although chimase-positive mast cells are increased within airway walls of severe asthmatic horses, their role remains unknown, probably contributing to lung tissue remodeling (22). The exacerbation

of the disease involves a delayed immune response mediated by CD4+ T-cells, leading to neutrophil accumulation into the airways about 5-6 hours after allergen exposure (23).

Several studies attempted to characterize the Th-type response with controversial results. Most evidences suggest a predominant Th2-type response, with increased levels of IL-4 and IL-5 and decreased IFNγ, characterizing both the acute and the chronic disease (24-26). Recently, increased levels of IL-17 mRNA expression have been demonstrated in the bronchoalveolar lavage fluid (BALF) of chronic severe asthmatic horses, suggesting that an involvement of the Th17-type response may follow the Th2 type, contributing to maintenance of pulmonary neutrophilia (27, 28). Nevertheless, other studies have detected increased levels of IFNγ but not of IL-4 and IL-13, suggesting a predominant Th1-type response or increased levels of all the cited cytokines, suggesting a mixed Th1/Th2 -type response (29-31). The disagreement among the results may reflect the complexity of the immune response in severe equine asthma, suggesting that different T-cell sub-populations could be involved in different phases. Furthermore, individual genetic susceptibility and exposure to variable mixtures of exacerbating allergens may affect the predominant type response, supporting the hypothesis that several immunological pathways could be subtended to similar disease phenotypes (2).

Although the involved sub-population and related cytokine expression may vary, T-cell activation results in a consistent both airway and systemic inflammatory reaction. Airway inflammation is characterized by massive neutrophil chemotaxis as well as enhanced proteolytic activity and increased oxidative stress. Early neutrophil recruitment into the airways is related to up-regulation of IL-8 gene expression, a well-known neutrophil chemotactic factor, in bronchial epithelial cells, BALF derived cells and pulmonary endothelial cells. In vitro evidence suggests that IL-4 induces IL-8 gene expression up-regulation as well as peripheral blood neutrophils priming and activation, supporting the predominant role of a Th2-type response in the early pathogenesis of severe equine asthma (28, 32). Both proteolytic activity and reactive oxygen species (ROS) production are enhanced during any inflammatory process. Increased release into the airway *lumen* of neutrophil-derived oxidative

enzymes such as myeloperoxidase as well as elevated levels of metalloproteinases (MMPs, including MMP-1, MMP-8, MMP-9, MMP-13) secreted by immune and epithelial cells have been detected in respiratory secretions of severe asthmatic horses (33-36). A systemic inflammatory reaction is also detectable as shown by increased *serum* levels of some acute phase proteins such as haptoglobin and *serum* amyloid A (37).

Even though further investigation is required to characterize specific pathways and neutrophil relative contribution, the ongoing massive inflammatory reaction induces bronchospasm, increases mucus production and initiates airway remodeling, eventually resulting in overall airway obstruction (18). Different mechanisms may contribute to airway smooth muscle hyperresponsiveness and bronchospasm in severe asthmatic horses, including defections in the noradrenergic and nonadrenergic-noncholinergic (NANC) inhibitory systems, hypercontractility related to increased expression of neurokinin receptors and enhanced neurokinin-induced bronchoconstriction as well as increased release of endothelin-1(38-41). Excessive mucus secretion into airway *lumen* significantly contribute to peripheral airway obstruction in severe equine asthma. Furthermore, its accumulation into central airways represents a diagnostic finding that could be clinically detected by endoscopy. Both qualitative and quantitative abnormalities are responsible for mucus accumulation. Goblet cells hyperplasia and metaplasia are associated with mucus hypersecretion and altered mechanical viscoelasticity that prevent effectiveness of the physiological mucociliary *clearance* (42-44). Both mechanical stimulation and inflammatory infiltration contribute to airway remodeling, resulting in airway wall thickening, *lumen* narrowing and bronchoconstriction amplification. Epithelial hyperplasia, airway smooth muscle (ASM) cell phenotype switching and enhanced turnover, increased collagen deposition represent the most consistent bronchial remodeling events in severe asthmatic horses (7, 10, 45, 46).

Antigen avoidance induces clinical symptom as well as airway neutrophilia remission, however subclinical inflammation and airflow obstruction persist. A persistent subclinical inflammatory response is detectable not only at the airway level but also at a systemic level, since inflammatory

markers remains elevated both in the respiratory secretions and in the *serum* (9, 34). Deposition of disorganized collagen and elastic fibers within the bronchial walls appears not reversible with disease remission and contribute to persistent airflow obstruction by decreasing lung elastic compliance (10).

CLINICAL FINDINGS AND DIAGNOSIS

CLINICAL PRESENTATION

The evolutive stage of the disease as well as the complexity of the pathogenesis influence clinical presentation, leading to variable degree of symptom severity. Clinical findings include non-specific poor performance or exercise intolerance, mild respiratory clinical signs such as cough or more rarely mucopurulent nasal discharge and expiratory dyspnea culminating in severe respiratory distress. In chronic affected horses, persistent expiratory effort may cause abdominal muscle hypertrophy (heaves line). Generalized cachexia may occur, due to significant mismatch between peripheral tissue oxygenation and breathing work energy consumption (2).

The diagnosis of severe equine asthma is easily achieved by clinical examination, supported by history of exposure to exacerbating allergens. The most consistent finding detectable at clinical examination of severe asthmatic horses is the abnormal breathing pattern, characterized by increased expiratory effort, also revealed by nasal flaring and recruitment of accessory abdominal muscle during the expiratory phase. In advanced cases, tachypnea may also be present as a reflex response to hypoxaemia. Nasal flaring and abdominal effort have been used to elaborate a clinical score attempting to estimate the degree of airway obstruction, as shown in table 1 (47). This score reliably estimates worsening or post-treatment improving of airway obstruction only in advanced cases, when lung dysfunction is severe enough to determine hypoxaemia (total score greater than 5). Although

significant lung function deterioration occurs, clinical estimation of airway obstruction is not reliable for total scores lower than 5 and lung function tests are required to assess treatment efficacy (48). During clinical examination, thorax auscultation usually detects presence of end-expiratory wheezes, reflecting the increasing end-expiratory airflow resistance, as well as early inspiratory crackles reflecting opening of collapsed alveoli. Auscultation area may result wider because of lung hyperinflation and air trapping (2).

ASSIGNED VALUE	NASAL FLARING	ABDOMINAL EFFORT	TOTAL SCORE
1	No flaring	No abdominal component to breathing	-
2	Slight, occasional flaring	Slight abdominal movement	**No signs**
3	Moderate flaring	Moderate abdominal movement	**Mild signs**
4	Severe, continuous flaring	Severe, marked abdominal movement	
5	-	-	**Moderate signs**
6	-	-	
7	-	-	**Severe signs**
8	-	-	

Table 1: Clinical score for severe equine asthma according to Rush et al. (47). Both nasal flaring and increased abdominal effort aim to minimize expiratory airflow obstruction. The total clinical score is obtained as the sum of the two scores and estimates the severity of airway obstruction.

ANCILLARY DIAGNOSTIC TESTS

Although diagnosis is easy, assessing disease severity and its evolutive stage, evaluating response to treatment and defining long-term prognosis still represent a challenge for the clinician and often require multiple ancillary diagnostic tests. Furthermore, since importance of airway remodeling in the pathogenesis of severe equine asthma has recently emerged, increasing interest has been directed to develop diagnostic techniques to assess and quantify the ongoing remodeling process.

Lung function assessment

Lung function assessment is particularly useful in those cases of mild-moderate clinical airway obstruction, although technical difficulties, such as invasiveness and expensiveness, limit its use during routinely practice (2). The most traditional system to assess lung function measures the trans-pleural pressure variation (ΔPpl), by means of an intra-esophageal catheter and airflow rates, by means of a heated pneumotachograph attached to a mask. Lung function indexes are then derived and usually include lung resistance, dynamic compliance and related elastance (49). In severe asthmatic horses, lung function deteriorates progressively as the degree of airway obstruction worsens. Initially, the obstruction limits flow through large and small airways as well as causes irregular ventilation of alveoli, inducing an increase of resistance and elastance respectively. Subsequently, to avoid excessive airflow reduction, an increase in ΔPpl occurs. Further increase in resistance and elastance reflect airway obstruction worsening related to the disease progression (mucus accumulation and airway remodeling) (48).

Blood gas analysis

Arterial blood gas analysis allows evaluating arterial oxygen tension (P_aO_2)as an estimation of the ventilation/perfusion (V/Q) mismatch severity (2). Hypoxaemia is a frequent finding in severe asthmatic horses during exacerbation, depending on increased dead space ventilation and related pulmonary gas exchange compromise. Increased dead space results from alveolar hyperinflation and subsequent compression on pulmonary capillaries that prevents adequate perfusion (50, 51). Hypercapnia is not an usual finding in severe asthmatic horses since significant increase in respiratory rate and total ventilation ensure maintenance of PCO_2 levels within normal ranges (50).

Endoscopy

Endoscopic examination of the lower airways is almost systematically performed when severe equine asthma is suspected. Mucus accumulation in the tracheal *lumen* and thickness of the tracheal *carina* could be visually examined during tracheoscopy, even though clinical significance of these findings should be critically evaluated. An endoscopic score for mucus accumulation has been elaborated, showing a good correlation with airway neutrophilic inflammation (52). However, one should remind that mucus is a non-specific marker and other disorders resulting in lower airway inflammation may be associated with mucus accumulation. An endoscopic scoring system has been proposed also for carina thickness but no significant correlation with the degree of airway inflammation has been demonstrated (53). In addition to allowing direct visualization of the airways, transendoscopic techniques are available to collect cytological and histological samples and more recently to investigate airway structure by ultrasonography.

Bronchoalveolar lavage fluid (BALF) cytology

BALF cytology still represent the gold standard to assess presence of lower airway inflammation and to characterize the inflammatory profile (2). Although BALF cytological composition may vary according to several individual and environmental factors, severe asthmatic horses in exacerbation usually show a typical cytological differential count characterized by elevated neutrophil percentage (usually greater than 25%) and decrease of lymphocyte and macrophage relative counts (54). Nevertheless, BALF cytology and particularly neutrophil count does not correlate with severity of clinical airway obstruction and lung dysfunction. This is further supported by response to

corticosteroid therapy, characterized by significant improvement of clinical signs and airway obstruction without reduction of airway neutrophilia, achieved only with antigen avoidance (8).

Endobronchial biopsy

Endobronchial biopsies could be easily collected during bronchial endoscopic examination using a transendoscopic smooth oval forceps. Endobronchial biopsy evaluation allows estimation of ongoing central airway remodeling by assessing histological changes within airway ephitelium, basal membrane, sub-mucosal and smooth muscle layers. A semiquantitative histological score has been recently developed, showing a good correlation between severity of central airway remodeling and degree of airway obstruction (55).

Imaging techniques

Thoracic radiography may show non-specific diffuse increased bronchial and interstitial pattern. However, diagnostic value of this technique is questionable since similar radiographic changes may occur related to ageing. Furthermore, presence of severe equine asthma may be associated to normal thoracic radiography. Recently, transendoscopic endobronchial ultrasonography (EBUS) has been validated in horses. This technique allows non-invasive and reliable estimation of airway smooth muscle remodeling in severe equine asthma. However, equipment expensiveness and technical difficulties related to procedure realization and data interpretation limit the use of this technique to the research field (56).

THERAPY

Severe equine asthma is an incurable disease and all the available therapeutic options are aimed to control clinical signs typical of exacerbation and to decelerate the disease progression. The main therapeutic goals are reducing airway inflammatory response and limiting airway obstruction (2). Two main groups of strategies are available: the antigen avoidance strategy and the pharmacological strategy.

ANTIGEN AVOIDANCE STRATEGY

The antigen avoidance strategy consists of management changes aimed to minimize the risk of exposure to exacerbating allergens. Ideally, severe asthmatic horses should be maintained at pasture (with the exception of those horses affected by pasture-associated forms). When stabling is unavoidable, managerial efforts should be pursued to limit exposure to airborne dust, including use of low-dust bedding, administration of forage other than hay (such as haylage, chopped dry forage, alfalfa, silage or cubed diets), improvement of stable ventilation (2). The antigen avoidance strategy represents the most effective and complete therapeutic approach because allows visible reduction of clinical signs within 3 days, progressive improvement of lung function, regression of airway neutrophilic inflammation, reduction of IL-8 mRNA expression and gradual reversibility of ASM remodeling (although the ASM remains about 30% increased compared to healthy horses) (8).

PHARMACOLOGICAL STRATEGY

Corticosteroids

Corticosteroids remain the elective drug choice to control severe equine asthma. Effective routes are parenteral (intravenous or intramuscular) administration and inhalation. The most common parenterally administered corticosteroid is dexamethasone (0.01-0.08 mg/kg) (57). Other corticosteroids which efficacy has been assessed after parenteral administration include triamcinolone acetonide (0.09 mg/kg) and isoflupredone acetate (0.03 mg/kg) (58, 59). Administration via inhalation limits systemic side effects (laminitis and adrenal suppression), while providing high drug concentrations within the airways (60). The most studied inhaled corticosteroids are beclomethasone dipropionate and fluticasone propionate. Beclomethasone dipropionate (500 µg-1320 µg q12 h) induces a dose-dependent adrenal suppression, showing variable efficacy compared to parenteral dexamethasone according to different studies (47, 61, 62). Fluticasone propionate (6 mg q 12h) administration, although resulting in a slightly reduced efficacy in improving airway obstruction is associated with significant lower side effects on adrenal function compared to parenteral dexamethasone and represent the most used inhaled corticosteroid (63). Corticosteroid treatment allows similar reduction of clinical signs compared to antigen avoidance, although horses remain exposed to exacerbating allergens. Furthermore, corticosteroid administration result in faster improvement of lung function, although incomplete, because an additional improvement could be obtained only by the antigen avoidance strategy. The reduction of ASM achieved by corticosteroid administration is similar to that obtained with antigen avoidance but is more rapid. However, corticosteroids are not efficacious in controlling airway inflammation, since pulmonary neutrophilia and level of IL-8 mRNA expression remain elevated even after long-term fluticasone administration (6 months) (8).

Bronchodilators

Bronchodilators have been largely employed in severe equine asthma treatment. An effective inhibition of bronchospasm and related airway obstruction may be achieved by adrenergic stimulation (sympathomimetics) or cholinergic/muscarinic antagonization (parasympatholytics) respectively. Bronchodilators are often administered as emergency drugs in severely affected horses or as pre-treatment that facilitates subsequent deep penetration and deposition of inhaled corticosteroids (64).

Sympathomimetics mainly include β_2-adrenergic agonists that may be administered systemically or via inhalation. Clenbuterol is usually administered orally and combined to corticosteroid, to avoid β_2-receptor downregulation and response decrease, a typical side effect of alone administered clenbuterol (tachyphylaxis) (65). Alternatively, some sympathomimetic bronchodilators may be administered via inhalation, including albuterol sulfate and salmeterol xinafoate. Albuterol sulfate is a short-acting β_2-adrenergic agonist having good efficacy in inducing rapid bronchodilatation (within 5 minutes after administration) with negligible systemic side effects. However, its effect is short, lasting variably between 30 minutes and 3 hours and requiring frequent administration (720 µg 3-4 times/day) (66). Salmeterol xinafoate (210 µg once-twice/day) is a long-acting β_2-adrenergic agonist which bronchodilation effect onset is delayed compared to albuterol (30-60 minutes after administration) but lasts longer (about 6 hours) (67). It is more indicated for long-term control of severe equine asthma, especially whether combined to fluticasone. A recent study showed that long term treatment with combinations of inhaled salmeterol and fluticasone are more effective than fluticasone alone in controlling clinical signs and airway neutrophilia, while no differences were detected regarding the effect on airway remodeling (68).

Parasympatholytic drugs which bronchodilator effect has been assessed in severe asthmatic horses include both anticholinergics and antimuscarinics. Anticholinergics include atropine and the anti-spasmodic N-*butylscopolammonium* bromide that are both administered systemically, providing rapid and efficacious bronchodilation. However, their clinical use is limited for different reasons.

Atropine has severe and significant systemic side effects while N-*butylscopolammonium* bromide has fewer side effects, however showing too short effect last (69, 70). The most used antimuscarinic is the non-selective ipratropium bromide (1.8-3.6 mg twice-three times/day). It could be administered via inhalation inducing efficacious bronchodilation with a quite long-lasting action and no significant systemic side effects (71). The selective M1-M3 antimuscarinic revatropate (1.2-7 mg) has been also assessed in horses, inducing significant bronchodilation. However, no significant advantages are shown compared to non-selective drugs (72).

Other drugs

Several molecules have been evaluated aiming to discover alternatives that could be as efficacious as corticosteroids in controlling clinical signs, but also capable to reduce airway inflammation. Unfortunately, most molecules targeting specific immunological pathways, such as p38 MAPK inhibitors, PDE4 inhibitors, cysteinyl-leukotriene antagonists appearing promising in experimental rodent models of asthma, did not show any significant efficacy in spontaneous severe equine asthma (4). Considering the emergent importance of ASM remodeling in determining disease progression, future therapeutic investigation could be target ASM proliferation, with the aim of reducing its mass (7).

CHAPTER 2: PULMONARY HYPERTENSION

In severe equine asthma, pulmonary hypertension (PH) is described as complication occurring during clinical exacerbation of the disease. However, accurate assessment of pulmonary artery pressure in horses is challenging, limiting clinical investigation about PH occurrence, its clinical impact and pathophysiology. In humans, PH represents a typical end-stage complication of some chronic respiratory disorders and its pathophysiology has been extensively investigated in cardiorespiratory medicine. Pulmonary vascular remodeling, defined as structural and compositional change of the pulmonary vessel wall, represents a key determining factor in the pathophysiology of PH, particularly in those forms associated with chronic respiratory disorders. An overview of human PH, especially concerning PH associated with lung disease, is provided in this chapter, highlighting the role of pulmonary vascular remodeling in its pathophysiology, supporting the hypothesis that similar mechanisms may be involved in severe equine asthma.

PULMONARY HYPERTENSION AND CHRONIC RESPIRATORY DISORDERS IN HUMAN MEDICINE

DEFINITION

Pulmonary hypertension (PH) is a pathological hemodynamic condition characterized by an increase in the mean pulmonary artery pressure (mPAP) above the range of physiological values. The mPAP is the mean between systolic and diastolic PAP, normally varying between 20 and 30 mmHg and

between 8 and 12 mmHg, respectively. In humans, the mPAP at rest is about 14.0 ±3 mmHg, with a normal upper limit of about 20 mmHg both for standing and supine positions (73).

