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REVERSIBILITY OF AIRWAY REMODELING IN EQUINE ASTHMA

Contribution of anti-inflammatory and bronchodilator therapies

par
Michela Bullone

Département de sciences cliniques
Faculté de médecine vétérinaire

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Abstract

Airway remodeling and inflammation are the hallmarks of asthma. Both airway smooth muscle (ASM) mass and extracellular matrix (ECM) deposition are increased in the central and peripheral airways of asthmatic patients, which contribute to airway obstruction. Few studies have investigated the ability of current asthma medications to reverse airway remodeling, especially the increased ASM mass. Inhaled corticosteroids (ICS) and long-acting β_2-agonist combinations (ICS/LABA) are more effective than ICS monotherapy to control asthma exacerbations. However, their efficacy at modifying bronchial inflammation and remodeling at the peripheral level of the lung is not well-described. In fact, most work has been performed using endobronchial biopsy samples obtained from asthmatic subjects, which completely disregard the alterations occurring in peripheral airways. Ethical considerations limit the possibility of biopsying the peripheral airways in humans due to the invasiveness of the procedure. Equine asthma, or heaves, is a naturally-occurring disease of adult horses and a recognized animal model of human asthma characterized by neutrophilic inflammation as well as ASM and ECM remodeling of peripheral airways.

This thesis has assessed the contribution of ICS and LABA, alone or combined, to the reversal of remodeling and inflammation in central and peripheral airways using the equine asthma model. To attain this goal, we have first optimized and validated the application of endobronchial biopsy and endobronchial ultrasound (EBUS) in the equine species. EBUS reliably estimates the bronchial ASM. Subsequently, asthmatic horses with ongoing airway remodeling and inflammation were treated with ICS, LABA, ICS/LABA, or antigen avoidance. Lung function, airway remodeling and inflammation were then weekly assessed for 3 months. Our results demonstrated a 30% decrease of peripheral ASM remodeling attained with ICS and ICS/LABA pharmacological treatment. A decrease of a similar magnitude of peripheral ASM was previously reported after 6 and 12 months of ICS monotherapy and antigen avoidance, respectively. A synergistic effect of ICS/LABA was observed on ECM deposition and alveolar neutrophils. ICS/LABA decreased the ECM fraction of the ASM layer both peripherally and centrally, while the same effect on the lamina propria was observed only

in central airways. Both ICS/LABA and ICS monotherapy decreased submucosal inflammation in central airways, while only ICS/LABA and antigen avoidance decreased bronchoalveolar neutrophilia.

In conclusion, our results suggest that the enhanced therapeutic effect of ICS/LABA over ICS monotherapy in asthmatic patients was associated with a reduction of ECM deposition, mainly observed within the large airways, and possibly also with a decreased alveolar neutrophilia. However, ICS/LABA did not provide any additional benefit to ICS monotherapy in terms of peripheral ASM remodeling as both induce a 30% decrease of the ASM mass in 3 months.

Keywords: asthma, animal model, horse, remodeling, airway smooth muscle, extracellular matrix, corticosteroids, long-acting β-agonists.

Résumé

L'asthme bronchique est caractérisé par un remodelage et une inflammation des voies aériennes. La masse du muscle lisse ainsi que la déposition de matrice extracellulaire sont augmentées dans la paroi des bronches asthmatiques, ce qui contribue à l'obstruction respiratoire. Peu d'études ont évalué les effets des traitements utilisés dans l'asthme sur le remodelage bronchique, et surtout peu de données sont disponibles concernant les effets sur le muscle lisse. La combinaison de corticostéroïdes et de β_2-agonistes à longue durée d'action administrée par inhalation permet de mieux contrôler les crises d'asthme par rapport à la monothérapie avec des médicaments corticostéroïdes. Cependant, l'action spécifique de la combinaison sur le remodelage et sur l'inflammation des bronches périphériques n'est pas décrite. Surtout, il reste à clarifier si l'administration de la combinaison est avantageuse par rapport à la monothérapie corticostéroïde. La plupart des études réalisées chez l'homme utilisent des tissus bronchiques obtenus par biopsie endobronchique, qui ne sont pas représentatifs du processus pathologique affectant les voies respiratoires périphériques. Leur inaccessibilité par des méthodes non invasives est la raison pour laquelle si peu de données existent sur la pathophysiologie des voies périphériques chez les patients asthmatiques. L'asthme équin, aussi connu comme « le souffle », est une pathologie obstructive des chevaux adultes considérée comme un modèle animal d'asthme humain. Elle est caractérisée par un remodelage des bronches périphériques et par une inflammation bronchoalvéolaire de type neutrophilique.