According to the European Society of Cardiology and the European Respiratory Society guidelines, to define clinically the presence of PH, the mPAP must respect three different criteria: 1) it must be higher than 25 mmHg, 2) assessed at rest and 3) measured by right heart catheterization (74). Although the normal upper limit of mPAP is 20 mmHg, patients whose mPAP is between 21 and 24 mmHg are not considered hypertensive but they should be monitored, particularly when belonging to risk categories for development of pulmonary arterial hypertension (PAH) (75).

Diagnosis of PH is based on mPAP measured at rest because a reliable threshold between physiological and pathological increase in mPAP values during exercise is still impossible to be defined. Therefore, according to the current guidelines, a definition of "PH on exercise" is not accepted anymore (74). In any case, a resting mPAP between 21 and 24 mmHg seems closely associated with PH on exercise, when a combination of a mPAP greater than 30 mm Hg and a total pulmonary resistance, calculated as mPAP/cardiac output *ratio*, greater than 3 Wood units (WU) is detected at maximal exercise (76, 77).

Although right heart catheterization is invasive, it is the gold standard for the diagnosis of PH, especially when treatment is considered. Echocardiographic continuous wave Doppler technique allows to assess peak tricuspid regurgitation, from which an estimation of the mPAP could be obtained and used to determine the likelihood of PH. This technique is noninvasive and useful in clinical settings where it is performed as a screening test for patients suspected of PH. However, it does not provide the accurate measurement needed to support a treatment decision on an individual basis.

CLASSIFICATION

The classification of PH proposed in 1973, differentiated primary and secondary PH, according to the presence of identified causes or risk factors (78). In 1998, a more clinical approach was elaborated, aiming to classify the disease into groups sharing similar clinical presentations, pathological findings, and therapeutic strategies (79). This general classification scheme is still accepted today, having been revalidated in the most recent guidelines (74). Five groups of clinical disorders associated to PH are defined:

- Group 1: pulmonary arterial hypertension (PAH)
- Group 2: pulmonary hypertension due to left heart disease
- Group 3: pulmonary hypertension due to chronic lung disease/hypoxia
- Group 4: chronic thromboembolic pulmonary hypertension
- Group 5: pulmonary hypertension due to unclear multifactorial mechanisms

However, different clinical groups share similar hemodynamic characteristics, therefore a wider distinction between pre-capillary and post-capillary PH is also reported. For this classification, different hemodynamic parameters obtained by right heart catheterization are considered in addition to mPAP, including the cardiac output (CO), the pulmonary arterial wedge pressure (PAWP), the pulmonary vascular resistance (PVR) and the diastolic pressure gradient (DPG). The CO represents the blood volume pumped by the ventricle per minute and it is calculated by multiplying the stroke volume by the heart rate. The physiological CO varies between 4 and 8 liters per minute. The PAWP indirectly estimates the left atrial pressure and it measured by wedging the catheter with an inflated balloon into a small pulmonary arterial branch. Normal PAWP values vary between 4 and 12 mmHg. The PVR reflects the right ventricle afterload and it is calculated as the difference between the mPAP and the PAWP, divided by CO. Normally, PVR range is 0.3-1.6 Woods Units (WU). The DPG is a novel parameter calculated as the difference between the diastolic PAP and the mean PAWP, showing

low sensitivity to changes in loading conditions thereby serving as a robust marker for pre-capillary pulmonary vascular remodeling (80).

Pre-capillary PH includes all the PH conditions characterized by a PAWP value lower than 15 mmHg, with normal or reduced CO. Group 1, group 3, group 4 and some group 5 disorders are associated with pre-capillary PH. Conversely, post-capillary PH include all the PH conditions characterized by a PAWP value greater than 15 mmHg, with normal or reduced CO. Group 2 and some group 5 disorders are associated as post-capillary PH. A further sub-classification of post-capillary PH can be made according to the PVR and DPG values. Isolated post-capillary PH (Ipc-PH) refers to those post-capillary PH disorders characterized by a DPG value lower than 7 mmHg and/or a PVR value lower than 3 WU. In these cases, the primary left-sided cardiac condition is the only responsible for the PH and no pulmonary vascular remodeling is occurring. Combined post-capillary and pre-capillary PH (Cpc-PH) occurs when DPG value is equal or greater than 7 mmHg and/or PVR value is equal or greater 3 WU. In these cases, a primary left-sided cardiac condition inducing post-capillary PH, is associated to evidence of pulmonary vascular remodeling that add a pre-capillary PH component (81). Table 1 summarizes classification criteria of PH, according to the most recent guidelines (74).

A more detailed description of group 3 disorders will be provided below, including epidemiology, pathophysiology and clinical implications of respiratory diseases associated to PH.

HEMODYNAMIC CLASSIFICATION			CLINICAL CLASSIFICATION
Pre-capillary PH	mPAP > 25 mmHg PAWP < 15 mmHg		Group 1: Pulmonary Arterial Hypertension Group 3: Chronic lung disease/hypoxia PH Group 4: Chronic thromboembolic PH
.			Group 5: Unclear multifactorial PH
Post-capillary PH	mPAP > 25 mmHg PAWP > 15 mmHg	**Ipc-PH**: DPG < 7 mmHg PVR < 3 WU	Group 2: Left heart disease PH
		Cpc-PH: DPG > 7 mmHg PVR > 3 WU	

Table 2: Classification of pulmonary hypertension (PH). *mPAP =mean pulmonary artery pressure; PAWP= pulmonary arterial wedge pressure; DPG =diastolic pressure gradient; PVR = pulmonary vascular resistance; Ipc-PH = isolated post-capillary pulmonary hypertension; Cpc-PH = combined pre-capillary post-capillary pulmonary hypertension (74).*

CLINICAL IMPLICATIONS

Persistent PH predominantly increases the right ventricle (RV) afterload, that is the load against which the right ventricle must eject its volume of blood during contraction. The "RV afterload" depends on the pulmonary vascular load, such as the pulmonary vascular resistance and the blood volume, flow pulsatility, wave reflections through the pulmonary vascular system (82). The RV afterload is estimated by the pulmonary arterial elastance (Ea), defined as the change in pressure for a change in

volume, effectively representing the arterial load that the ventricle should overcome during ejection (82). Persistent increase in afterload affects RV structure and function, determining its remodeling and adaptation with progressive clinical evolution of PH (83). Progressive RV remodeling usually consists of an initial compensatory hypertrophy, that later progresses to maladaptive enlargement, until right heart failure (84). In case of group 3 PH, right heart involvement characterized by hypertrophy, dilatation or both is known as *cor pulmonale* (85). Right ventricle dysfunction is the most important determinant of PH long-term prognosis, since PH patients with higher mean right atrial pressures and lower cardiac output have significantly poorer survival rate (86). The evolution time from RV adaptation to RV failure is variable and is related to the response of myocardial tissue more than the amount of pressure overload (84).

In early PH stages, there is no RV dysfunction at rest and clinical evidence of cardiac involvement is limited to exercise intolerance, due to impaired "right ventricular exertional contractile reserve". The "right ventricle contractility" is an estimation of right ventricle pump performance, such as the degree to which myocardial fibers can shorten, independently from preload and afterload. It can be expressed quantitively by the right ventricle-end systolic elastance (Ees), i.e. the slope of the linear relationship between end-systolic pressure and volume, obtained by changes in ventricular filling (82, 83). The ratio between right ventricle contractility, expressed as Ees, and right ventricle afterload, expressed as Ea, and, is defined as "right ventricle-pulmonary arterial coupling" and represents a marker of ventricular efficiency (87). The adaptive compensatory mechanism to increased right ventricle afterload in early PH stages, is an increase of right ventricle contractility, via an enhancement of intrinsic cardiomyocyte contractile properties and via muscular hypertrophy (83, 84). The activation of sympathetic and renin angiotensin aldosterone system (RAAS) in response to short term pressure-overload play a pivotal role for maintaining in enhancing RV contractility. It has been suggested that the RV first adapt occurs through concentric hypertrophy and eventually evolves into eccentric hypertrophy (84). At rest, PH affected patients show similar right ventricle-pulmonary arterial coupling, preserved ventricular efficiency and unaltered ventricle volumes, compared to healthy

controls (87). Since no chamber dilatation occurs, the increase in contractility is defined as "homeometric" RV adaptation (Naijé et al. 2014). Right ventricle hypertrophy may be reversed by a decrease of RV afterload, although it is not clear whether the RV function is also restored (84).

During exercise, the increase of metabolic demand induces concomitant increase in heart rate and stroke volume. In healthy subjects, the increased stroke volume stimulates pulmonary vessel passive recruitment and distension, preventing excessive increase of the right ventricular afterload and resulting in increased cardiac output, enhanced pulmonary perfusion and gas exchanges. The "ventricular exertional contractile reserve" is defined as the magnitude from basal to maximal cardiac power output in response to exercise (88). In PH affected patients, a greater increase in afterload occurs during exercise because of limited passive distension of pulmonary vessels, decreasing right ventricle-pulmonary arterial coupling and ventricular efficiency. The impairment of the exertional contractile reserve prevents adequate increase of cardiac output, appearing as clinical exercise intolerance (87).

In more severe PH stages, the increase of right ventricle contractility is not sufficient to match the increased afterload. As the load progressively increases, the stroke volume and the ejection fraction decrease, with inactivation of the hypertrophic response and systolic dysfunction (82, 84). Failure of systolic function is associated to the RV "heterometric" maladaptive state, characterized by progressive RV dilatation and remodeling leading to right ventricle and chronic right heart failure (83). Several mechanisms, including genetic predisposition, neurohormonal overactivation and RV ischemia could trigger the transition from an adaptive to a maladaptive state, characterized by cardiomyocyte contractile dysfunction, progressive myocardial inflammation and fibrosis (82, 84). The sarcomeric stiffening associated to myocardial fibrosis is responsible for further deterioration of RV diastolic function and progression to end-stage right heart failure. Systolic and diastolic dysfunctions limit RV flow output, increase right-sided filling pressures, with the appearance of a congestive right heart failure clinical syndrome, and decrease the left-sided filling, with eventual lowering of the systemic arterial blood pressure (83).

DIAGNOSIS

Diagnosis of pulmonary hypertension may be challenging since most frequent clinical symptoms are related to progressive failure of the right ventricle and they are often non-specific. Furthermore, early clinical signs are not detectable at rest but mainly appear as a general exercise intolerance, with shortness of breath, fatigue, weakness, angina and syncope occurring during the effort and less commonly exercise-induced nausea and vomiting. With the progression of the disease severity, these symptoms are also detectable at rest and the end-stage disease consists of a congestive right heart failure, associated with abdominal distension and peripheral edema.

As suggested by the most recent guidelines, when PH is suspected, a systematic approach should be adopted aiming to confirm the haemodynamic changes, to identify the clinical classification group with the possible etiology, and to assess the condition severity (74). First, diagnostic confirmation of PH requires assessment of mPAP and other haemodynamic parameters. As mentioned above, the initial screening test is performed using echocardiography while the definitive diagnosis is achieved by right heart catheterization. Other ancillary diagnostic tests that may be useful to investigate the PH-associated clinical disorders, include routine hematology and biochemistry, electrocardiography, pulmonary function tests, arterial blood gas analysis, immunology, basic and advanced imaging techniques (chest radiography, ventilation/perfusion lung scans, high-resolution computed tomography, cardiac magnetic resonance imaging).

Transthoracic echocardiography is the least invasive tool to estimate PAP and is recommended as preliminary diagnostic test in patients suspected of PH, based on history and clinical signs. In absence of pulmonary valve outflow obstruction, systolic PAP corresponds to right ventricular systolic pressure (RVSP) and may be estimated from continuous wave Doppler measurements. The RVSP can be calculated by measuring the peak tricuspid regurgitation velocity (TRV) and estimating the right atrial pressure (RAP), according to the simplified Bernoulli equation $RVSP=4(TRV)^2+RAP$

(89). However, RAP could be only approximately estimated, therefore the direct TRV measurement is more accurate than the RVSP calculation to assess the level of PH probability in suspected symptomatic patients. When TRV is not measurable, lower than or equal to 2.8 m/s using transthoracic echocardiography, the probability of PH is low; when TRV is greater than 3.4 m/s probability of PH is high. When the TRV varies between 2.9 and 3.4 m/s the PH probability level may be intermediate or high depending of the presence of additional echocardiographic abnormalities affecting the ventricles (such as increased right ventricle/left ventricle *ratio* and flattening of the interventricular septum), the pulmonary artery (such as decreased right ventricle outflow Doppler acceleration time, increased early diastolic pulmonary regurgitation velocity and increased pulmonary artery diameter) or the right atrium/inferior vena cava (such as increased inferior vena cava diameter with decreased respiratory collapse, increased right atrial area) (74). Criteria for echocardiographic PH probability level assignation are resumed in table 2. However, inaccuracy in estimating the PH risk could occur. In patients with severe tricuspid regurgitation, the TRV may be technically difficult to measure and significantly underestimated, therefore it could not be used to rule out PH. Conversely, overestimation could also occur, therefore echocardiographic TRV alone is not a reliable screening tool for PH in asymptomatic or mild symptomatic patients (90, 91). Furthermore, although a presumptive diagnosis of PH may be based on echocardiographic findings in symptomatic patients, PH definitive diagnosis and treatment decision always requires confirmation by right heart catheterization. From a clinical point of view, echocardiography is useful to select those symptomatic patients that are likely to be affected by PH and should undergo further invasive investigation by right heart catheterization. In addition, some echocardiographic findings are suggestive for specific etiology in case of confirmed PH, especially for group 2 cardiac disorders.

The PH probability level is used to further orient the diagnostic approach in symptomatic patients. When the probability is low, causes of exercise intolerance other than PH should be considered and ruled out. When the probability of PH is intermediate or high, the suspicion merits investigation. In this case, one should investigate whether some clinical disorders typically associated to PH occur or

not. Chronic lung diseases (group 3) or left side chronic heart failure (group 2) could be detected by electrocardiography, pulmonary function tests, arterial blood gas analysis, chest radiography and high-resolution computed tomography. If group 2 or group 3 clinical disorders are diagnosed, the severity of PH-associated clinical signs should be evaluated. If they are mild or negligible, treatment could be focused only on the underlying disease; if symptoms are severe, PH itself should be treated and right heart catheterization is required to optimize the treatment choice. If group 2 and group 3 disorders are ruled out, the presence of other clinical disorders (group 1,4 and 5) should be investigated. An V/Q scan is the method of choice to assess group 4 CTEPH. This technique has an excellent sensitivity; therefore, a normal V/Q scan can rule out CTEPH. However, unmatched perfusion defects may be present also in group 1 clinical disorders (92). Definitive differential diagnosis must rely on other diagnostic test results, such as contrasted computed tomography angiography of the pulmonary artery, that can delineate the typical angiographic findings of CTEPH, including complete obstruction, bands and webs and intimal irregularities (93-95). If diagnosis of CTEPH is confirmed, traditional pulmonary angiography is required to evaluate which patients may benefit of elective lung surgery. Similarly, right heart catheterization might be also necessary for those patients already showing severe PH clinical signs, for prognosis and therapeutic approach evaluation. If CTEPH is ruled out, suspicion of group 1 PAH disorders becomes consistent. Serology, genetics and other specific diagnostic tests are required to differentiate etiologies of disorders belonging to group 1. When PH is confirmed with right heart catheterization, but etiology remains undiagnosed, patients are assigned to clinical group 5.

Right heart catheterization remains the gold standard to confirm PH diagnosis, to assess the severity of haemodynamic impairment and to perform vasoreactivity testing of the pulmonary circulation in to identify suitable candidates for treatment with high-dose calcium channel blockers. In patients showing post-capillary PH (elevated PAWP) in absence of echocardiographic signs of left heart disease (preserved ejection fraction, normal left atrial size and absence of markers of elevated left

ventricle filling pressures), a left heart catheterization may also be required to determine whether inaccuracy of PAWP occurs, by directly measuring the left ventricle end-diastolic pressure.

TRV measurement (m/s)	Other echocardiographic signs	Level of PH probability
≤ 2.8 or not measurable	NO	Low
	YES	Intermediate
2.8 < TRV < 3.4	NO	
	YES	High
>3.4	NOT REQUIRED	

Table 3: Echocardiographic criteria for assignation of pulmonary hypertension (PH) probability level. *Echocardiographic additional signs may be detected from at least two of the three echocardiographic districts including the ventricles, the pulmonary artery and the inferior vena cava/right atrium.*

CHRONIC LUNG DISEASE/ HYPOXIA-ASSOCIATED PULMONARY HYPERTENSION

Definition, classification and epidemiology

Group 3 PH occurs when the mPAP increase greater than 25 mmHg is related to respiratory abnormalities, such as chronic parenchymal lung diseases or persistent hypoxia. The condition may be further classified as "severe" when one of two following situations occurs: the mPAP is greater than 35 mmHg or the mPAP is lower than 35 mmHg, but associated with a low cardiac index (inferior than 2.5L/min/m^2) not explained by other causes (74, 96). Lung diseases that are associated with PH include Chronic Obstructive Pulmonary Disease (COPD), Interstitial Lung Diseases (ILD), Connective Tissues Disease (CTDs) involving the lung, Sarcoidosis, Idiopathic Pulmonary Fibrosis (IPF), Lymphangioleiomyomatosis (LAM) and Combined Pulmonary fibrosis and Emphysema

(CPFE) (97). All these disorders may evolve without the appearance of PH. However, development of PH is negative prognostic factor that is significantly associated with deterioration of exercise capacity, worsening of hypoxemia, reduced response to treatment and shorter survival (98, 99). Pulmonary hypertension represents the most common end stage complication of both severe ILD and severe COPD. However, there is no correlation between the severity of the lung disorder and the severity of the associated PH. In most cases, PH is mild or moderate while severe PH is rare, mainly occurring in the CPFE, where the overall prevalence of PH is high. In one survey study, among PH affected patients showing mPAP greater than 40 mmHg, only 9.7% belonged to clinical group 3 (97). Therefore, it is unlikely that a mild lung disease is associated to severe PH. If this combination is detected, other groups of clinical disorders (particularly group 2 and 4) causing PH could be present in addition to lung disease and should be investigated. In patients with COPD, serotonin transporter gene polymorphism appears to be related the severity of PH. Patients carrying genotypes associated with high serotonin transporter expression in pulmonary artery smooth muscle cells show significantly higher smooth muscle hyperplasia, with development of more severe PH (100).