En étudiant le modèle équin, cette thèse a évalué la contribution des médicaments corticostéroïdes et de β_2-agonistes à longue durée d'action, administrée comme monothérapies ou en combinaison, sur la réversibilité du remodelage et de l'inflammation de voies aériennes dans l'asthme bronchique. A cette fin, nous avons d'abord optimisé et validé l'application de la biopsie endobronchique et de l'échographie endobronchique chez le cheval adulte. Nos résultats indiquent que les échantillons obtenus par biopsie endobronchique sont inadéquats pour l'évaluation quantitative de la masse du muscle lisse chez le cheval. Cependant, ils permettent d'étudier les changements quantitatifs des structures épithéliales et de la lamina

propria, ainsi que les aspects qualitatifs du muscle lisse. L'échographie endobronchique, quant à elle, permet d'estimer la masse du muscle lisse bronchique, et ce, chez des chevaux sains et chez des chevaux asthmatiques. Cette thèse démontre aussi que le traitement de 12 semaines avec des corticostéroïdes induit une diminution significative de la masse du muscle lisse périphérique, qui n'est pas amélioré davantage par l'administration concomitante d'un β_2-agoniste à longue durée d'action. Cette diminution est toutefois incomplète. Un effet positif et synergique de la combinaison a également été observé au niveau de la déposition de matrice extracellulaire. La combinaison a produit une diminution significative de la quantité de matrices déposées dans la lamina propria et dans la couche du muscle lisse dans les bronches centrales, alors que l'effet été limité à la couche du muscle lisse dans les bronches périphériques. La combinaison n'améliore pas le contrôle de l'inflammation bronchique ni bronchiolaire par rapport aux monothérapies ; cependant, elle diminue la neutrophilie bronchoalvéolaire de façon synergique.

Mots-clés : asthme, modèle animal, cheval, remodelage, muscle lisse bronchique, matrice extracellulaire, corticostéroïdes, β_2-agonistes à longue durée d'action.

Table of contents

Tables

Figures

Annex I

Abbreviations

A_{ASM}: non-corrected area of the airway smooth muscle (expressed in mm^2)

AHR: airway hyperresponsiveness

ASM: airway smooth muscle

ASM%: percentage of the endobronchial biopsy occupied by airway smooth muscle

AP-1: activator protein 1

A_{tot}: total area of the endobronchial biopsy (expressed in mm^2)

BAL: bronchoalveolar lavage

BALF: bronchoalveolar lavage fluid

BM: basal membrane

C: control horses

C_{dyn}: lung compliance

COPD: chronic obstructive pulmonary disease

CT: computed tomography

ΔP_L: variation of transpulmonary pressure

EBB: endobronchial biopsy

EBUS: endobronchial ultrasound

ECM: extracellular matrix

E_L: pulmonary elastance

EUS: endoscopy ultrasound

FeNO: fractional exhaled nitric oxide

FEV_1: forced expiratory volume in 1 second

HRCT: high-resolution computed tomography

IAD: inflammatory airway disease

ICS: inhaled corticosteroids

ICS/LABA: inhaled corticosteroids and long-acting β_2-agonist combination

HE: horses with heaves (asthmatic horses) in exacerbation

HR: horses with heaves (asthmatic horses) in remission

Ig: immunoglobulin

IL: interleukin

IOS: impulse oscillometry

L: endobronchial echographic layer

LABA: long-acting β_2-agonist

LYVE-1: lymphatic vessel endothelial hyaluronan receptor-1

MAPK: mitogen-activated protein kinase

MMPs: metalloproteinases

MRI: magnetic resonance imaging

NF-kappa β: nuclear factor kappa-light-chain-enhancer of activated B cells

OCT: optical coherence tomography

PCNA: proliferating cell nuclear antigen

PDE: phosphodiesterase

PET: positron-emission tomography

Pi: internal perimeter (of the airway)

P_L: transpulmonary pressure

SMA: smooth muscle actin

SMMHC: smooth muscle myosin heavy chain

SPAOPD: summer-pasture associated obstructive pulmonary disease

SPECT: single photon emission computed tomography

RAO: recurrent airway obstruction

R_L: pulmonary resistance

Th-1, Th2, or Th-17: polarized inflammatory response characterized by increased lymphocytes T-helper 1, 2, or 17

TGF-β: transforming growth factor beta

TIMPs: tissue inhibitor of metalloproteinases

TNF-α: tumor necrosis factor alpha

TSLP: thymic stromal lymphoprotein

US-TBNA: ultrasound-guided transbronchial needle aspiration

$\dot{V}$: airflow

V: volume

VEGF: vascular endothelial growth factor

VEGFR: vascular endothelial growth factor receptor

V_T: tidal volume

V_V: volume fraction

« On résiste à l'invasion des armées; on ne résiste pas à l'invasion des idées. »

Victor Hugo

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Ad maiora!