Physiopathology

In physiology, the pulmonary circulation is defined as “low pressure circulation system” because vascular pressure values are much lower compared to systemic circulation, although both systems receive the same cardiac output flow. The blood driving pressure is directly influenced by vascular resistance which regulation mechanisms highly differ between the two circulation systems. Pulmonary vascular resistance (and the associated driving pressure) is inversely proportional to the cardiac output flow, because, unlike the systemic circulation, the vasomotor activity of pulmonary vessels is limited.

Regulation of pulmonary regional blood flow mainly occurs at pulmonary capillaries which represent the largest percentage of the pulmonary vascular surface. No smooth muscular layer is detectable within the pulmonary capillary wall, therefore vascular resistance regulation occurs through passive vasoconstriction and vasodilation, depending on alveoli inflation status and blood flow through the lung. In physiological conditions, pulmonary circulation adapts to large changes in cardiac output by distension or recruitment of under-perfused pulmonary capillaries, meaning that as the pulmonary blood flow increases, the pulmonary vascular resistance and pressure decrease.

Increase in pulmonary vascular resistance by active vasoconstriction may occur as an adaptive mechanism only for extra-alveolar precapillary vessels (arterioles and muscular pulmonary arteries), in response to specific nervous, humoral and particularly gaseous *stimuli* such as hypoxia (101). Hypoxic pulmonary vasoconstriction (HPV), also known as von Euler-Liljestrand mechanism, is a reflex mechanism that optimizes the ventilation/perfusion matching, in case of regional acute or sustained hypoxia. Alveolar hypoxia as well as a drop in pulmonary arterial oxygen tension (PaO_2) directly induce local pulmonary artery vasoconstriction, diverting blood flow from unventilated hypoxic areas toward better ventilated lung regions. HPV is triggered by intrinsic mechanisms, involving pulmonary artery smooth muscle cells (PASMC) and endothelial cells. In acute HPV, the oxygen sensing-signaling pathway is completely located in PASMC and adapts perfusion to ventilation in seconds, on a breath-to-breath basis. A change in PASMC redox state due to a modification in reactive oxygen species (ROS) content of PASMC mitochondria is the most likely transduction pathway, since ROS production is an oxygen-dependent process that is rapidly modified according to oxygen tension. However, whether the cause of HPV is an increased or a decreased ROS production and how the cellular redox state may activate smooth muscle contraction cascades are not clear and still debated (102-106). Sustained HPV, occurring when hypoxic ventilation lasts more than 10 minutes, involves transduction mechanisms related to PASMC energetic metabolism, particularly a shift from aerobic mitochondrial ATP production to predominant anaerobic glycolysis (107, 108). Definitively, both a change in the PASMC redox status and/or an enhanced PASMC anaerobic

metabolism trigger PASMC intracellular calcium release, resulting in myosin light chain phosphorylation and contraction. The myosin light chain phosphatase mediates de-phosphorylation with myorelaxation and vasodilation. Sustained hypoxia also activates endothelial signaling pathways, such as endothelial membrane depolarization in alveolar capillaries. The signal is propagated upstream through the endothelial layer in a connexin-40-dependent manner and is necessary for pre-conditioning PASMC to execute sustained HPV. An endothelium-dependent rho-kinase activation could inhibit the myosin light chain phosphatase, inducing sensitization and persistent vasoconstriction in sustained HPV (109). Although triggered by PASMC or endothelium intrinsic mechanisms, HPV can be modulated by extrinsic systemic neural, humoral and physical factors, which play a role in pathological conditions (110).

During parenchymal chronic lung disease, pathological changes involve mechanical, structural and biochemical/reflexive factors, as summarized in table 3. Although the contribution of single factors depends on the specific underlying disease, these changes enhance active vasoconstriction and prevent passive vasodilation, resulting in an overall increase of pulmonary vascular resistance and onset of PH (97, 101).

Mechanical factors include alveolar hyperinflation and increased blood viscosity. Alveolar hyperinflation significantly contributes to PH in advanced COPD (111) Chronic bronchitis reduces airway lumen, limits expiratory airflow and determines lung air trapping, increasing intra-alveolar volume and pressure. Increase of alveolar air volume stretches capillaries lying within the alveolar wall, with reduction of their diameter and increase in their length. The consequence is a great increase in capillary resistance, because vascular resistance is directly proportional to vascular length and inversely proportional to vascular radius to the fourth power. Furthermore, increased intra-alveolar pressure directly compresses pulmonary capillaries, limiting passive recruitment and distension in response to pulmonary blood flow augmentation (112). Chronic hypoxemia occurs in several chronic lung diseases and stimulates secondary polycythemia, with increased blood viscosity that is associated to increased vascular resistance in a directly proportional manner (113).

Structural factors refer to remodeling and re-distribution of pulmonary vessels. Remodeling is the predominant cause of increase in vascular resistance in all chronic lung disease associated to PH. Furthermore, remodeling represents the main cause of PH unresponsiveness to oxygen therapy in these disorders. The common consequence of different remodeling processes is vascular wall thickening that involves predominantly small pulmonary arteries and, in conditions such as sarcoidosis, also pulmonary venules (114, 115). Wall thickening increases vascular resistance by reducing vascular *lumen* diameter and favoring vascular stiffness, with the limitation of passive distension. Some respiratory disorders (such as COPD and pulmonary fibrosis) and chronic hypoxia (116) are also characterized by vascular smooth muscle hypertrophy/hyperplasia and distal arteriolar neo-muscularization. These events contribute to PH onset, by reducing distal vessel compliance but also enhancing global lung response to HPV. Re-distribution of pulmonary vessels include loss of pulmonary vascular bed due to emphysema or fibrosis associated to abnormal angiogenesis (101). Emphysema typically occurs in COPD and is defined as an irreversible abnormal enlargement of lung air space due to progressive destruction of alveolar walls, that also involve destruction of alveolar capillaries. In IPF, severe fibrosis also involves intimal layer of pulmonary capillaries, leading to vascular obliteration. Furthermore, chronic hypoxia, lung parenchymal destruction and inflammation stimulate vascular pericytes to release pro-angiogenetic mediators, such Vascular Growth Endothelial Factor (VGEF), Transforming Growth Factor β (TGFβ) and Platelet Derived Growth Factor (PDGF). These factors induce angiogenesis by stimulating proliferation of endothelial cells (117). However, newly produced vasculature is structurally abnormal, having less capability of passive distension compared to normal pulmonary capillaries. Destruction or obliteration of pulmonary capillaries and abnormal angiogenesis prevent adequate vascular recruitment when cardiac output increases, contributing to enhanced vascular resistance and effort intolerance during exercise conditions (118). Re-distribution of pulmonary vessels plays a minor role compared to vascular remodeling in the pathophysiology of PH at rest, especially in mild-moderated disease. It has been shown that the extent of emphysema in COPD, evaluated as lung density *in vivo* by CT scans, is not correlated to the

severity of PH at rest, assessed as measured mean pulmonary arterial pressure, cardiac output, or calculated total pulmonary vascular resistance. However, in end-stage disease, emphysema represents the major determinant of PH and significantly affects gas exchange surfaces and arterial oxygenation.(111, 119).

Biochemical/reflexive factors include pathological sustained HPV and pulmonary vascular endothelial dysfunction. Local acute HPV is a protective mechanism, but becomes detrimental when sustained and activated in the whole lung. This situation occurs at high altitude or during chronic parenchymal lung diseases, because of persistent global hypoxia and hypoxemia (110). In these disorders, sustained global HPV increases pulmonary vascular resistance, contributing to PH progression, that may be prevented by long term oxygen therapy. However, oxygen administration does not achieve a complete PH reversibility, demonstrating that the role of HPV in PH physiopathology is strictly related to presence of VSM remodeling. In fact, increase in VSM mass results in enhanced vasoconstriction due to HPV, with a proportionally more significant increase in vascular resistance. Furthermore, sustained vasoconstriction stimulates VSM hypertrophy, favoring advancement of vascular remodeling and disease progression (101). Pulmonary vascular endothelial dysfunction involves an imbalance of endothelial vasoactive mediator production. An increased endothelial expression of endothelin-1, the most potent vasoconstrictive mediator, is associated to a reduced expression of nitric oxide (NO) and prostacyclin synthases, with impairment of endothelium-derived vascular relaxation mechanisms and overall increased vascular resistance. Change in endothelial-derived vascular tone regulation occurs as consequence of endothelial remodeling. In fact, in advanced COPD, impairment of NO release is correlated to the severity of hypoxemia and to the degree of pulmonary artery intimal layer thickening. Direct endothelial injury also affects endothelial mediator production. In smokers, cigarette smoke directly mediates endothelial injury, therefore endothelial dysfunction may also precede both COPD and PH onset (117, 120).

Mechanical factors	Air trapping (alveolar hyperinflation) • Increased intra-alveolar volume (capillary stretching) • Increased intra-alveolar pressure (capillary compression) Increased blood viscosity • Secondary polycythemia
Structural factors	Vascular remodeling • Wall thickening (increased vascular stiffness and reduced lumen) • Vascular smooth muscle mass increase (smooth muscle hypertrophy, hyperplasia and neo-muscularization) Pulmonary vasculature re-distribution • Capillary destruction (emphysema) • Capillary obliteration (fibrosis) • Abnormal angiogenesis
Biochemical/reflexive factors	Sustained global hypoxic pulmonary vasoconstriction Endothelial dysfunction • Increased endothelin-1 production • Impaired nitric oxide and prostacyclin mediated vasorelaxation

Table 4: Factors contributing to pulmonary hypertension physiopathology in chronic lung disorders (group 3).

Diagnosis and treatment

In patients with chronic lung disease, the onset of PH may be difficult to detect clinically because symptoms do not differ from those of the respiratory disorder alone. Furthermore, concomitant left heart disease is frequent and may also contribute to the PH. All patients affected by chronic lung disease routinely undergo pulmonary function testing and arterial blood gas analysis: those patients showing clinical signs more severe than what expected according to results of these analyses should be suspected of PH. A disproportionally low lung diffusion capacity for carbon monoxide (DLCO) and pCO2 also suggest presence of PH in patients with chronic lung disease. All these patients must undergo echocardiographic screening evaluation for PH. Right heart catheterization should be performed to definitively confirm or rule out PH in cases of inconclusive echocardiography, especially when probability of PH is high, when patients are suitable candidates for surgical treatments (transplantation, lung volume reduction) or when other concomitant causes of PH (such as left heart disease) could be present, with the potential impact on PH therapeutic approach (74).

So far, PH due to chronic lung disease has no specific therapy. Drugs that are currently administered for treating group 1 PAH, such as conventional calcium channel blockers vasodilators (CCBs), are not recommended in chronic lung disease because there is no evidence suggesting that they could improve pulmonary haemodynamics, clinical signs or prognosis. Furthermore, CCBs inhibit HPV, worsening ventilation/perfusion mismatch and gas exchange, being more deleterious than beneficial particularly in hypoxaemic patients (97, 121-125). The therapeutic approach should optimize the management of the underlying respiratory disorder. In COPD, long-term O2 administration represents the elective treatment and seems to prevent further progression of PH. However, normalization of mPAP is rarely obtained, because the increase in PVR due to pulmonary vascular remodeling persist after oxygen administration (126).

PULMONARY HYPERTENSION AND SEVERE EQUINE ASTHMA

Pulmonary hypertension and related cardiovascular complications are reported in severe equine asthma. As human respiratory conditions associated with group 3 PH, severe equine asthma is characterized by alveolar hyperinflation, hypoxaemia and related HPV, all factors that could contribute to development of PH (50). However, disadvantages of available diagnostic techniques as well as unknown clinical significance has limited the interest in investigating PH in severe asthmatic horses. An overview about experimental case-control studies (51, 127-130) and case reports (131-133) that focused on PH and related cardiovascular complications in severe asthmatic horses will be provided.

PHYSIOPATHOLOGY AND CLINICAL PRESENTATION

In horses, PH is diagnosed in presence of systolic and/or mean PA pressure values greater than 40 mmHg and/or 35 mmHg respectively (133). Severe equine asthma is typically associated with recurrent reversible PH. A significant increase in mPAP occurs during exacerbation, leading to severe PH, with mPAP values that frequently reach 80 mmHg. However, disease remission or oxygen administration rapidly induce normalization of mPAP, even in horses with a long-term history of severe asthma. During remission, mPAP values of severe asthmatic horses, although within the normal range, remain significantly higher than those of healthy horses (51, 127).

The main etiological factor contributing to PH onset during exacerbation is hypoxaemia. Although PH develops only in severe asthmatic horses, experimentally-induced hypoxaemia results in increased mPAP also in healthy horses, due to reflexive HPV (51). Furthermore, the severity of PH appears inversely correlated to PaO_2, evaluated with arterial blood gas analysis (129). The

determinant role of HPV is supported by the rapid decrease of mPAP values observed after oxygen administration (51).

Nevertheless, oxygen administration is only partially efficacious in reversing PH of severe asthmatic horses and some horses in remission still show elevated mPAP even though PaO_2 values are normal. Furthermore, in response to a similar PaO_2 level drop, asthmatic horses but not healthy subjects develop PH, suggesting the involvement of other etiological factors that are still uninvestigated in severe equine asthma-associated PH (51, 129). Possible contributing factors include compression of capillaries due to positive intrapleural pressure and alveolar hyperinflation as well as vasospasm induce by inflammatory mediators (51, 129).

CARDIOVASCULAR IMPLICATIONS

Although severe equine asthma-associated PH is reversible, it could lead to onset of cardiovascular complications, particularly when the disease is advanced and the response to treatment is incomplete. The cardiovascular complications of PH described in severe asthmatic horses include *cor pulmonale* and dysrhythmias (atrial fibrillation).

Cor pulmonale and cardiac remodeling

The overall incidence of *cor pulmonale* appears to be low in severe equine asthma, compared to human chronic respiratory conditions. In severe equine asthma, cardiac overload is not persistent because PH is reversible, therefore it is likely that advanced stages as well as frequent exacerbations are required to induce significant cardiac remodeling. (127). However, it is not clear whether the low incidence reflects a real low occurrence or just a low sensitivity of the available diagnostic methods. In 1982, Dixon et al. systematically investigated presence of clinical right heart failure and *post-*

mortem macroscopic findings suggestive of *cor pulmonale*, such as right ventricle (RV) hypertrophy and dilatation, in a group of severe asthmatic horses. Cardiac dilatation and clinical heart failure were not observed, while mild RV hypertrophy was detected in only few cases (127). More recently, two case reports described the occurrence of severe *cor pulmonale* associated with right-sided heart failure, showing clinical signs of mild distal limb edema and jugular vein distension, echocardiographic findings of severe tricuspid valve regurgitation and RV enlargement as well as marked increase of right ventricular pressure (85 mmHg) (131, 133). One case failed to respond to combined corticosteroid-bronchodilator therapy and was euthanized. At *post mortem* examination, lung tissue appeared extensively damaged with significant emphysema that was considered a more likely cause than right heart failure for the treatment unsuccess (131). The other case showed good response to the same treatment and complete regression of the related cardiac changes (133). Therefore, in severe asthmatic horses the presence of *cor pulmonale* do not represent a negative prognostic factor and adequate treatment of the primary respiratory condition may reverse not only PH but also related cardiac impairment.

However, these observation does not rule out a significant clinical impact of recurrent PH and *cor pulmonale* (127). Recent evidence suggest that relative short periods of PH induce subclinical structural myocardial remodeling that persist during disease remission. These structural changes are probably underestimated because they could not be detected by conventional echocardiography or *post-mortem* macroscopic examination (129). Although subclinical, cardiac remodeling may significantly affect cardiac function during exercise as occurring in human athletes, where it is associated with increased risk ventricular dysrhythmias (134). As in human COPD, PH may early develop only during exercise in asthmatic horses, determining poor performance (128). Although mild increase in *serum* level of troponin physiologically occurs after exercise, it has been reported that asthmatic horses have abnormally increased troponin after slight exercise, reflecting possible myocardial stress due to exercise PH (129).

A description of the most significant findings suggestive of morphologic or functional cardiac abnormalities detected in severe asthmatic horses is provided below.

Morphologic abnormalities

Cardiac morphologic changes typical of *cor pulmonale* include RV hypertrophy, dilatation or both (85). During exacerbation, dilatation of right atrium and right ventricle as well as associated interventricular *septum* abnormalities may be detected by echocardiography, but are rarely appreciable at *post mortem* examination. Increase in right heart size may be associated with normal or reduced left ventricle size (127, 128, 132, 133). Morphologic septal abnormalities include flattening of the septal curvature, leftward deviation, hypokinesis or paradoxical motion (128, 129, 132, 133). Increase in right ventricle overload may prevent physiological movements of the *septum* that normally contribute to the activity of the left ventricle (133). Other findings that suggest right heart dilatation are significant tricuspid or pulmonic valve regurgitations that could be detected as a right-sided systolic or left-sided diastolic murmurs respectively (132). Although significant murmurs may not be audible, regurgitations could be also assessed by color Doppler echocardiography (128, 132, 133). Hypertrophy of the RV is more difficult to evaluate, since measurement of RV wall thickness is not enough sensitive, both at traditional echocardiography and at macroscopic *post-mortem* examination (127, 128). In some cases, the visualization of a rounded heart apex at echocardiographic examination may suggest non-specific ventricular hypertrophy (133). Mild RV hypertrophy is more often detected at *post-mortem* examination, as a decreased weight *ratio* between left and right ventricles, indicating presence of heavier right ventricle (127, 131). A recent study detected a significant increase of RV wall thickness in severe asthmatic horses during remission, compared to healthy horses, suggesting that hypertrophy may persist also after normalization of mPAP values. The same study focused the attention on histological examination, a useful tool to assess presence of myocardial damage and cardiac remodeling induced by pressure overload and

particularly their persistence after PH regression. In one remission horse, endomyocardial biopsy revealed a focal interstitial neutrophilic infiltration. This finding could reflect a persistent inflammatory process induced by mechanical tension stress occurred during exacerbation (129).