Michela

Introduction

Central and peripheral airways sustain remodeling and inflammation in asthma (Bergeron, Al-Ramli et al. 2009). In particular, the airway smooth muscle (ASM) is increased and has the ability to induce an exaggerated bronchoconstriction in response to normally non-harmful stimuli, which is the main cause of airflow obstruction in asthma (Martin, Duguet et al. 2000). The amount, composition, and distribution of extracellular matrix (ECM) proteins within the bronchial wall can also profoundly affect lung function (Bosse, Riesenfeld et al. 2010). Multiple studies performed on asthmatic patients have investigated the effect of treatments on the reversibility of airway remodeling and inflammation, and most of them have studied samples of central airways obtained by endobronchial biopsies (Hoshino, Takahashi et al. 1999, Chakir, Shannon et al. 2003, Vignola, Riccobono et al. 2005). However, a growing body of evidence supports a central role of the small peripheral airways in disease pathobiology (Sturton, Persson et al. 2008, Contoli, Bousquet et al. 2010, Burgel 2011, Manoharan, Anderson et al. 2014). There is also increasing awareness that current therapeutic guidelines for asthma do not guarantee an adequate control of peripheral airway dysfunction (namely remodeling and inflammation) (Anderson, Zajda et al. 2012, Lipworth 2013). Peripheral airway assessment is challenging due to their anatomical inaccessibility, which is why their role in asthma has been overlooked for a long time. Equine asthma, or heaves, is a naturally-occurring disease of adult horses and a recognized animal model of human asthma (Leclere, Lavoie-Lamoureux et al. 2011). It is characterized by peripheral remodeling of the ASM and ECM, and by bronchoalveolar neutrophilic inflammation (Leclere, Lavoie-Lamoureux et al. 2011, Setlakwe, Lemos et al. 2014). Peripheral lung biopsies can be harvested from asthmatic horses for direct evaluation of small airway remodeling and inflammation (Lugo, Stick et al. 2002, Relave, David et al. 2008, Relave, David et al. 2010). Using this technique, our laboratory has previously shown that airway remodeling can be reversed by inhaled corticosteroids (ICS) monotherapy (Leclere, Lavoie-Lamoureux et al. 2012). However, the effect (a 30% reduction of ASM mass) was only partial as asthmatic horses still remained with twice as much ASM compared to control subjects even after 1 year of treatment. An insufficient deposition of the drug peripherally may explain the incomplete effect, and is

supported by the residual bronchoconstriction observed in ICS-treated animals (Leclere, Lavoie-Lamoureux et al. 2012). In human asthma, ICS and long-acting β_2-agonist combinations (ICS/LABA) are more effective than ICS monotherapy to control disease exacerbations, but their efficacy compared to ICS on bronchial remodeling and inflammation are not well-described, particularly at the peripheral level of the lung. The objective of this thesis was to investigate and compare the effects of common anti-asthma medications on aspects of peripheral airway remodeling that cannot be studied in man due to ethical constraints. Specifically, we studied whether ICS/LABA enhances the reversibility of peripheral remodeling and the control of airway inflammation compared to ICS or LABA monotherapy. Of note, we simultaneously monitored the effect of these treatments on central airways, in order to identify whether the central remodeling or inflammation are related and may predict peripheral alterations. Due to the paucity of information available on the pathophysiology of peripheral airway remodeling in asthma and its reversibility with treatments, we believe that the results of this thesis are important and can be translated to human asthma.

What this study adds to the field

Airway wall remodeling is a characteristic finding in human asthma. It involves several components of the bronchial wall, with ASM and ECM likely mostly contributing to airway obstruction. These changes have been reported in both the central and peripheral airways in asthma, but few studies have investigated the effectiveness of commonly administered asthma treatments at inducing their reversal, especially in peripheral airways. The involvement of small airway dysfunction in asthma is increasingly recognized, but tools for studying the progression of disease at this level are not readily available. Also, how (and whether) peripheral remodeling and inflammation are reflected by changes observable in central airways remains unsettled. The studies described in this thesis have employed an equine model of neutrophilic asthma to address these questions. The results obtained provide evidence that the combination of ICS/LABA is not superior to ICS monotherapy for ASM remodeling reversibility or bronchial inflammation control. Bronchoalveolar neutrophilia is inhibited by ICS/LABA, while ICS and LABA monotherapies were ineffective. It remains to be determined whether ICS/LABA effectively increases peripheral deposition of the drug compared to ICS alone, or whether oral corticosteroids improve the reversibility of peripheral remodeling, before concluding that a portion of ASM remodeling is resistant to treatment. We have also shown that, peripherally at least, ASM remodeling is reversible in a shorter period of time compared to ECM remodeling, and that the reversibility of ECM remodeling appeared to be determined by the degree of airway wall inflammation. We could not identify remodeling or inflammatory features of central airways reflecting peripheral structure or function, indicating that central and peripheral bronchi act as separate compartments of the lung and not as a continuum. Finally, we have also significantly contributed to the advancement of veterinary medicine by optimizing respiratory research tools that can be employed in future studies and by describing new physiopathological mechanisms associated with equine asthma.

Literature review

Normal anatomy and physiology of the lower respiratory tract

« Among the various cell types that populate the body, we might think of airway smooth muscle as the Hell's Angel of cells, sitting on a Harley-Davidson, unshaven, a cigarette in one hand, a can of beer in the other, and a tattoo on its arm reading *Born to Lose*. »

C.Y. Seow and J. J. Fredberg,
J Appl Physiol (2001) 91:938-52

The lower respiratory tract extends from the larynx to the most distal portions of the lung parenchyma. Its role is to provide the tissues with oxygen and to remove their volatile metabolites as carbon dioxide, contributing to the maintenance of a balanced blood pH. Also, important metabolic and endocrine functions are accomplished in the lungs such as the activation of angiotensin I, or the removal of serotonin, bradykinin, as well as some prostaglandins and leukotrienes from the blood (Joseph, Puttaswamy et al. 2013).

Structure and function of the airways

Airways of the lower respiratory tract are considered as conducting airways when their walls do not contain alveoli and do not allow gas exchange, or as transitional airways when there are alveoli protruding from their walls and gas exchanges can occur. The formers comprise trachea, bronchi and bronchioles until terminal non-respiratory bronchioles, while the latter comprises the alveolar ducts. Distally to alveolar ducts there are alveolar sacs and alveoli.

These are not considered airways but, together with transitional airways and vessels, they form the respiratory tissue or lung parenchyma (West 2008). Airways can further be classified as cartilaginous (bronchi) or membranous (bronchioles) depending on the presence or absence, respectively, of cartilage within their wall. Also airways are classified as intra- or extra-pulmonary based on their anatomical location.

The basic microscopic anatomy of the conducting airways is similar along the bronchial tree, consisting of a pseudo-stratified epithelium overlying subepithelial tissue where connective tissue, vessels, glands and smooth muscle are present, with cartilage and adventitia located outermost (**Figure 1**). The bronchial epithelium is pseudo-stratified and ciliated. It is mainly composed of columnar ciliated and mucus-secreting goblet cells in trachea and proximal bronchi. Columnar ciliated cells and goblet cells decrease in quantity in smaller bronchi, and most of them are replaced by cuboidal epithelial cells and by Club cells (previously named Clara cells (Winkelmann and Noack 2010, Irwin, Augustyn et al. 2013)), respectively, in the bronchioles. The cuboidal bronchial respiratory epithelium completely disappears in the alveolar ducts. The bronchial epithelium lies on a basement membrane that provides attachment for epithelial cells, mainly composed of collagen IV, laminin, and proteoglycans, with elastic fibers within it.