Morphologic abnormalities may be suggestive not only of *cor pulmonale* but also of PH. Dilatation of PA is considered as an indicator of PH and may be detected at traditional echocardiography as an increased PA diameter or more easily as a decreased *ratio* between aortic and PA diameters (128, 132). Also at *post- mortem* examination, PA dilatation is more easily appreciated by calculating the *ratio* between aortic and PA circumferences. A decreased *ratio* suggests PA enlargement (127, 131).

Functional abnormalities

Although cardiac morphologic changes are not detected in all cases, presence of PH systemically induces altered RV function during exacerbation. During remission, as mPAP decreases, also normalization of cardiac function occurs. However, it is unknown whether subtle changes persist, contributing to exercise intolerance in severe asthmatic horses (128, 129).

Most findings of altered RV functions are detected by echocardiography, particularly using color-flowc and pulsed-wave Doppler evaluation or new developed techniques, such as tissue Doppler imaging (TDI) or 2D speckle tracking echocardiography (STE). Increase in PA pressure and volume may cause pulmonary insufficiency, altered RV systolic and diastolic function, as well as reduced RV contractility. Pulmonic valve insufficiency associated with PH is end-diastolic and characterized by high-velocity flow. In one case report, clinical signs of right heart failure were detected associated pulmonary insufficiency, indicating that this finding is typical of advanced disease stages (131). Altered RV systolic function is reflected by abnormal Doppler PA systolic trace, reflecting an altered RV ejection pattern that become more similar to that of the LV and decrease of systolic time intervals, including the acceleration time (AT), the RV-ejection time (ET), the AT:ET *ratio*, as a consequence of the increase in RV pressures and PH (132). Decrease and increase of early and late diastolic filling

velocities respectively suggest an impairment of the RV diastolic function, with prolonged isovolumic relaxation and pre-ejection period. Increased PA pressures and subclinical myocardial damage due to repeated hypoxaemia are considered as the most likely cause of altered relaxation (129). The longer pre-ejection period associated with a reduced segmental longitudinal strain also suggest an impaired RV contractility (129).

Altered left ventricle (LV) function is also possible. A reduction of the contractility (shortening fraction) as well as of the estimated stroke volume may occur (128, 133). This is determined by a decreased left ventricular pre-load, due to the reduced pulmonary venous return associated with PH. However, an increase of the heart rate usually allows maintenance of a normal cardiac output (128). Interestingly, according to Decloedt et al., RV pressure and contractility of severe asthmatic horses in remission significantly differ from those of healthy horses, suggesting the presence of myocardial remodeling responsible for persistent RV subclinical altered function (129).

Dysrhythmias (atrial fibrillation)

In 2015, Hanka et al. described a case of paroxysmal atrial fibrillation in a severe asthmatic horse, occurring during exacerbation in association with evidence of reversible PH and *cor pulmonale*. Administration of combined corticosteroid and bronchodilator therapy resulted in normalization of both cardiac morphologic as well functional abnormalities and cardiac rhythm (133). The authors hypothesized that atrial enlargement due to cardiac morphologic abnormalities associated with PH may have been the cause of atrial fibrillation. However, dysrhythmias may also appear as a consequence of myocardial hypoxia, as a consequence of hypoxaemia and impaired gas exchanges related to the primary respiratory disorder (129).

DIAGNOSTIC ASSESSMENT

Diagnostic assessment of PH and *cor pulmona*le in horses has several limitations that are related to the low sensitivity of available non-invasive techniques as well as to the invasiveness and expensiveness of the diagnostic gold standard (right heart catheterization). However, recent development of advanced echocardiographic-assisted techniques has improved diagnostic sensitivity, increasing the attention towards significance of sub-clinical structural and functional abnormalities that were previously undetectable (129).

Right heart catheterization

Right heart catheterization represents the gold standard to diagnose PH in equine as in human medicine. The procedure is invasive but is performed in standing non-sedated horses. In an area corresponding to the lower one-third of the jugular groove, a catheter is introduced through the skin, into the jugular vein and then advanced through the right *atrium* (RA), the RV and eventually about 2 cm into the main PA trunk. The catheter is connected to a manometer placed approximately at the level of the shoulder point (level of the RA), then to a pressure transducer and a digital recording system (51). The technique allows estimation of systolic, diastolic and mean pressures of the RA, the RV and the PA. Normal ranges in the horse are reported in table 5 (131, 133, 135). During exacerbations, it is common to detect significant increase of mean RA pressure, systolic RV pressure, mean and systolic PA pressure (51, 129, 131, 133). Detection of increased RV pressures has been associated with clinical signs of right-sided heart failure (131, 133). Although invasive, right heart catheterization is the most sensitive technique, being able to detect significant differences in mPAP values between asthmatic horses in remission and healthy horses (51).

Pressure mmHg	Right atrium	Right ventricle	Pulmonary artery
Systolic	-	40-60	32-38
Diastolic	-	10-14	15-22
Mean	5-10	19-25	25-30

Table 5: physiological ranges for right atrium (RA), right ventricle (RV) and pulmonary artery (PA) pressure in horses (135).

Echocardiography

Echocardiography is a non-invasive diagnostic method providing useful tools to investigate both presence of PH and cardiac abnormalities suggestive of *cor pulmonale*. Different echocardiographic techniques are available, allowing assessment of multiple parameters.

Bi-dimensional echocardiography

Bi-dimensional echocardiography requires a phased-array or micro-convex transducer, with a frequency of 2.5-3.5 mHz and a depth of 25-27 cm. Standard images are obtained both from the right and from the left side. Measurements should be collected at a standard moment of the breathing cycle, such as the end of inspiration, because of variations related to intrathoracic pressure changes (128). Measurements suggestive of both pulmonary hypertension and *cor polmunale* may be collected.

- *RV, LV, RA luminal diameters and LV/RV ratio*

  These measurements estimate the size of the respective cardiac chambers. Increase in RV and RA diameter may suggest the presence of right heart (and particularly right ventricle) dilatation, as an indication of *cor pulmonale*. Decrease of LV diameter reflects reduction of LV size, due to RV overload and increased pressure, as an indirect indication of PH (128,

129, 132, 133). Measurements should be taken always at the end of the inspiration because of variations related to the breathing cycle. During inspiration, the ΔPpl increases, facilitating the venous return to the right heart with increase in size of the RV. At the same time, the LF size reduces because an increase in the whole heart volume is prevented by the *pericardium* stiffness. These variations are negligible in a horses and in asthmatic horses during remission, however they become consistent during exacerbation of severe equine asthma, due to the significant increase of ΔPpl (128).

- *RV free wall thickness*

  An increase of RV wall thickness, assessed using the left side views, reflects RV hypertrophy, as indication of *cor pulmonale* (128). However, this parameter has a very low accuracy and sensitivity, being unable to detect mild RV hypertrophy, as often occur even in cases of consistent RV dysfunction (128, 131, 133). Although a significant increase in RV thickness is rarely detected during exacerbation, RV wall is significantly more thickened in asthmatic horses compared to healthy horses, also during remission, suggesting presence of persistent cardiac remodeling (129).

- *Heart apex morphology*

  An abnormal cardiac apex that appears round-shaped is consistent with ventricular hypertrophy. In those clinical cases suggestive of PH, a rounded apex may reflect RV hypertrophy and therefore suggest presence of *cor pulmonale* (133).

- *Interventricular septum morphology and motion*

  Interventricular *septum* abnormalities include flattening, leftward deviation, hypokinesis and paradoxical septal motion. These abnormalities are related to the excessive RV afterload that impair the physiological synergic role between the *septum* and the left ventricle. Septal

abnormalities represent a frequent finding in severe asthmatic horses affected with *cor pulmonale* (128, 131-133).

- *PA, AO diameters and their ratio*

  Increased PA diameter as well as an increase in the ratio between the PA and the AO diameters indicate dilatation of PA and are quite systematically detected during exacerbation (129). In severe cases, the PA diameter may become equal or greater than the AO diameter (131-133). Pulmonary artery dilatation represent the main echocardiographic finding allowing indirect, non-invasive, qualitative assessment of PH in horses (128).

- *Index of cardiac performances*

  The most important indexes of global cardiac performances that could be assessed with bi-dimensional echocardiography include the LV shortening fraction (and shortening area), the "estimated" stroke volume and the cardiac output. As mentioned above, during exacerbation a decrease in the LV shortening fraction reflects decreased LV contractility, while a reduced stroke volume is related to a decrease of LV preload associated with PH (128, 133)

Color-flow Doppler

Color-flow Doppler may be used to investigate presence of tricuspid or pulmonic valve regurgitation, that could appear as a consequence of right heart dilatation. In case of significant regurgitation, the subtended jet area may be measured as an indirect estimation of the PA pressure (128). Pulmonic valve insufficiency may be detected in case of severe *cor polmunale* and impending right-sided heart failure (131, 133). Presence of tricuspid regurgitation should be interpreted with caution. Mild or moderate tricuspid regurgitation often represents an incidental finding in older horses and is not associated with clinical signs of cardiac disease. Therefore, if mild-moderate tricuspid regurgitation

is found in severe asthmatic horses both during exacerbation and during remission, its significance as a marker of *cor pulmonale* is unlikely (128). Conversely, when moderate-severe tricuspid regurgitation is associated with consistent RV and RA enlargement and clinical signs of right-sided heart failure, it may be considered as an indication of ongoing *cor pulmonale* (133).

Pulsed-Wave Doppler

Pulsed-Wave Doppler is used to assess flow velocity and wave-form. Assessment of PA blood flow velocities, wave form and systolic time intervals, such as the AT, ET and the AT:ET *ratio*, gives information about the RV ejection pattern and related severity of PH, as shown in figure 1 (132). In case of moderate PH, a decrease of the AT may be observed, with subsequent steep appearance of the wave ascending phase. In case of severe PH, the AT may further decrease and a notch may appear in the middle of the descending phase, that looks relatively prolonged due to AT decrease (132). One should remind that Pulsed-Wave Doppler evaluation is accurate only if the beam is aligned with the blood flow. In case that a good alignment could not be achieved, the technique may fail in detecting typical findings suggestive of PH, although PH occurs (128). Furthermore, a decrease in systolic time intervals occur also in tachycardic horses, therefore more importance should be given to the AT:ET parameter, that is less influenced by heart rate (132).

In case of significant tricuspid regurgitation, Pulse-Wave Doppler could be used to evaluate its maximum velocity and eventually to estimate the PA pressure, in absence of pulmonary outflow obstruction, using the same approach described by human PH diagnostic guidelines (74, 132). However, as for PA flow, the accuracy of estimation significantly depends on the alignment between the beam and the regurgitant flow (128).

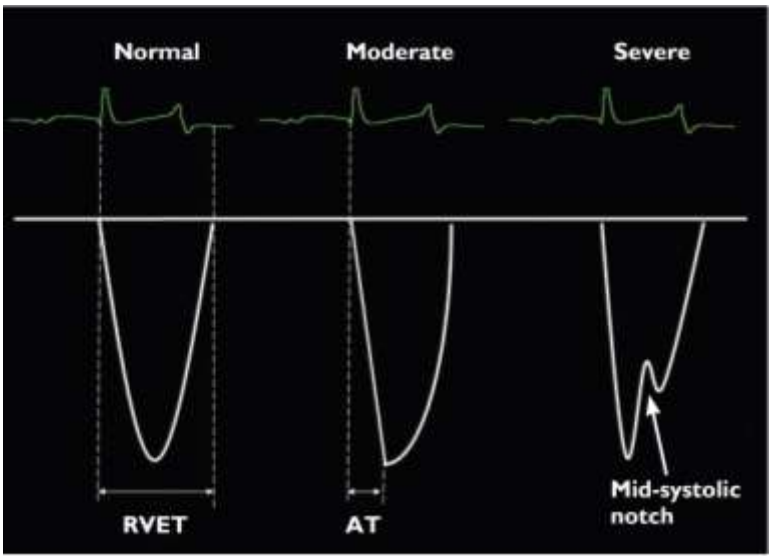


Figura 1: Schematic representation of PA flow wave forms (132). *In physiological conditions (Normal), the wave is symmetric, with similar acceleration time (AT) and deceleration phase. In case of moderate PH (Moderate), the AT significantly decreases: the wave loses its symmetric shape and the ascending phase becomes steep. In severe PH (Severe) the AT further decrease with an almost vertical ascending phase and a notch appear in the middle of the descending phase which appearance becomes biphasic. RVET=ET=right ventricular ejection time*

Novel techniques: tissue Doppler imaging (TDI) or 2D speckle tracking echocardiography (STE)

Tissue Doppler imaging (TDI) and 2D speckle tracking echocardiography (STE) are recently developed techniques that allows a sensitive evaluation of the RV function. Their application in investigating PH and *cor pulmonale* in severe equine asthma gave interesting results, especially in comparing asthmatic and healthy horses.

Also during remission, asthmatic horses may show greater left ventricular end-systolic eccentricity index, decreased longitudinal strain and longer RV pre-ejection period. All these findings suggest reduced RV contractility in asthmatic horses, probably reflecting the presence of structural subclinical cardiac remodeling.

During exacerbation, employment of these techniques allows detecting functional abnormalities that could not be detected by traditional echocardiography. Severe asthmatic horses in exacerbation show prolonged pre-ejection period and isovolumic relaxation time, that are correlated with increase in PA

pressure and are suggestive of PH. Furthermore, presence of RV pressure overload may be indicated both by the presence of higher LV end-systolic eccentricity index, reflecting septal flattening, and by the presence of RV volume overload, reflected by a high LV end-diastolic eccentricity index (129).

Endomyocardial biopsy and myocardial enzymes

These methods are usually employed to investigate presence of myocardial damage. Myocardial enzyme (troponin) *serum* dosage has a low sensitivity, since myocardial damage may occur significantly, accompanied only by a slight increase of *serum* troponin (128). Endomyocardial biopsy is a recently described promising techniques allowing assessment of myocardial histological changes. Presence of structural remodeling may be subclinical but persistent during remission and whether it could have an impact on athletic performances of asthmatic horses still remains unknown (129).

***Post mortem* examination**

Presence of PH and *cor pulmonale* may also be investigated at *post mortem* examination. One should measure the RV free wall thickness, the LV free wall thickness, the external circumferences of the AO and PA and the weight of RV and LF + *septum* (LV+S). The LV:RV free wall thickness *ratio*, the AO:PA circumference *ratio* as well as the LV+S:RV *ratio* should then be calculated (127, 131).

- *RV free wall thickness, LV free wall thickness*

  The measurements must be collected about 20 minutes after death, as both ventricle are similarly fully contracted. RV wall thickness is obtained midway on a line joining the subauricular papillary muscle origin and the tricuspid valve. LV wall thickness was measured midway between the origins of the sub-auricular and sub-atrial papillary muscles. The LV:RV wall thickness *ratio* must be calculated. This parameter has low sensitivity and specificity. In

most cases, mild RV hypertrophy do not result in significant RV free wall thickening. Furthermore, great variations may occur in wall thickness according to the site of assessment and to the contraction state, also in healthy animals (127).

- *AO, PA external circumferences (and their ratio)*

  External circumferences are evaluated at the vessel origin. An increase in PA external circumference as well as a decrease of AO:PA circumference ratio are often detected in severe asthmatic horses and reflect presence of PH (127, 131). In human, severe PH result also in PA muscle hypertrophy and endothelial arteriosclerotic-like changes, but usually these changes are not detectable in horses (127).

- *LV+S, RV weights (and their ratio)*

  The most accurate assessment of RV hypertrophy (and related *cor pulmonale*) is by comparing the RV and LV +S weights, by calculating their *ratio*. The RV should be dissected separately free from the interventricular septum. The atria are removed from both ventricles. Usually, asthmatic horses have an increase in RV weight, resulting in decreased LV+S:RV *ratio* (127, 131)

PART 2: RESEARCH PROJECT

INTRODUCTION

Severe equine asthma is a chronic, recurrent, non-infectious, inflammatory disorder affecting the equine lower respiratory tract, sharing pathophysiological similarities with human asthma (4). A multifactorial etiology has been proposed, involving a genetic predisposition to T2 helper lymphocyte-mediated immune response activation and exacerbating exposure to some environmental allergens, especially those found in moldy hay (6, 24).

Clinical exacerbations are characterized by airway obstruction caused mainly by reversible bronchospasm but also by mucus and exudate accumulation, with increased pulmonary resistance, enhanced expiratory effort at rest and progressive alveolar hyperinflation (136, 137). Pulmonary neutrophilic inflammation, with a percentage of neutrophils at bronchoalveolar lavage fluid (BALF) cytology superior to 25% and systemic inflammation, with increased serum levels of haptoglobin are also typical features of the disease (37, 54). The inflammatory response induces lung tissue remodeling, involving both peripheral and central airways. Remodeled airways show increased smooth muscle mass, deposition of extracellular matrix and hyperplasia of mucous producing cells (7, 55). Airway remodeling worsens airflow obstruction, being responsible for its subclinical persistence also during remission (7, 10).

Diffuse airway obstruction and alveolar hyperinflation increase dead space regions of high ventilation/perfusion (V/Q) ratios, due to pulmonary capillary mechanical compression. The resulting ventilation/perfusion (V/Q) mismatch impairs normal pulmonary gas exchanges, leading to

hypoxemia, clinically detectable as low arterial PO_2. Magnitude of V/Q mismatch and hypoxemia correlates with severity of clinical signs during disease progression (50, 137).

It is reported that chronic hypoxemia exacerbates sustained global hypoxic pulmonary vasoconstriction (HPV), increasing pulmonary vascular resistance and contributing to pulmonary hypertension (PH) onset and progression (110).

Pulmonary hypertension and reversible *cor pulmonale* are reported as clinical complications during exacerbation of severe equine asthma. At echocardiographic examination, severe asthmatic horses may show increased pulmonary artery (PA) diameter, enlarged right ventricle and atrium with high-velocity end-diastolic pulmonary insufficiency, rounded heart apex, hypokinetic and flattened interventricular septum, reduced left ventricle size and contractility (128, 131, 133). Advanced techniques such as tissue Doppler and speckle tracking echocardiography may detect altered right ventricular function and paroxysmal atrial fibrillation may appear as further complications (129, 133). Abnormal increased PA pressures may be measured by invasive right heart catheterization and pressure values are inversely correlated with arterial oxygen tension, suggesting a significant role of HPV (51, 129). However, PH is only partially reversed by oxygen administration and asthmatic horses have a significantly higher PA pressure compared to healthy horses, also during disease remission (51, 129). Therefore, other factors likely contribute to PH onset in asthmatic horses but they are not yet investigated.