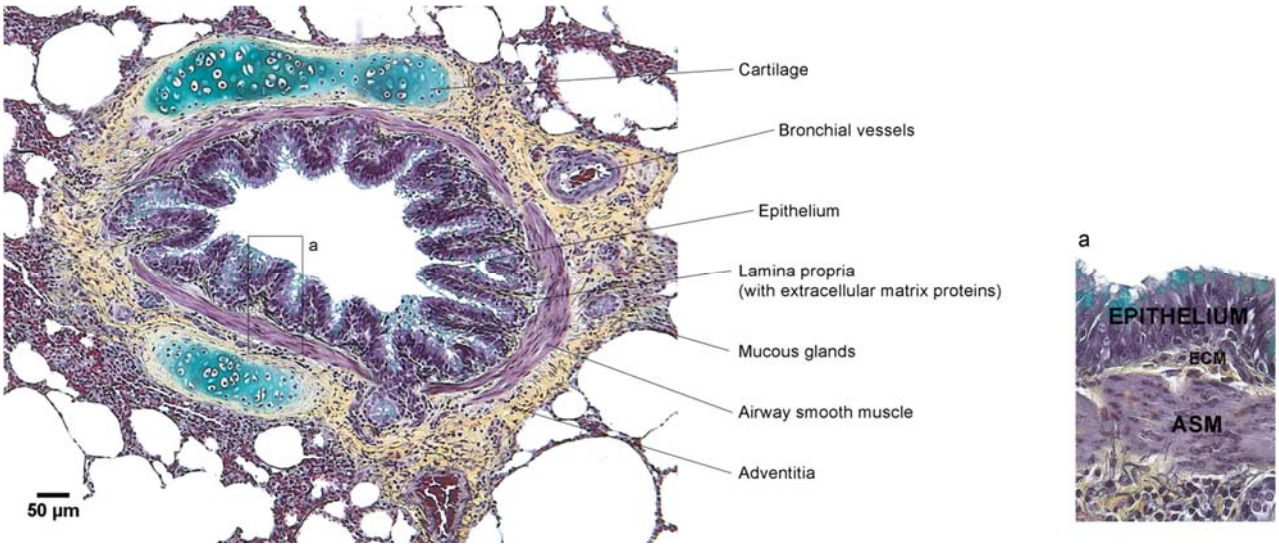

Figure 1. Bronchial anatomy. ECM: extracellular matrix; ASM: airway smooth muscle.

The lamina reticularis is the deepest portion of the basal membrane, adjacent to the lamina propria of the submucosa. Collagen I, III, V, and tenascin amongst others form the lamina reticularis, which is strongly connected to the other portions of the basal membrane (namely the lamina rara and the lamina densa) by strands of collagen VII (Liesker, Ten Hacken et al. 2009). The lamina propria is made up mainly of connective tissue, in which vessels, nerves and elastic fibers are dispersed (Brinkman, Brooks et al. 1969). Airway vessels from the bronchial circulation are arranged in two plexuses within the bronchial wall: a submucosal plexus, running through the lamina propria between the ASM bundles and epithelium, and a peribronchial plexus, supplying the adventitia externally to the ASM layer (Carroll, Cooke et al. 1997). Collagen I and III are the main components of the lamina propria, with several proteoglycans and other glycoproteins. In the bronchial submucosa, beneath the lamina propria, there are smooth muscle fibers, cartilage, and tracheobronchial glands, all surrounded by supporting connective tissue. Caudally to terminal non-respiratory bronchioles, the lamina propria disappears, and only ASM and the elastic network continue in a spiral fashion surrounding the alveoli. Cartilage is disposed as parallel "C-shaped" rings along the trachea and main bronchi, and it becomes smaller and more irregular in segmental and lobular bronchi, completely disappearing in bronchioles (Fraser 2005).

Smooth muscle fibers are disposed as bundles either in the open portion of the cartilage rings (in the trachea, where they are recognized as the *trachealis muscle*), or between the lamina propria and the cartilage. Smooth muscle bundles make up roughly 3-5 and 14-18% of the central and peripheral bronchial wall in adult patients, respectively, with their orientation varying along the bronchial tree (Hale, Olsen et al. 1968, Dunnill, Massarella et al. 1969, Takizawa and Thurlbeck 1971, Sobonya 1984, Saetta, Di Stefano et al. 1991). Overall, they are more regularly and circumferentially oriented in the proximal airways, whilst they are arranged in branching and anastomosing bundles forming oblique and irregular spirals in the lower airways (Smiley-Jewell, Tran et al. 2002). Their disposition around smaller airways has been described as helical or geodesic (James and Carroll 2000). Of note, the orientation of the ASM bundles along the bronchial axis depends on the degree of lung inflation, with higher lung volumes increasing the angle of orientation as a consequence of the longitudinal stress

imposed on the airways. Taking this into account, the estimated angle of orientation of the ASM bundles has been described as small in central airways (<5th generation) and approximately 15 degrees in membranous airways. Furthermore, the distribution is often asymmetric, particularly in the more proximal airways, so that, in case of bronchospasm, ASM orientation tends to favor one direction of coiling over the other (Ebina, Yaegashi et al. 1990, Lei, Ghezzo et al. 1997). Ebina and colleagues (1990) showed that the thickness of ASM relative to airway diameter increases towards the periphery in man. Although the absolute volume of smooth muscle decreases with decreasing airway size, the volume fraction of smooth muscle in the airway wall increases in smaller peripheral airways, and this in spite of a reported tendency for the density of the bronchial smooth muscle bundles to diminish as one moves from the central to the peripheral airways (Lei, Ghezzo et al. 1997). The same group also showed that the thickness of ASM layer relative to the airway diameter could vary up to 10-fold within the same group of airways, possibly in relation to changes in smooth muscle thickness within a bronchial segment, subject size, sex or age, site sampled, degree of muscle contraction and possibly even racial-related differences. The bronchial smooth muscle bundles are surrounded by connective tissue whose ECM is composed of both collagenous and non-collagenous elements, such as elastin, proteoglycans, and other glycoproteins. They are secreted by structural cells such as fibroblasts, myofibroblasts, and smooth muscle cells themselves. Although the mechanisms regulating their turnover (synthesis, deposition, and degradation) within the lung are not completely elucidated, a strong body of evidence supports the balance of metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) within the bronchial tissue as the major determinant of ECM homeostasis.

Within the airways, each structural component has a recognized role contributing to the normal physiology of the lung (summarized in **Table 1**) with the only exception of the ASM, whose function, if any, is still unknown. A few theories have been proposed concerning the role of bronchial smooth muscle in ontology (Murphy, Summer et al. 2008):

1. in the developing fetal lung, the ASM has been shown to contract with a regular peristaltic rhythm, generating distending pressures that could promote the development and maturation of the lung;
2. the contraction of ASM could serve to improve ventilation/perfusion matching and/or decrease dead space ventilation in case of reduced blood flow to a specific district of the lung;
3. during coughing episodes, ASM contraction may increase the velocity of gas movement to promote the expulsion of foreign bodies or noxious particulate materials;
4. the ASM may act as a damper, by balancing the hysteresis between small airways and alveolar units;
5. mucus propulsion (Bullowa and Gottlieb 1922);
6. lastly, the ASM may simply be an ‘‘evolutionary oversight,’’ that is, a vestige of the lung’s origin from an organ, the foregut, already programmed to develop smooth muscle, with no true physiologic purpose.

Furthermore, at present, there is no disease entity or appreciable physiological deficit associated with loss of ASM contraction (Seow and Fredberg 2001).

The lung is a relatively stable organ with low rates of cell turnover, particularly in the airways. With less than 1% of epithelial cells proliferating at any given point in time (Ayers and Jeffery 1988, Demoly, Simony-Lafontaine et al. 1994, Leigh, Kylander et al. 1995), there appears to be little need for local cellular self-renewal under normal circumstances. Nevertheless, following injury, the epithelium has great ability to repair in a very short time by means of processes of dedifferentiation, migration and proliferation of adjacent cells (Erjefalt and Persson 1997, Yahaya, Baker et al. 2011). Proliferation and possibly migration of subepithelial cells seem to be involved in this process as well, as documented by studies of induced epithelial damage in a sheep model and by the observation of increased quantity of subepithelial myofibroblasts in asthmatic patients after experimental allergen exposure (Gizycki, Adelroth et al. 1997, Yahaya, Baker et al. 2011). The proliferation rate of ASM cells in normal conditions is considered as negligible (Chernyavsky, Croisier et al. 2014).

Subepithelial cells proliferating in normal conditions are scarce and mostly represented by endothelial cells and cells in close proximity to the mucus glands (Yahaya, Baker et al. 2011). On the other hand, turnover of the ECM is impressively rapid; it is estimated that 10-15% of the lung ECM is renewed every day (Dunsmore, Lee et al. 1996, Roberts and Burke 1998).

Table 1. Normal function of airway components.

Airway structure	Normal function
Epithelium	Physical barrier against inhaled particles
Lamina reticularis	Structural support for epithelial attachment
Lamina propria	Structural support of the bronchial wall Determines mechanical properties of the bronchial wall May regulate ASM proliferative/synthetic function
Submucosal vessels	Trophic support of the airways
Smooth muscle	?
Adventitia	Sustains structure Guarantees airway-parenchyma interdependence

Structure and function of the lung parenchyma

Within the functional respiratory tissue, which is composed of alveolar ducts, alveolar sacs, and alveoli, alveolar units are demarcated by septa composed of a continuous layer of squamous epithelial cells overlying a thin interstitium. Capillaries as well as connective tissue and several cells responsible for maintaining alveolar shape and defense lie within the septal interstitium. The alveolar epithelium consists primarily of type I and type II alveolar cells.

Type I cells are more numerous, they cover approximately 95% of the alveolar surface, and their function is related to the transport of substances in either direction across the air-blood barrier. Type II alveolar cells are generally located close to the junctions among different alveoli, they are responsible for production of surfactant and renewal of the alveolar epithelium by differentiation into type I cells. A basement membrane is present underneath the alveolar epithelial cells, intimately connected with the basement membrane of the capillary endothelium. Elastic and collagen fibers, as well as myofibroblasts, are irregularly distributed within the connective matrix between epithelial and endothelial basement membranes (Fraser 2005).