In experimental rodent models, chronic hypoxia and hypoxemia induce pulmonary vascular remodeling that mainly involves small pulmonary arteries (116, 138-140). Remodeled muscular arteries show increased vascular smooth muscle (VSM) mass because of intimal muscularization and medial smooth muscle hypertrophy (138, 139). Muscularization of distal arterioles also occurs (116, 140). Pulmonary vascular remodeling may also develop in chronic inflammatory respiratory disorders. In human chronic obstructive pulmonary disease (COPD), remodeling of pulmonary arteries is well documented while it is poorly investigated in human asthma (117). Increased eosinophilic adventitial infiltration was detected in muscular pulmonary arteries of human patients

dead because of acute asthma attack (141). Furthermore, studies on asthma mouse models showed that allergic airway inflammation can induce pulmonary vessel remodeling, with increased VSM mass that persist during short-term disease remission (142-144). The overall increase in VSM mass results in artery wall thickening, lumen narrowing and vasoreactivity enhancement, with subsequent pulmonary vascular resistance augmentation (139). In human COPD, pulmonary artery remodeling is recognized as a determining factor in the onset of pulmonary hypertension and *cor pulmonale*, that represent typical end-stage disease complications (101).

We therefore postulated that hypoxemia and chronic airway inflammation occurring in severe equine asthma may induce remodeling of small pulmonary arteries, similarly to what is reported in human chronic lung diseases. If present, pulmonary artery remodeling could then contribute to both PH onset during clinical exacerbation of the disease and persistence of higher PA pressure values during the disease remission. According to our hypothesis, the present study had the following objectives: to 1) determine the presence and anatomical location of pulmonary artery remodeling in severe equine asthma, 2) assess whether differences are detectable between remission and/or exacerbation state of the disease, 3) investigate the remodeling pattern and whether an increase of VSM mass is present and, 3) assess remodeling reversibility after a long-term asthma treatment.

STUDY DESIGN

A blinded histomorphometric assessment was conducted in three phases, i.e. a preliminary *post-mortem* case-control study, an *in vivo* case-control study and a prospective randomized *in vivo* clinical trial.

The preliminary *post-mortem* case-control study aimed to determine presence of pulmonary artery remodeling, to evaluate its anatomical location in the lung, to assess differences between remission and exacerbation of the disease. The study was performed on lung histological samples collected *post-mortem* from 12 asthmatic horses and 6 age-matched controls. Among asthmatic horses, 6 were in exacerbation (exposition to the exacerbating antigen) and 6 were in remission (short-term antigen avoidance) at the moment of euthanasia. Pulmonary artery wall area was measured by histomorphometry, as an estimation of the degree of wall thickening. Measurements were compared between asthmatic horses and controls for the whole lung and for different lung regions. A comparison between asthmatic horses in remission and exacerbation was also performed.

The *in vivo* case-control study was aimed to confirm *post-mortem* results and to characterize the vascular smooth mass involvement in the remodeling process. Peripheral lung biopsies were collected via thoracoscopy from the caudodorsal lung of 6 asthmatic horses in remission and 5 age-matched controls. Pulmonary artery wall area was measured similarly to the preliminary *post-mortem* study on histological samples. Furthermore, the intimal and medial areas were evaluated, to assess presence of specific intimal and/or medial thickening. The VSM mass and the extracellular matrix (ECM) were quantified by a point counting technique on sections immunostained for smooth muscle specific α-actin (α-SMA). Measurements were compared between asthmatic horses and controls.

The *in vivo* prospective randomized clinical trial was performed on 11 asthmatic horses to assess reversibility of wall and VSM changes after long-term asthma treatment. As a baseline, lung biopsies

were collected during clinical exacerbation. Pulmonary artery wall area and VSM mass were quantified by histomorphometry, similarly to the *in vivo* case control study. Then, horses were randomly divided into 2 groups and treated for 12 months with two different protocols. Five horses were treated only with the antigen avoidance strategy for the whole experimental period. Six horses were treated with the only administration of inhaled corticosteroids for 6 months and then with a combination of corticosteroid inhalation and antigen avoidance for the remaining 6 months. After treatment, peripheral lung biopsy collection and histomorphometry were repeated as a follow-up evaluation. For each horse and each group, a comparison between the wall area and VSM mass amounts at baseline and follow-up was performed.

MATERIALS AND METHODS

Animals

For the *post-mortem* study, horses were selected from the caseload available in our equine respiratory tissue biobank (http://www.btre.ca). For the *in vivo* studies, horses belonged to the experimental court of a larger project aiming to assess lung tissue remodeling and its reversibility in severe equine asthma (7, 8). All the asthmatic horses had a well-documented 3–10-year history of largely reversible airway obstruction and inflammation. Clinical remission of the disease was experimentally obtained by keeping asthmatic horses on pasture for 2-4 months before recruitment for the study. Clinical exacerbation was experimentally induced by stabling asthmatic horses indoor and exposing them to moldy hay. Control horses were selected according to absence of previous respiratory disease in their history and age-matched to asthmatic horses. Animal manipulation and euthanasia were performed in accordance with the guidelines of the Canadian Council for Animal Care.

Treatments

The antigen avoidance strategy consisted in keeping horses on pasture during the summer months, and in a paddock during the winter. A pelleted diet was administered as supplementation to cover nutritional requirements while avoiding exposition to exacerbating allergens. Corticosteroid treatment consisted in inhaled fluticasone propionate (Flovent HFA; GlaxoSmithKline, Montreal, PQ, Canada) administered at the dose of 2-3 mg twice a day.

Lung function and BALF cytology

Lung function and BALF cytology were assessed before euthanasia or lung biopsy collection, as previously described (24, 145). Briefly, pulmonary resistance and elastance were calculated from flow rates obtained from a heated pneumotachograph attached to a mask and transpulmonary pressure (ΔPpl) was derived from an esophageal catheter. For BALF collection, a 2.50 m endoscope (Olympus Medical Systems Corp., Tokyo, Japan) was used to instill and recover two boluses of 250-ml isotonic saline in the main bronchi. Cytospins were obtained and stained with Wright-Giemsa.

Lung sampling techniques

In vivo sampling

Lung biopsy was performed via thoracoscopy in standing sedated horses and peripheral lung tissue (8-12 cm^3) was obtained from the caudodorsal region of the lung as previously described (146).

Post-mortem sampling

After euthanasia and blood removal by gravity after carotid incision, the whole lungs were removed from the thoracic cavity. Lung samples of 8-12 cm^3 were collected from different regions of the left lung. Samples were named cranio-caudally from “A” to “D”. A further sample named “E” was collected from the region corresponding to the biopsy site during thoracoscopy procedures. Specific sites of collection are shown in figure 1.

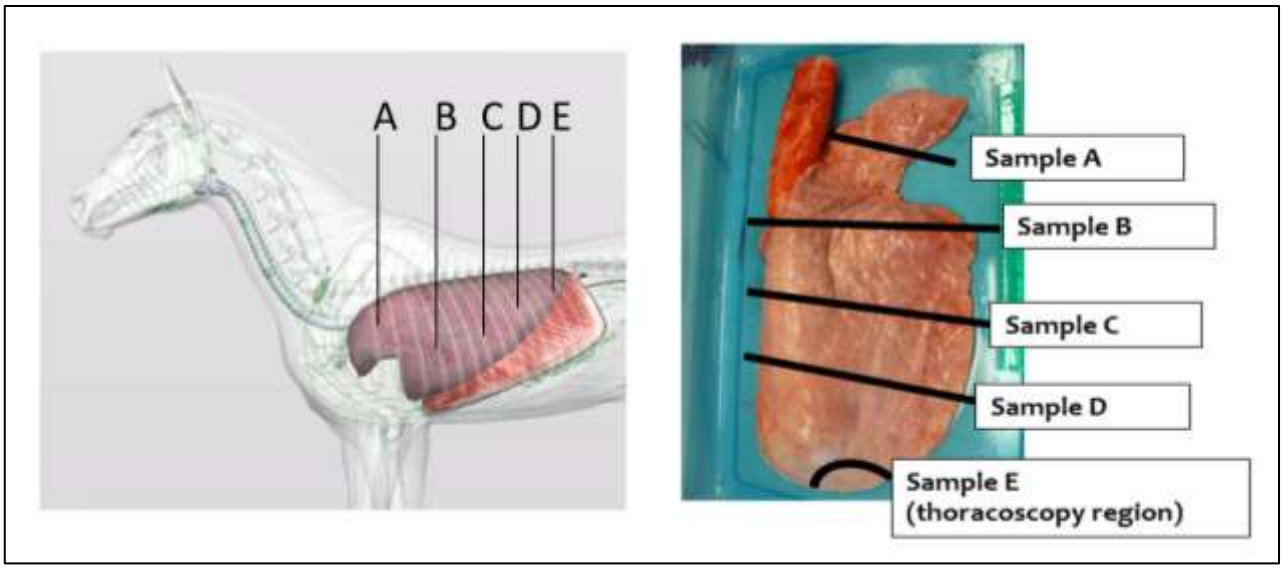


Figure 1: lung sites of post-mortem sampling. Sample "A" was collected from the lung apex. Sample "B" was collected at the emergence of the main bronchus. Sample "C" was collected from the center of the main lung lobe. Sample "D" was collected from the caudodorsal part of the main lung lobe. Sample "E" was collected from the peripheral caudodorsal part of the main lung lobe and corresponds to the specific biopsy site during thoracoscopy.

Processing and staining

Lung samples were fixed for 24 hours in 4% formaldehyde, embedded in paraffin, cut, placed on glass slides and stained. *Post-mortem* samples were stained for histology with hematoxylin eosin saffron. *In vivo* samples were stained for histology with Movat Russel Pentachrome. This staining highlights the internal elastic lamina, allowing differentiation between intimal and medial layers within the artery wall. *In vivo* samples were also immunostained for α-SMA, as previously described (45).

Histomorphometry

Histology

Post mortem and *in viv*o histological sections were scanned and digitalized at 40X magnification as TIFF images with PanOptic software version 1.4.3; morphometric measurements were collected on TIFF images using Image-Pro Plus software version 1.4g (MediaCybernetics, Carlsbad, CA). For each horse, all the artery sections having enough preserved integrity to allow clear distinction between the artery wall and the *lumen*, were evaluated. Both linear (1D) and bidimensional (2D) parameters were measured or derived, as previously reported for human pulmonary vascular histomorphometry (147-149). In brief, "1D (µm) measured parameters" included length of external artery diameter (ED), greatest longitudinal axis (GLA), external perimeter (EP), internal perimeter (IP) and *lumen* perimeter (LP). In horses, muscular pulmonary arteries have a minimal ED of 40 µm therefore, arteries with lower ED length were excluded (150). 2D measurements (μm^2) included the total area (TA), the internal area (IA) and the *lumen* area (LA). Definition and collection methods of 1D and 2D "measured parameters" are summarized in table 1 and figure 2 respectively. The IP and the IA were measured on Movat Russell Pentachrome stained sections (*in vivo* case-control study), only if the artery internal elastic lamina appeared well defined in the whole artery. "Derived parameters" included the wall area (μm^2), the intimal area (μm^2) and the medial area (μm^2), the *ratio* between greatest longitudinal axis and external diameter (GLA/ED *ratio*) and the narrowing index. "Derived parameters" were calculated from "measured parameters", according to definitions resumed in table 2 (Office Excel software v. 2016; Microsoft Corporation, Redmond, USA). The intimal area and the medial area were obtained only if IA measurement was available. To allow comparison among data of different sized-artery sections, these measurements were also expressed as percentages of the total area (wall area %, intimal area % and medial area %). Longitudinal sections of vessels (GLA/ED *ratio* > 3) were excluded, as previously suggested (149).

1D measured parameters (µm)	
External artery diameter (ED)	Widest distance between external elastic laminas, perpendicular to the greatest longitudinal axis
Great longitudinal axis (GLA)	Longest distance between external elastic laminas
External perimeter (EP)	Outline of the external elastic lamina
Internal perimeter (IP)*	Outline of the internal elastic lamina
Lumen perimeter (LP)	Outline of the inner aspect of the intima
2D measured parameters (µm^2)	
Total area (TA)	Area encompassed by the external perimeter
Internal area (IA)*	Area encompassed by the internal perimeter
Lumen area (LA)	Area encompassed by the lumen perimeter

Table 6: 1D and 2D "measured parameters". These parameters were already defined for pulmonary vascular histomorphometric studies in human COPD (149). Those parameters marked with (*) were assessed only on sections stained with Movat Russel Pentachrome (*in vivo* case-control study).

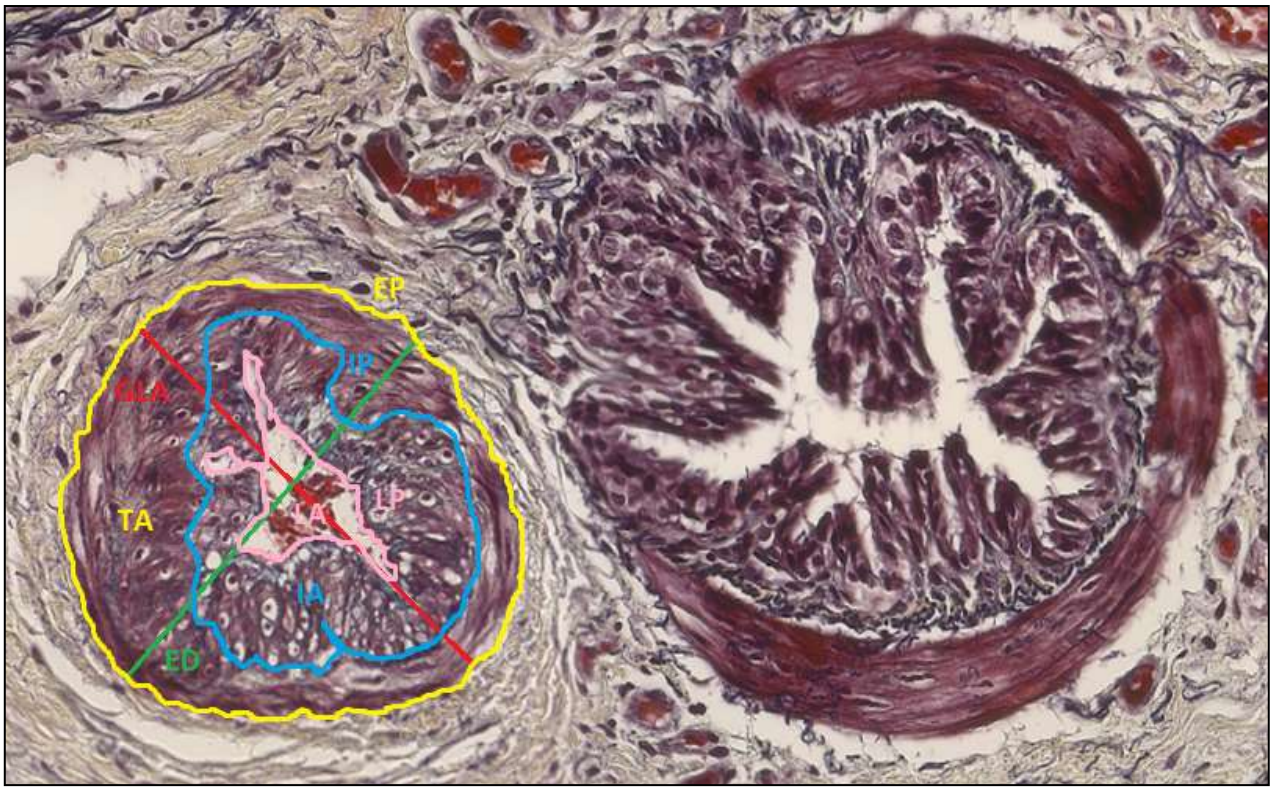


Figure 2: histomorphometric 1D and 2D measured parameters. Muscular pulmonary artery and annexed bronchus histological sections were scanned at 40X and stained with hematoxylin eosin saffron (for the *post-mortem* study) or Movat Russel Pentachrome (for the *in vivo* study, as in this picture). Elastic fibers are stained black with Movat Russel Pentachrome, outlining the internal elastic lamina and allowing differentiation between intimal and medial layers. GLA (red outline) =great longitudinal axis; ED (green outline) =external diameter; EP (yellow outline) =external perimeter; IP* (blue outline) =internal perimeter; LP (pink outline) =lumen perimeter; TA (within yellow outline) =total area; IA* (within blue outline) =internal area; LA (within pink outline)=*lumen* area.

Derived parameters	
Wall area (μm^2)	Area included between the external perimeter and the lumen perimeter, calculated as difference between total measured area and lumen area
Medial area (μm^2)*	Area included between the external perimeter and the internal perimeter, calculated as difference between total measured area and internal area
Intimal area (μm^2)*	Area included between the internal perimeter and the lumen perimeter, calculated as difference between internal area and lumen area
GLA/ED *ratio*	Ratio between greatest longitudinal axis and external artery diameter
Narrowing index	Index of artery collapse estimated as the *ratio* between the total measured area (TA) and the theoretical area. The theoretical area represents the area of the fully distended artery obtained from a theoretical diameter calculated as follows: EP/π

Table 7: 2D derived parameters. These parameters were defined for pulmonary vascular histomorphometric studies in human COPD (149). Those parameters marked with (*) were assessed only on sections stained with Movat Russel Pentachrome (*in vivo* case-control study). Narrowing index and GLA/ED *ratio* were used to assess differences due to histological processing and cut sections respectively.