Age-related changes of pulmonary structure and function

The physiological aging process undoubtedly impacts the structure and function of the lungs. Anatomically, the airways and their components increase in volume during infancy until complete development of the body, with the smooth muscle occupying a slightly greater proportion of the airway wall in children compared to adults (James and Carroll 2000). The aging lung is characterized by higher levels of collagen deposition and decreased elastin expression, which contribute to the decreased elastic recoil observed with age (Thannickal, Murthy et al. 2015). Lung function decline with aging in healthy individuals is associated to alterations of the lung parenchyma and terminal conducting airways. Reduced mucociliary clearance and altered surfactant composition are also reported in aged individuals (Miller 2010, Ramly, Kaafarani et al. 2015).

Mechanisms of airway patency

When the lungs are removed from the thoracic cage, they immediately collapse due to their elastic recoil. Bronchial lumen is also significantly reduced when lungs are collapsed, and this independently of the presence or absence of cartilage in the airway wall, because of the highly

developed elastic network connecting all structures within the lung. Several mechanisms contribute to maintain airway patency *in vivo* and to oppose the collapsing force induced by reduced lung elastic recoil at low lung volumes. First of all, it is the intimate relationship between airway dimension and lung volume that prevents bronchial collapse even at very low lung volumes. This is known as airway or parenchymal tethering, or airway-parenchymal interdependence (Bates and Lauzon 2007). Airway stiffness itself as well contributes to airway patency *in vivo*. Lastly, the lining fluid exerts surface tension acting inwardly to close the airways, especially the smallest ones. The secreted surfactant, on the other hand, reduces the surface tension of the lining fluid and thereby contributes to airway patency (Veldhuizen and Haagsman, 2000).

Mechanics of breathing

The act of breathing is accomplished by respiratory cycles. For each human breath, one active inspiratory and one passive expiratory process occur, during which the air is forced in and out of the alveoli as a result of the changing intrapulmonary (or alveolar) pressures. Indeed, the contraction of the diaphragm (and other respiratory muscles) during inspiration expands the thorax which, in turn, induces lung expansion. Following the increase in lung volume, the alveolar pressure drops as a consequence of Boyle's law and produces a pressure gradient that makes the air move from the mouth to the alveoli. As stated by Ohm's law: the airflow between two points is directly proportional to the pressure difference across the two points. Similarly, during expiration, the lung passively recoils due to its elastic properties and its volume decreases. Alveolar pressure then increases compared to the pressure at the mouth, which allows air to move from the alveoli towards the atmosphere. Any alteration of the airway lumen (bronchoconstriction, space-occupying lesion or mucus) between two points of the bronchial tree is an obstacle for the (theoretically[1]) laminar airflow generated by the

[1] In the airways there is usually a combination of both laminar and turbulent flow (called "transitional flow") in the center of the airway and near the bronchial wall, respectively. True laminar flow probably only occurs in the smallest airways, where linear velocity of the air is very low.

pressure difference existing between them. In order to maintain a constant airflow ($\dot{V}$), the pressure gradient between the two points (ΔP_L) must be increased proportionally to the resistance of the lung (R_L):

$$R_L = \frac{\Delta P_L}{\dot{V}}$$

Lung resistance is expressed in cm H_2O/L/s, and it is the sum of airway resistance (normally about 80% of R_L) and tissue resistance (lung parenchyma and chest wall, counting for 20% of R_L). The Poiseuille's law states that airway resistance is directly proportional to the length of the airway and the viscosity of the gas, and inversely proportional to the radius to the fourth power (i.e.: a 2-fold decrease in bronchial radius increases airway resistance by 16-fold). Thus, the main determinant of airway resistance is airway diameter which, in turn, results from:

- ASM contraction (bronchospasm),
- airway wall thickening (edema and/or remodeling),
- airway lumen occlusion (i.e. by mucus and cell accumulation or space occupying lesions),
- lung volume (at higher volumes, the parenchyma pulls on the airways keeping them wide open).

For a given flow, resistance to air passage is thus greatest in individual small airways compared to large ones due to their small diameter. However, the total resistance to airflow contributed by the small airways taken together is very low (on average 10% in health) as they represent numerous parallel pathways, whose transversal area can be summed up. Under normal circumstances it has been calculated that the greatest resistance to airflow resides in the medium-sized bronchi (Macklem 1998).