Immunohistochemistry

Immunostained muscular pulmonary arteries were scanned at 40X magnification and digitalized as SVS images with PanOptic software version 1.4.3. Histomorphometry was then performed on the SVS images using the NewCAST software version 4.5.1.324 (Visiopharm Integrator System, Hoersholm, Denmark). The total vessel area (μm^2) and the α-SMA positive and negative wall area (μm^2) were estimated with a counting point technique. Briefly, on each uploaded SVS digital image, every visible pulmonary artery section was delimited as a region of interest (ROI), using the "ROI drawing" function. The parameters counted were the sum of the points falling on vessel lumen and wall surfaces ($\sum p_{vessel}$), the sum of the points falling on α-SMA positive wall surface ($\sum p_{\alpha\text{-}SMA+}$) and

the sum of the points falling on α-SMA negative wall surface ($\sum p_{\alpha\text{-SMA-}}$). A grid of 576 crosses per screen was applied since this point density provided a total count of *at least* 400 points for every counted parameter in each horse, an essential condition to ensure a reliable individual estimation (figure 3). The total vessel area, the α-SMA positive wall area and the α-SMA negative wall area were estimated by multiplying the $\sum p_{vessel}$, $\sum p_{\alpha\text{-SMA+}}$ or $\sum p_{\alpha\text{-SMA-}}$ respectively per the surface subtended to a single point (117,22 μm^2). The total vessel area reflects the overall surface occupied by the artery section (both *lumen* and wall surface), while the α-SMA positive wall area and the α-SMA negative area reflect respectively the VSM and ECM mass within the artery wall. Both α-SMA positive and negative wall areas were then expressed as a percentage of the total vessel area (α-SMA+ area %, α-SMA- area %), to allow comparison among data of different sized-artery sections.

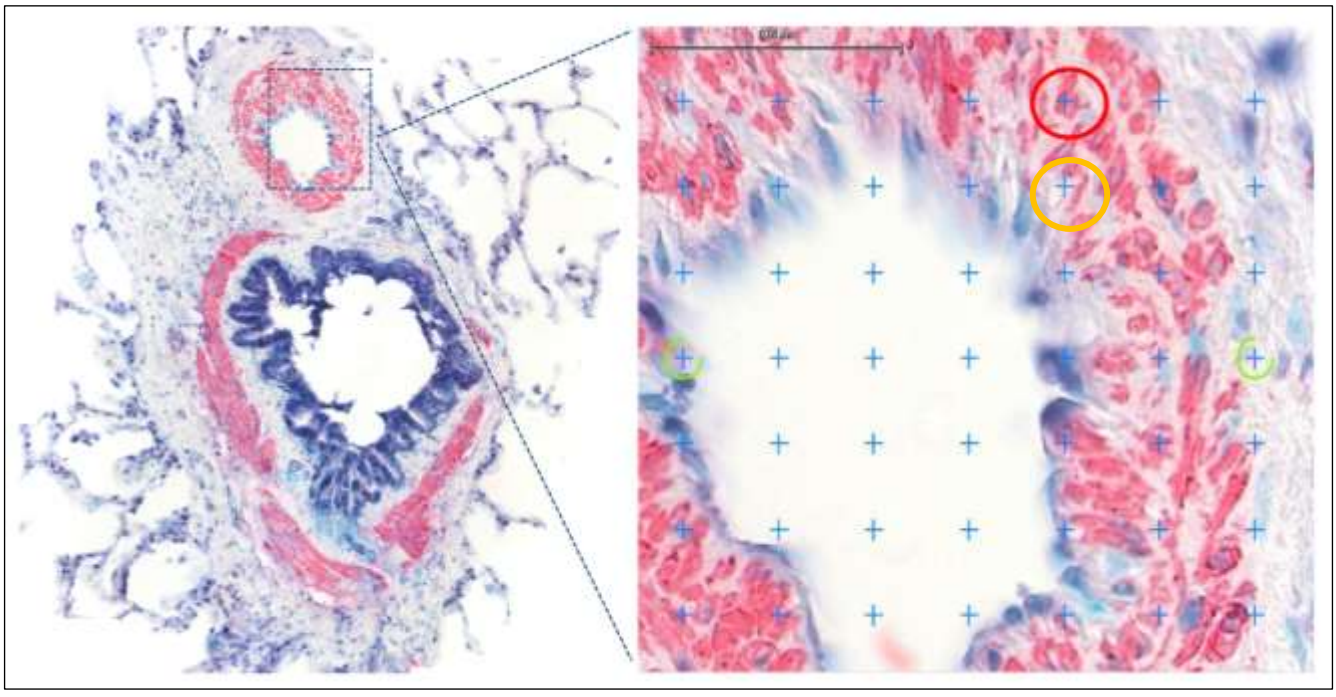


Figure 3: immunostaining for α-SMA and point counting analysis. Vascular smooth muscle (VSM) stained pink-red with immunostaining for α-SMA, while ECM did not stain. An example of ROI analysis with the point counting technique is provided. A grid of 576 crosses/screen was applied. Crosses falling on pink-red artery wall points (red circle) were counted as α-SMA+ (representative of VSM). Crosses falling on uncolored artery wall points (yellow circle) were counted as α-SMA- (representative of ECM).

Statistical analysis

Age, lung mechanics and BALF cytology

For the *post-mortem* study, mean values and standard deviations of age, lung function measurements (ΔPpl, resistance, elastance) and BALF neutrophil % were calculated for each group (controls, remission and exacerbation) (Office Excel software v. 2016; Microsoft Corp., Redmond, USA). Presence of significant differences among groups was investigated using the one-way Analysis of Variance (ANOVA) with Bonferroni's multiple comparison *post-hoc* test (GraphPad Prism software version 6; Graphpad software Inc., California, USA). Level of statistical significance was set at $p<0.05$. For the *in vivo* studies, details of statistical analysis are already described elsewhere (7, 8).

Histomorphometry

For the *post-mortem* and *in vivo* case-controls studies, mean and standard deviation (SD) of morphometric measurements were calculated for each horse (individual means). Case-control comparisons were performed using individual means. A two-tailed *t* Student test with Welch's correction was used in comparing groups for narrowing index and GLA/ED *ratio,* to assess differences related to histological processing. A one-tailed *t* Student test with Welch's correction was used to compare the two groups for the wall area %, intimal area %, medial area %, α-SMA+ area %, since greater individual means were expected in asthmatic horses. To compare asthmatic horses in remission and asthmatic horses in exacerbation a two-tailed *t* Student test with Welch's correction was used. For the *in vivo* prospective clinical trial, mean and standard deviation (ds) of morphometric measurements were calculated for each horse at baseline (individual means before treatment) and at the follow-up (individual means after treatment). A two-tailed paired *t* Student test was used to compare narrowing index and GLA/ED *ratio* while a one-tailed paired *t* Student test was used to

compare wall area % and α-SMA+ area % of each treatment group between baseline and follow-up, since lower individual means were expected at the follow-up. Means and SD were calculated with Office Excel software v. 2016 (Microsoft Corporation, Redmond, USA) and statistical tests were performed with GraphPad Prism software v. 6 (Graphpad software Inc., California, USA). The level of statistical significance was set at $p < 0.05$.

RESULTS

Preliminary *post-mortem* study

Age, lung mechanics and BALF cytology

Age, lung function measurements and neutrophil % in BALF are reported in table 3. All groups are age-matched, as shown in figure 4a. A significant increase of cytological neutrophil % in BALF is associated with asthma exacerbation, while no difference was detectable between controls and asthmatic horses in remission (figure 4b). Similarly, lung function was significantly deteriorated by disease exacerbation, while during remission values were comparable to those of controls (figure 4c).

Group	**Age**	**Lung mechanics**			**BALF**
	(years)	**ΔPpl**	**Resistance**	**Elastance**	**Neutrophils (%)**
		(cmH_2O)	**($cmH_2O/L/s$)**	**(cmH20/L)**	
Controls	24 ± 5	5.261 ± 3.448	0.6203 ± 0.2333	0.6558 ± 0.1576	1.19 ± 1.28
Remission	25 ± 5	11.270 ± 3.273	0.8335 ± 0.2965	0.6633 ± 0.2851	10.75 ± 10.28
Exacerbation	25 ± 5	37.550 ± 16.060	2.3480 ± 0.5611	2.0250 ± 1.3060	30.90 ± 14.55

Table 3: means and standard deviations of age, lung function measurements and BALF neutrophils % (*post-mortem* study). ΔPpl = transpulmonary pressure.

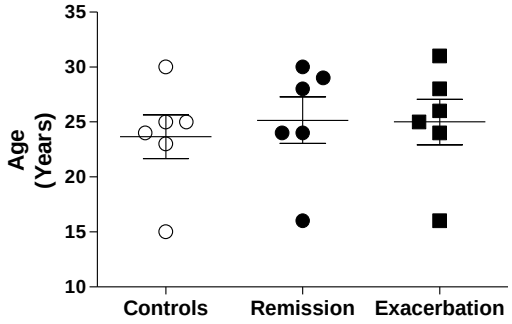


Fig. 4a: age (*post-mortem* study). Controls, remission and exacerbation horses did not significantly differ in age (p=0.853).

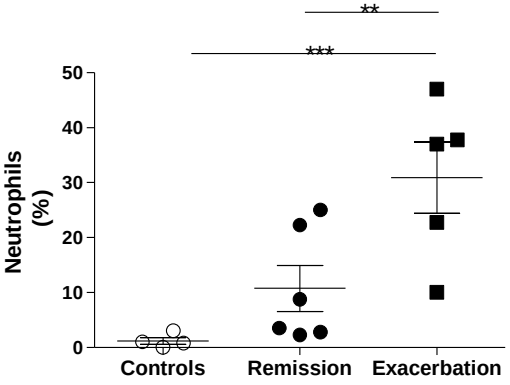
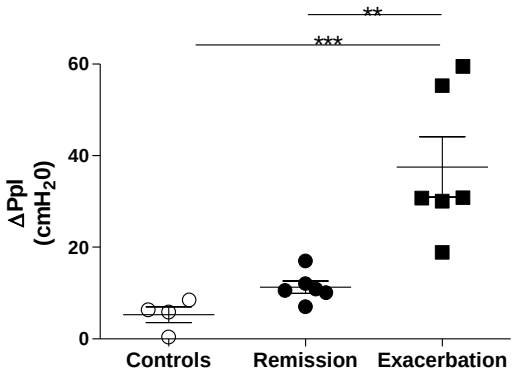
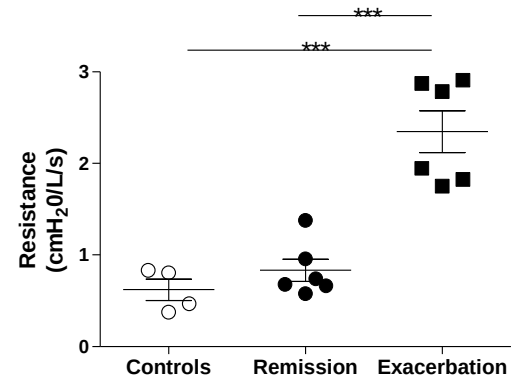


Fig. 4b: neutrophil % at BALF cytology (*post-mortem* study). Neutrophil % was significantly higher in exacerbation compared to both controls (p<0.001) and remission (p<0.01).





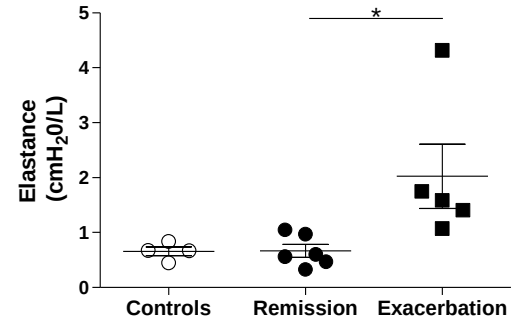


Fig. 4c: Lung mechanics (*post-mortem* study). During exacerbation, asthmatic horses showed deterioration of lung function, compared to controls and remission horses, with significant increase of transpulmonary pressure (ΔPpl; p<0.001), resistance (p<0.001) and elastance (p<0.05). No significant differences were detected between remission horses and controls.

Histomorphometry

The average number of histological pulmonary arteries assessed per horse was 42±14 in the asthmatic group (45±13 for the remission group; 38±14 for the exacerbation group) and 37±12 in the control group. Means and standard deviations of each morphometric parameter are resumed in table 4. The two groups did not significantly differ in respect to GLA/ED *ratio* (p=0.07), but differences were detected in the narrowing index that was significantly higher in the asthmatic group (p=0.0005) than in controls (figure 5a). Asthmatic horses had significantly increased % of wall area when compared to controls (p=0.03) and artery remodeling was similar in severe asthmatic horses either in exacerbation and in remission (p=0.70). These changes were due to significant artery wall thickening in sample A and D that respectively correspond to the apex (p=0.003) and caudodorsal lung fields (p=0.03). Significant remodeling was also detected in sample E, the specific region sampled via thoracoscopy in the subsequent *in vivo* studies (p=0.02).

Group	GLA/ED *ratio*	Narrowing index	Wall area (%)
Controls	1.80 ±0.07	0.58 ±0.04	38.31 ±5.70
	Sample A		35.39±7.06
	Sample B		40.22±7.50
	Sample C		39.41±11.51
	Sample D		38.13±10.18
	Sample E		42.03±7.50
Asthma	1.71 ±0.12	0.70 ±0.05	49.09 ±12.52
	Remission		50.58±14.10
	Exacerbation		47.60±11.87
	Sample A		50.66±13.16
	Sample B		47.23±13.16
	Sample C		46.19±11.44
	Sample D		50.56±15.01
	Sample E		55.55±14.84

Table 4: means and standard deviations of morphometric parameters (*post-mortem* case-control study). GLA/ED *ratio*=ratio between the great longitudinal axis and the external diameter.

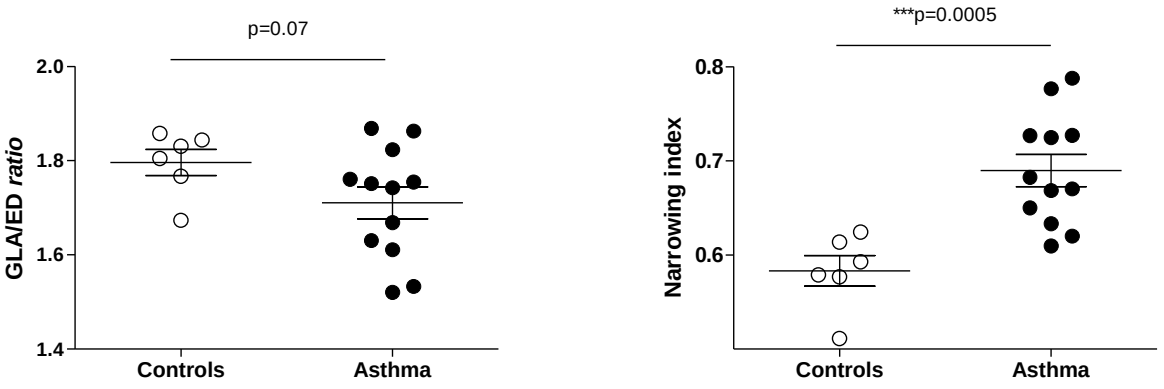


Figure 5a: GLA/ED *ratio* and narrowing index comparison (*post mortem* case-control study). Asthmatic horses and controls did not significantly differ in respect to histological cut section (GLA/ED *ratio*) but a significantly higher narrowing index was detected in asthmatic horses, indicating different *post-mortem* lung tissue reaction to histological processation.

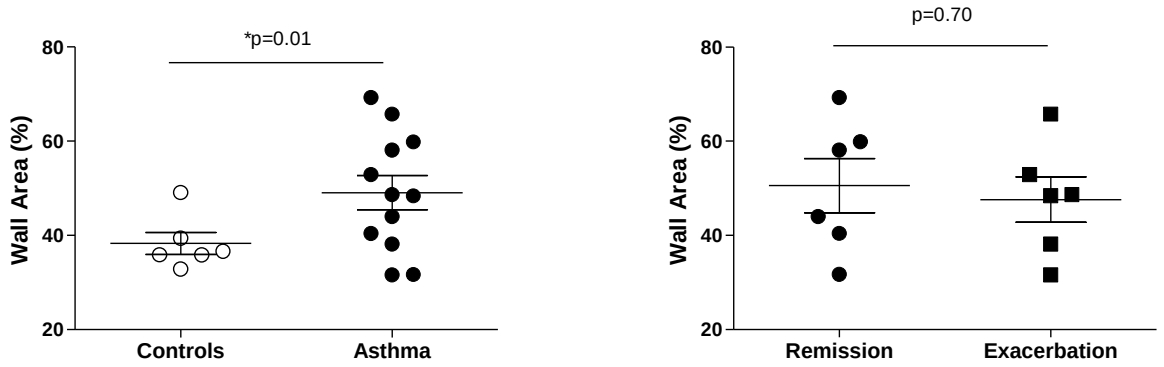


Figure 5b: histological morphometric remodeling comparison (*post mortem* case-control study). Asthmatic horses showed significant wall thickening (increased % of wall area), compared to controls. Remodeling did not significantly differ between asthmatic horses in remission and in exacerbation.

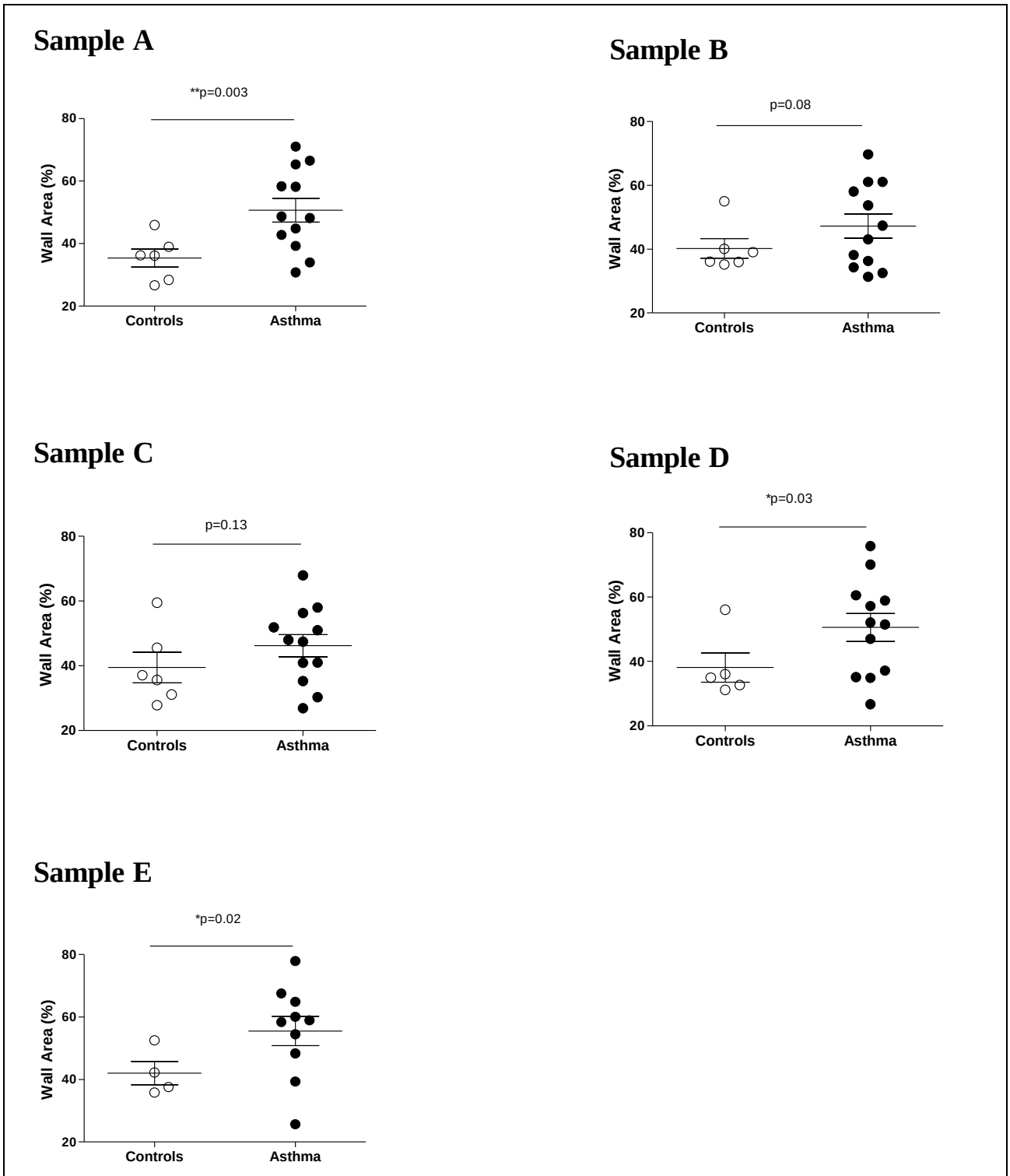


Figure 6: histological morphometric remodeling comparison of samples from different lung regions (*post mortem* case-control study). Asthmatic horses showed significant remodeling compared to controls in the apex (sample A) and in the caudodorsal lung field (sample D). Significant pulmonary artery remodeling was also shown in sample E that corresponds to the specific site of lung biopsy collected in the *in vivo* study. Remodeling was not detectable in the main lung lobe (sample B and C).

In vivo studies

Age, lung mechanics and BALF cytology

All details have been reported elsewhere (7, 8).

Histomorphometry

In vivo case-control study

The average number of histological pulmonary arteries assessed per horse was 19±14 in the asthmatic group and 19±10 in the control group. The average number of immunostained pulmonary arteries assessed per horse was 19±11 in the asthmatic group and 22±8 in the control group. Means and standard deviations of each morphometric parameter are provided in table 5. The two groups did not significantly differ in respect to GLA/ED *ratio* (p=0.19) and to the narrowing index (p=0.12), (figure 7a). Asthmatic horses had significantly increased % of wall area when compared to controls (p=0.03). However, it was not associated with a specific increase in % of medial area (p=0.44) or % of intimal area (p=0.46) (figure 7b). The immunohistochemistry revealed that in asthmatic horses, the increase in % of wall area corresponded to a significant increase in the % of VSM mass (α-SMA+ area %, p=0.04) but not in ECM (α-SMA- area, p=0.21), as shown in figure 7c.

Group	**GLA/ED *ratio***	**Narrowing index**	**Wall area (%)**	**Medial area (%)**	**Intimal area (%)**	**α-SMA+ area (%)**	**α-SMA- area (%)**
Controls	1.77 ±0.11	0.65 ±0.04	67.26 ±6.67	51.75 ±5.95	21.97 ±3.17	41.07 ±6.22	39.52 ±10.29
Asthma	1.66 ±0.14	0.70 ±0.04	74.51 ±2.78	52.21 ±4.60	22.13 ±3.33	47.17 ±3.17	35.06 ±5.30

Table 5: means and standard deviations of morphometric parameters (*in vivo* case-control study). GLA/ED *ratio*=ratio between the great longitudinal axis and the external diameter; α-SMA+ area (%) = vascular smooth muscle (VSM) %; α-SMA- area (%)= extracellular matrix (ECM) %.

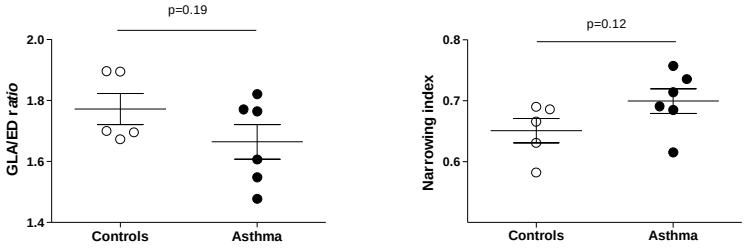


Figure 7a: GLA/ED *ratio* and narrowing index comparison (*in vivo* case-control study). Asthmatic horses and controls did not significantly differ in respect to histological cut section (GLA/ED *ratio*) and degree of artery collapse due to histological processation (narrowing index).

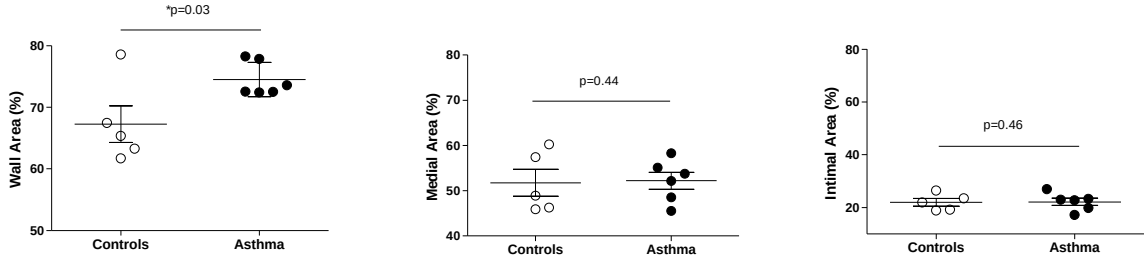


Figure 7b: histological morphometric remodeling comparison (*in vivo* case-control study). Asthmatic horses showed significant wall thickening (increased % of wall area), compared to controls. However, this did not correspond to specific medial and/or intimal thickening (% of medial and/or intimal area).

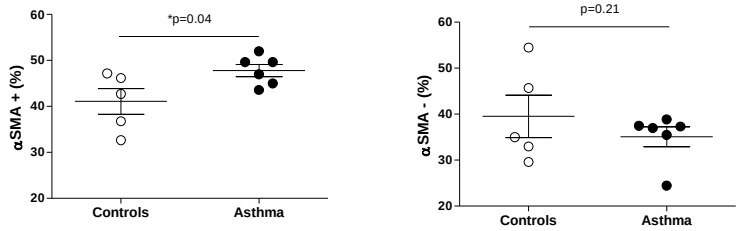


Figure 7c: immunohistochemical morphometric remodeling comparison (*in vivo* case-control study). Asthmatic horses showed significant increase in vascular smooth muscle (VSM) mass (increased % of α-SMA+ area), compared to controls. No signifcant increase in extracellular ,matrix (ECM) mass (% of α-SMA- area) was observed.

In vivo prospective clinical trial

At the baseline, 18±10 immunostained and 12±10 histological arteries were measured per horse in the group treated only with antigen avoidance; 12±7 immunostained and 8±3 histological artery sections were measured per horse in the group treated with corticosteroids (alone for 6 months, and combined with antigen avoidance for 6 months). At the follow-up, these numbers were 13±6 immunostained sections, 25±14 histological sections in the antigen avoidance group and 16±9 immunostained sections, 21±9 histological sections in the corticosteroid treated group respectively. One horse of the antigen avoidance group was not considered for the statistical analysis of the VSM quantification because at the baseline only one immunostained artery was measurable. Means and standard deviations of GLA/ED *ratio*, narrowing index, wall area (wall area %) and VSM mass (α-SMA+ area %) in the two groups at baseline and follow-up are resumed in table 6. The GLA/ED *ratio* did not differ between baseline and follow-up in both groups (p=0.46 and p=0.93), as shown in the figure 8a. The narrowing index was almost significantly reduced at the follow-up in the corticosteroid treated group (p=0.06) but was unaffected in the antigen avoidance group (p=0.35), as shown in figure 8b. Long-term treatment induced significant reduction of the wall area occurs both in antigen avoidance group (p=0.008) and in the corticosteroid treated group (p=0.04), as shown in figure 9a. The antigen avoidance strategy was associated with an almost significant VSM % reduction (p=0.05), while this does not happen with the steroid administration strategy (p=0.27) (figure 9b).

Time	**Group**	**Narrowing index**	**GLA/ED *ratio***	**Wall area (%)**	**α-SMA+ area (%)**
Baseline	Antigen avoidance	0.69±0.08	1.60±0.23	74.79±17.56	49.26±5.59
	Corticosteroids	0.68±0.03	1.79±0.10	69.73±10.17	49.83±7.65
Follow-up	Antigen avoidance	0.63±0.10	1.71±0.13	56.97±14.81	39.64±4.03
	Corticosteroids	0.61±0.06	1.79±0.15	53.42±14.81	45.35±15.00

Table 6: means and standard deviations of morphometric parameters (*in vivo* clinical trial).

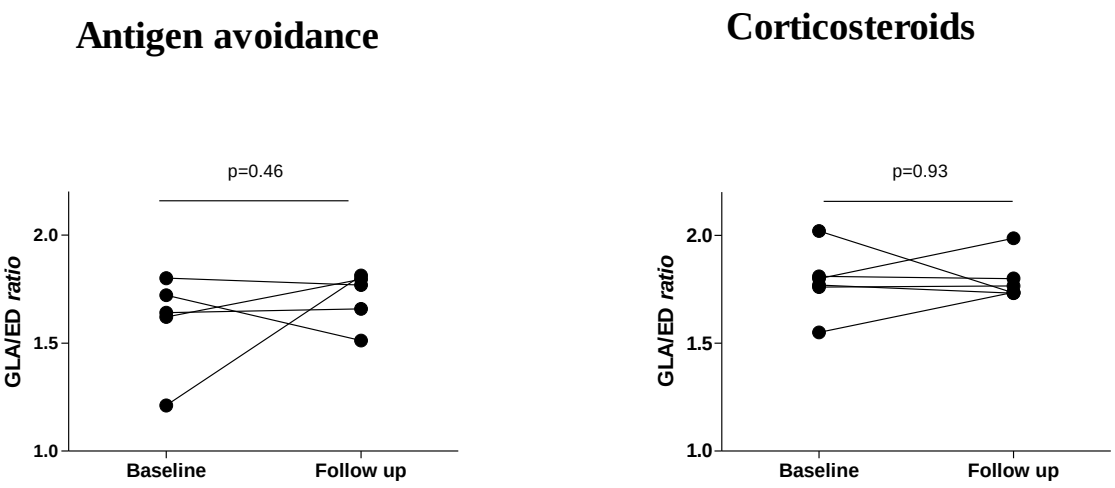


Figure 8a: GLA/ED *ratio* comparison (*in vivo* clinical trial). Both treatment group did not significantly differ in respect to GLA/ED *ratio*, showing similar histological cut section at baseline and follow-up.

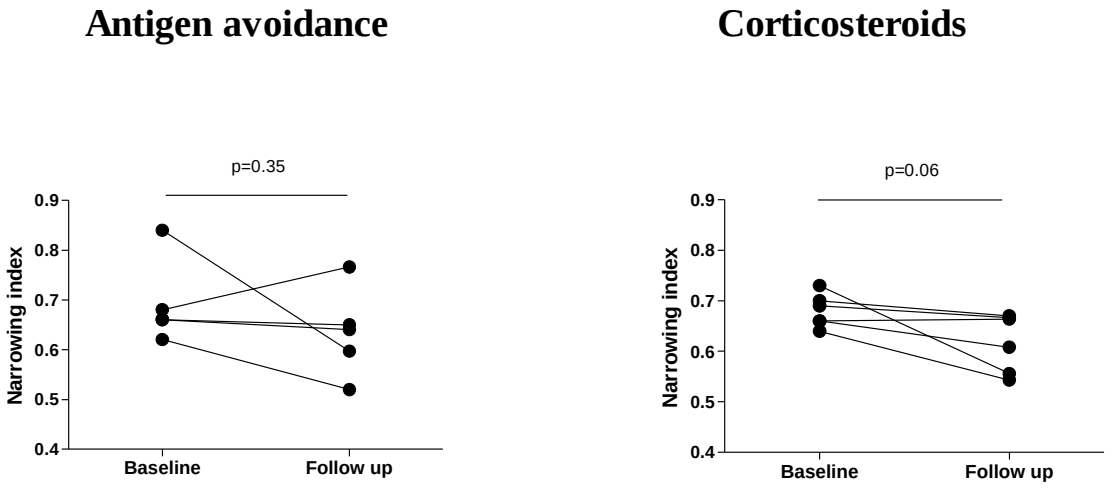


Figure 8b: narrowing index comparison (*in vivo* clinical trial). The antigen avoidance group did not significantly differ in respect to narrowing index between baseline and follow-up. Conversely, in the corticosteroid treated group the narrowing index was almost significantly reduced at the follow-up.

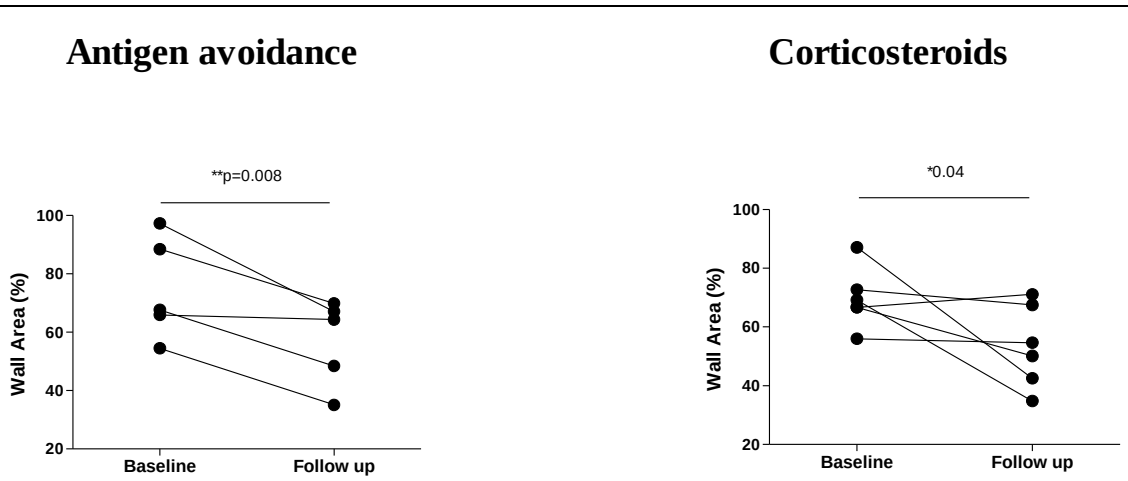


Figure 9a: histological morphometric remodeling comparison (*in vivo* clinical trial). In both groups, a significant reduction of the pulmonary artery wall area was observed at the follow- up. The reversibility of the wall area remodeling is more uniformely achieved with the antigen avoidance strategy than with the administration of corticosteroids.

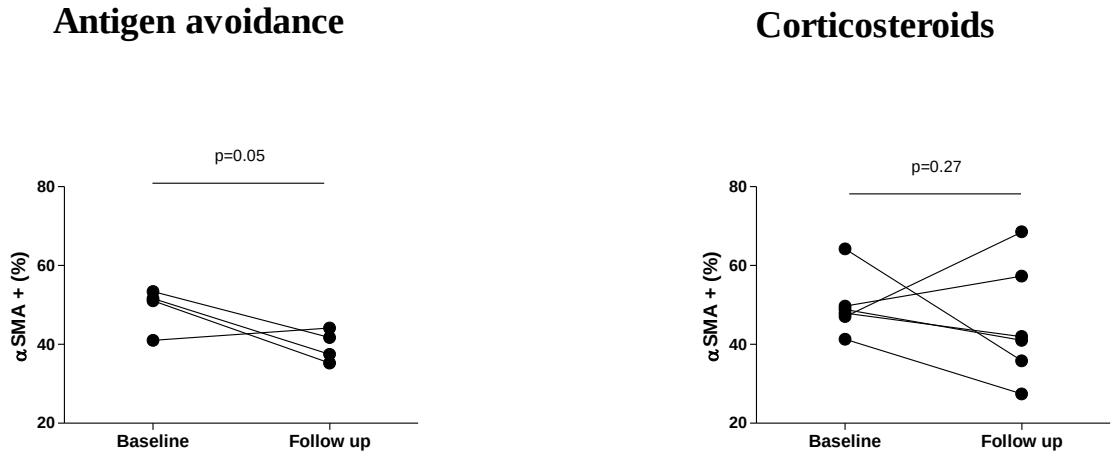


Figure 9b: immunohistochemical morphometric remodeling comparison (*in vivo* clinical trial). An almost significant and uniform reduction of the VSM was observed at the follow-up in the antigen avoidance group but not in the corticosteroid treated group.

Summary of in vivo results

The figures 10a and 10b graphically summarize the results of the *in vivo* studies, including both the case-control study and the clinical trial.

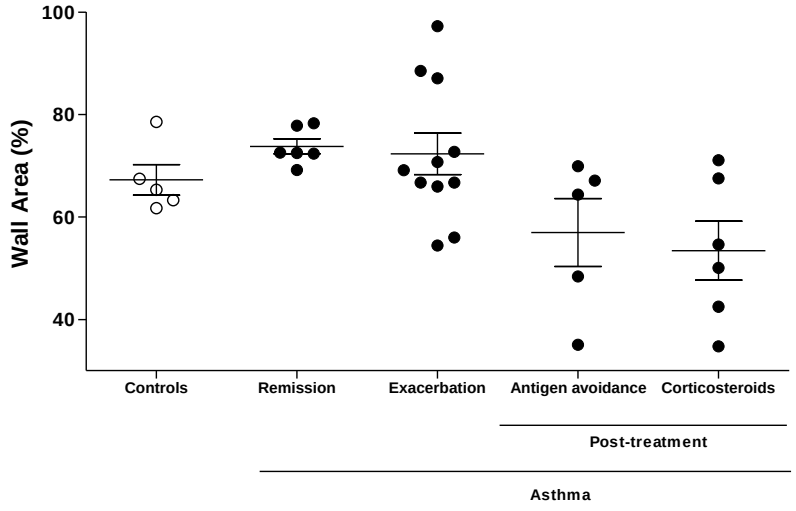


Figure 10a: summary of *in vivo* histological morphometric remodeling results. Asthmatic horses showed higher mean pulmonary artery wall area compared to controls, that was not modified by the disease status (remission or exacerbation). A normalization of the mean pulmonary artery wall area was observed after 11 month of treatment both with corticosteroids and with antigen avoidance.

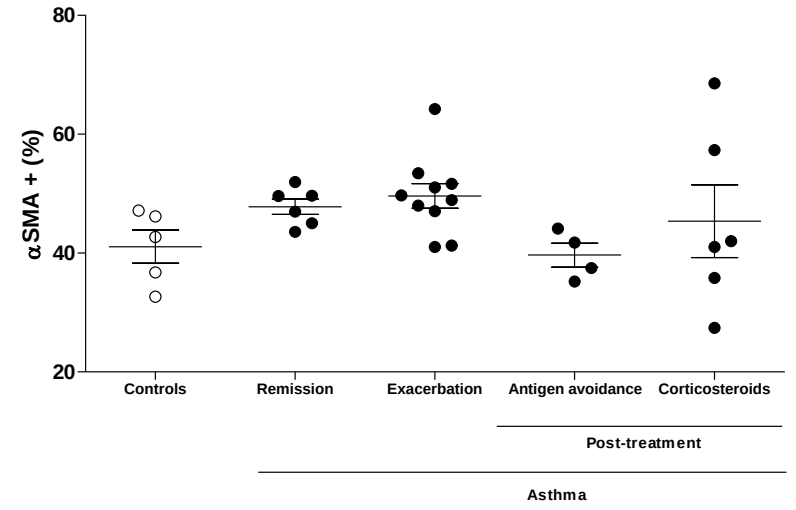


Figure 10b: summary of *in vivo* immunohistochemical morphometric remodeling results. Asthmatic horses showed higher pulmonary artery VSM compared to controls, that was not modified by the disease status (remission or exacerbation). A normalization of the mean pulmonary artery wall area was observed after 11 month of treatment with antigen avoidance but not with corticosteroids (less uniform response to treatment).

DISCUSSION

The present study investigated and characterized pulmonary artery remodeling in severe equine asthma using histomorphometry. Remodeling reversibility during short term disease remission and after long term treatment was also assessed, to evaluate whether structural alterations could contribute to the persistent increase of pulmonary artery pressure previously demonstrated during remission state of the disease (51). Both *post-mortem* and *in vivo* histomorphometry showed significant pulmonary artery wall thickening (increase in pulmonary artery wall area) in asthmatic horses compared to controls, that did not differ between exacerbation and remission. Furthermore, at *post mortem*, histomorphometric analysis revealed that pulmonary arteries of asthmatic horses appeared significantly less collapsed (higher narrowing index) than those of control horses. This difference was not remarkable in thoracoscopic lung biopsies, where both group showed similar low degree of pulmonary artery collapse (high narrowing index). *Post mortem* sampling of multiple lung regions showed that pulmonary artery wall thickening affected lung apex and caudodorsal lung fields, including the specific region sampled by *in viv*o thoracoscopy. In asthmatic horses, pulmonary artery wall thickening was due to a significant increase in VSM but not to an increase in ECM in then whole artery wall, as shown by the in *vivo* study. During exacerbation induced by antigen exposure, asthmatic horses showed significant airway neutrophilic inflammation (increase in neutrophil % at BALF cytology) and airway obstruction (significant lung function deterioration) compared to controls. Short term antigen avoidance alone (2-4 months of disease remission) significantly reduced BALF neutrophil count and ameliorated lung function but did not affect pulmonary artery remodeling changes in asthmatic horses. However, reversibility of pulmonary artery wall area and VSM was uniformly achieved after long term antigen avoidance (12 months). Combination of long term inhaled corticosteroids (12 months) and *medium*-term antigen avoidance (6 months) had less efficacy,

because pulmonary artery wall thickening and VSM were less uniformly reversed compared to long term antigen avoidance alone.

To the best of our knowledge, this is the first study that investigated pulmonary vascular remodeling in severe equine asthma. Investigation of pulmonary vascular remodeling also lack in human chronic asthma, with studies limited to experimental rodent models (142-144). Pulmonary artery remodeling had not been previously investigated in asthma, likely because the prevalence of pulmonary hypertension is unknown and unlikely to be clinically important at rest. However, would pulmonary hypertension may develop during effort, contributing to exercise intolerance in asthmatic patients and horses, it could then have important consequences to the quality of life of affected patients. Severe asthmatic patients rarely undergoing lung transplantation or peripheral lung biopsy procedure due to ethical limitations, limiting availability of lung histological samples may have also contributed to overlooking pulmonary arterial wall remodeling in severe asthma. Therefore, studying horses with severe equine asthma, as condition sharing many clinical and remodeling features with human asthma, may provide useful results for both species (4). A quantitative histomorphometric approach was extensively adopted to assess pulmonary vascular remodeling in human COPD (111, 120, 149, 151). Nevertheless, this is the first study that used a quantitative histomorphometric approach to assess pulmonary vascular remodeling in horses. Previous studies demonstrated pulmonary artery and vein remodeling in equine exercise-induced pulmonary hemorrhage (EIPH), using qualitative and semi-quantitative histopathology (152, 153).The present study showed that pulmonary artery wall area of asthmatic horses was about 7% and 12% higher than that of controls, *in vivo* and *post mortem* respectively. indicating presence of pulmonary artery wall thickening. Interestingly, similar ranges of increase (7-10%) were observed on explanted lungs of COPD-affected patients, compared to controls (120, 149, 151). A previous histomorphometric study of Saetta et al. on human fatal asthma detected increased eosinophilic infiltration of pulmonary artery adventitia but failed in demonstrating pulmonary artery wall remodeling (141). Nevertheless, the study was performed on lungs of patients died after a sudden asthma attack. Sudden fatal asthma prevents a chronic progressive evolution of

the disease, typical of severe equine asthma and COPD, required to induce vascular remodeling. Furthermore, Saetta et al. assessed remodeling using a 1D morphometric parameter (artery wall thickness) instead of a 2D morphometric parameter (artery wall area), as in the present study. Remodeling affects artery wall circumference in a patchy manner, therefore isolated linear measurements are less accurate and reproducible than bidimensional measurements (154).

The *post mortem* regional sampling showed that pulmonary artery remodeling did not involve the whole equine lung but affected the caudodorsal lung fields and the lung apex. It is interesting that the caudodorsal lung fields represent the main site of vascular remodeling also in equine EIPH (153). Preferential location of vascular remodeling in the caudodorsal regions depends on lung blood flow distribution pattern in the equine species (152). In standing horses at rest, lung blood flow distribution is related more to local differences in vascular resistance than to gravity, increasing in a dorsal and caudal direction (155). Therefore, higher perfusion of caudodorsal lungs means higher delivery and infiltration of inflammatory cells during acute lung inflammation. In rodent asthma models, remodeling of pulmonary arteries is correlated to increased eosinophilic infiltration, suggesting that blood-derived inflammatory cells can contribute to initiation and progression of vascular remodeling (142). Another factor that may contribute to regional distribution of vascular remodeling is the regional heterogeneity in mechanical properties and vasoreactivity of small pulmonary arteries, as recently demonstrated in the equine lung (156, 157). Involvement of cranioventral pulmonary arteries (lung apex) in pulmonary vascular remodeling of the equine lung represents a recent and surprising finding. Although blood flow distribution is lower in the lung apex, cranioventral small pulmonary arteries appear more prone to remodeling as they become stiffer than in caudodorsal regions in response to exercise. This suggests regional differences in vessel mechanical reactions in response to yet unidentified *stimuli* (157). Similar enhanced remodeling of cranioventral pulmonary arteries may be evoked also in severe equine asthma, nevertheless the determining factors merit further investigation. Also, endothelial dysfunction may occur in severe equine asthma as in human COPD, resulting in impaired production of vasodilating mediators such as prostacyclin and nitric oxide (NO)

(117). It has been recently demonstrated that prostacyclin production contributes to pulmonary vasomotor tone both in cranioventral and caudodorsal equine lung, while NO role is significant only in the cranioventral lung (156). In human COPD, endothelial dysfunction appears to play a role in inducing remodeling of pulmonary arteries and similar mechanisms may be implicated also in regional remodeling of the equine lung (120, 151). It is important to note that pulmonary artery remodeling was demonstrated *post mortem* in the same region sampled by *in vivo* thoracoscopy. Therefore, specimens collected with this technique are representative and adequate to investigate prospectively *in vivo* vascular remodeling evolution and response to treatment.

In the present study, *in vivo* wall area mean values were about 25-30% higher than those calculated *post mortem*, both in asthmatic horses (74.51 ±2.78 *in vivo* and 49.09 ±12.52 *post mortem* respectively) and in controls (67.26 ±6.67 *in vivo* and 38.31 ±5.70 *post mortem*). Therefore, independently from the group, pulmonary arteries were thicker when lung samples were collected *in vivo*. *In vivo*, both groups had similar negligible degree of artery collapse, with a high narrowing index (0.65 ±0.04 in controls and 0.70 ±0.04 in asthmatic horses). It has been shown in humans that minimally invasive techniques applying high traction force to harvest systemic vessels impair endothelium-dependent relaxation, without affecting contractile response (158). Methodological factors related to the cutting procedure and traction occurring during *in vivo* lung biopsy collection may have been the cause of the difference between *post-mortem* and in *vivo measurements*. Relaxation of pulmonary arteries could have been impaired by the sampling method, leading to a greater degree of collapse and lower thickness of the arterial wall in *post mortem* samples when compared to *in vivo* biopsies. *Post mortem* pulmonary arteries of asthmatic horses have a significantly higher narrowing index than those of controls (0.70 ±0.04 and 0.58 ±0.04 respectively). Pulmonary arteries showed increased tendency to collapse in controls compared to asthmatic horses at *post mortem* evaluation while degree of collapse was similar and negligible in both groups at *in vivo* evaluation. The fact that differences in degree of collapse were detected at *post mortem* but not *in vivo* suggests that *post mortem* alterations occurred differently in asthmatic and healthy horses.

Previous studies on experimental animal models documented that allergic lung inflammation is associated with hypercontractility and impaired relaxation of both pulmonary and systemic vasculature (159-161). Similarly, pulmonary arteries of asthmatic horses may show hypercontractility and impaired *post mortem* relaxation, compared to healthy horses. However, difference in wall area (expression of remodeling due to wall thickening) was not affected by methodological or reactivity differences between groups and was similarly detected both *in vivo* and *post mortem*, therefore suggesting consistent remodeling of pulmonary artery walls in asthmatic horses.

Presence of remodeling was further supported by point-counting results on immunostained pulmonary arteries, showing a significant increase in the VSM of asthmatic horses. This is the first study showing pulmonary VSM mass remodeling in a spontaneous asthma-like disease. However, involvement of VSM in pulmonary artery remodeling has been already demonstrated in rodent asthma models and in other chronic spontaneous lung disorders, such as human COPD or equine EIPH, using similar immunostaining technique with α-SMA (142-144, 153, 162-164). Increase in VSM mass of muscular pulmonary arteries could be induced by hypoxia-exposure, because persistent HPV results in VSM contraction and "work" hypertrophy, as shown by studies on experimental animal models (165). Chronic hypoxaemia is present in severe asthmatic horses and is able to evoke HPV as well, and therefore could have been contributing to the VSM mass increase detected by the present study (50, 110). In addition to hypoxaemia, airway inflammation may also contribute to the VSM mass increase. In rodent models, allergic airway inflammation increased pulmonary artery smooth muscle cells (PASMC) turnover inducing an overall increase of VSM mass with similar time course and magnitude to airway smooth muscle (ASM) remodeling, both being not reversible after one month of antigen avoidance (142, 143). An increase in ASM mass with increased myocyte proliferation rate is reported also in severe asthmatic horses (7, 45). Similarly, in severe equine asthma, although lung function and airway inflammation significantly improved after short term antigen avoidance (disease remission), smooth muscle remodeling changes are not affected, both in muscular pulmonary arteries, as shown in the present study, and in small peripheral airway, as previously shown (7).

However, magnitude of ASM remodeling appears greater than VSM remodeling in the equine species (45). Airway smooth muscle mass was previously shown to be two-folds increased, while the VSM mass increase in the present study was about 13% in asthmatic horses compared to controls. As the PASMC turnover was not evaluated, whether the increase in VSM mass is due to PASMC proliferation, hyperplasia or hypertrophy is still to be investigated. Time course of VSM remodeling development was not examined in the present study. However, consistent differences between ASM and VSM remodeling magnitude suggest that the two phenomena evolve in the same time but showing different rapidity. Acute episodes of bronchoconstriction and allergic airway inflammation may initiate both ASM and VSM remodeling after short-term antigen challenge, as shownin rodent models. Then, rapidity of progression may differ between ASM and VSM during the evolution of the spontaneous disease, although influencing factors are still unidentified (142). An hypothesis could be that proliferation of ASM maybe abnormally up-regulated due cellular intrinsic abnormalities involving asthmatic ASM cells but not PASMC (166).

In COPD, VSM mass increases in muscular pulmonary arteries mainly because of intimal longitudinal muscularization, with significant intimal thickening (162). A thickening of the medial layer is also detected in advanced stages because of circumferential VSM hypertrophy (163). In the present study, an overall increase in pulmonary artery VSM with significant wall thickening was detected, without specific thickening of intimal and/or medial layer was remarkable. Pulmonary artery remodeling could be patchy, affecting irregularly intimal and/or medial layer, therefore total wall VSM amount could be increased without increase of VSM amount within a single layer. Absence of specific intimal/medial thickening could be also related to methodological limitations. Intimal and medial area were not systematically evaluable because a well stained internal elastic lamina was not always present, reducing power of the histomorphometric assessment. Remodeling itself may cause loss of internal elastic lamina integrity and staining definition, as occurring in equine EIPH. In this equine lung disorder, pulmonary arteries remodeling pattern is similar to what was found for severe

equine asthma in the present study, showing artery wall thickening, medial smooth muscle hypertrophy, intimal hyperplasia with elastic laminas inconsistence and lack of organization (153).

In severe asthmatic horses, the increase in pulmonary artery VSM mass could contribute to PH onset during disease exacerbation, by enhancing magnitude of HPV. Experimentally-induced hypoxaemia increases mean pulmonary artery pressure (mPAP) both in normal and in asthmatic horses, however only asthmatic horses develop pulmonary PH (51). Furthermore, persistence of VSM mass increase and related artery wall thickening during remission may explain the higher mPAP values, although within physiological ranges, in asthmatic horses during remission than in controls (51). It is interesting that the present study detected a percentages of VSM mass increase in severe asthmatic horses in remission was about 13% when compared to controls, while an earlier report showed that the percentages of mPAP increase in severe asthmatic horses in remission compared to controls was about 16% (51).

In the present study, pulmonary artery remodeling did not affect the amount of vascular ECM. This result is somewhat surprising when compared with pulmonary artery remodeling observed in other spontaneous human or equine chronic lung diseases. The increased in the ECM mass in pulmonary artery of patients with COPD is mostly affecting the intima (162). In equine EIPH, there is increased ECM deposition in all layer of the pulmonary artery wall (153). Nevertheless, these discordances between COPD, EIPH and severe equine asthma may be related to differences in the etiological factors involved. In humans, intimal fibrosis is detectable in smokers before COPD onset, therefore the endothelial damage directly due to smoke chemical *stimulus* represents the most likely cause (162). Lung hemorrhage is followed by hemosiderin accumulation and is associated with progressive and massive lung fibrosis, likely contributing to the increased in vascular ECM typical of equine EIPH (153, 167). Furthermore, results of the present study are partially concordant with previous studies on experimental models of asthma. In rodents, an increased collagen content due to enhanced pro-collagen deposition is detected during sustained exacerbation but newly deposed pro-collagen content in the medial layer was marginal (142, 144). Pro-collagen deposition in rodents was mainly

in the perivascular layer, that was not examined in the present study. Therefore, perivascular changes and especially ECM deposition merit to be further investigated in severe equine asthma, using specific collagen and procollagen immunostainings.

The present study examined reversibility of pulmonary artery remodeling after long term asthma treatment. We observed that 12 months antigen avoidance strategy, known to control both airway obstruction and inflammation in affected horses, reversed the increase of pulmonary artery wall area and VSM mass. However, VSM decrease obtained in horses treated long term (12 months) with inhaled corticosteroids was less predictable, even if it was combined with antigen avoidance in the last 6 months of the study. Based on these findings, we postulated that airway inflammation may play an important role in the development of pulmonary artery remodeling, as VSM mass reversibility is achieved only with long term antigen avoidance strategy that normalizes both lung function and airway neutrophilic count (8). However, after long term treatment with inhaled corticosteroids, a significant decrease of pulmonary artery wall area was detected in association with a significantly higher degree of pulmonary artery collapse during histological processing (lower narrowing index). These results suggest a vasorelaxation in asthmatic horses following corticosteroid administration that is not related to airway inflammation modulation. Although this remains to be confirmed, both *in vitro* and experimental *in vivo* studies have shown that corticosteroids may counteract oxidative stress in asthma and improve the relaxation response of pulmonary arteries by decreasing oxidative stress, up-regulating expression of NO synthases and increasing bioavailability of NO in pulmonary endothelial cells (168, 169).

In conclusion, results of the present study indicate that there is a remodeling of pulmonary arteries in severe equine asthma independently from the disease status (exacerbation or remission) determining artery wall thickening and involving an increase of VSM mass. It was possible to study prospectively the reversibility of VSM mass increase in the same animals, which was achieved only with long term antigen avoidance strategies, suggesting a role for inflammation in the persistence of these structural vascular changes. Mechanisms of VSM remodeling, and endothelium and adventitial remodeling

remains to be investigated as well as the involvement of distal arterioles, pulmonary capillaries and veins. However, presence of pulmonary artery remodeling represents a new fascinating discovery in spontaneous asthma-like disease. Further clinical studies may investigate the role of hypoxaemia and inflammation in inducing VSM remodeling as well as its impact on pulmonary artery pressure and cardiovascular complications of severe equine asthma.

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