The elastic recoil of the lung is fundamental for breathing. The slope of the pressure-volume curve represents lung compliance (C_{dyn}, expressed in L/cm H_2O), which is a measure that

describes the ease by which the lungs stretch and expand in response to a given pressure. It is calculated using the formula:

$$C_{dyn} = \frac{\Delta V}{\Delta P_L}$$

where V is lung volume and P_L is pleural pressure. The compliance of the lungs demonstrates hysteresis; that is, the P_L is different on inspiration and expiration for identical volumes. Also, compliance is volume dependent. Elastance (E_L, expressed in cm H_2O/L and also known as elastic resistance) is, by definition, the reciprocal of compliance ($E = 1/C_{dyn}$), and describes the pressure change that is required to elicit a unit volume change of the lungs. This is a measure of the resistance of a system to expand or to shrink. Elastance is an indirect expression of the work that has to be exerted by the inspiratory muscles to expand the lungs. An increased elastance needs to be counteracted by an increased power of the muscles during inspiration, leading to an increased work of breathing (work of breathing is the physical work that has to be carried out by the muscles of respiration to overcome the elastic and non-elastic resistance of the respiratory system). The elastance of the whole respiratory system depends on the elastance of the chest wall and that of the lungs. Changes in the elastance (and therefore the compliance) of the chest wall are uncommon. In contrast, the elastance of the lungs is affected by many respiratory diseases. Thus, variations in the elastance of the respiratory system are mainly due to alterations of the elastance of the lungs, which is governed by two main factors:

- the elastic recoil forces of the lung (mainly regulated by the properties of the pulmonary elastin network, but also by the altered deposition of fibrous tissue),
- the surface tension at the air-alveolar interface (depending on the surfactant quantity and composition).

Conversely to resistance, compliance and elastance of the lungs are mainly determined by the physical properties of the small airways and lung parenchyma.

Even in healthy subjects, heterogeneity exists among the behavior of different airways and alveoli. However, taken together, the behavior of the lungs can be compared to that of a balloon sealed over the end of a pipe, which can be simplified using a mathematical approach called the linear single-compartment model (Bates 1993). This model has a clear anatomical analogy to the lung; the pipe represents the conducting airways and the balloon represents the elastic parenchymal tissue. There is also a functional analogy, as the balloon can be inflated and deflated through the pipe in the same way that a lung inspires and expires. Mathematically, is it expressed as:

$$\Delta P_L = \left(R_L \cdot \dot{V}\right) + \left(E_L \cdot V_T\right)$$

where V_T is tidal volume (in L) and K is the pulmonary end expiratory pressure (in cm H_2O). This equation states that the pressure gradient required to increase lung volume by V_T is proportional to the resistive and elastic properties of the lung, and it will increase at increasing R_L and E_L.

Functional anatomy of the lower respiratory tract

During breathing, the airway structures undergo dynamic morphological changes due to physical constraints and mechanical stress imposed by the changing intrathoracic pressures. Airways are subjected to two types of stretches or stresses: a radial stretch, acting outwardly all around the airway wall, and a longitudinal stretch, related to the elongation of the lung; both occurring during inspiration. The opposite occurs during expiration. Intra-thoracic airways reach a maximal and minimal cross-sectional area at end-inspiration and end-expiration, respectively. This is of particular interest during dynamic measurement of airway wall thickness or airway size.

Until recently, dynamic measurements of airway size or length were not possible, and general knowledge on this topic has been increasing exponentially in the last decade. With the advent of new imaging technologies, reliable measurements can now be performed. Airway wall area remains unchanged or undergoes minimal changes across the breathing cycle (Coxson,

Quiney et al. 2008), while airway wall thickness and lumen area vary. Specifically, airway lumen increases and bronchial thickness decreases during inspiration, as a result of bronchodilation. Significant changes are observed in the airway lumen area during quiet breathing in large (8-25 mm in diameter), intermediate (2-8 mm), and small airways (< 2 mm), with the latter being the site where this phenomenon is most remarkable (McLaughlin, Williamson et al. 2008). This occurs probably because they are more compliant than larger airways, and because of their strict interdependence with the pulmonary parenchyma. In patients with COPD, variations of the bronchial lumen up to 150-200% have been reported during deep breathing maneuvers (Matsuoka, Kurihara et al. 2008).

Mechanisms implicated in asthma pathobiology

« Breathing remains unaccountably easy. Indeed, it is this *lightness of breathing* in the healthy challenged lung, rather than the labored breathing that is characteristic of the asthmatic lung, that in many ways presents the greater challenge to our understanding of the determinants of acute airway narrowing. »

J.J. Fredberg and S.A. Shore
"The Unbearable Lightness of Breathing"
J Appl Physiol (1999) 86:3-4

Asthma is a chronic disease, considered as multifactorial, diffuse, and heterogeneous in nature. Different pathophysiological mechanisms are involved in disease development, both intrinsic and extrinsic to the patient. Specifically, genetic predisposition or dysregulated immunity, as well as several environmental triggers have been shown to take part in asthma pathogenesis. Whether the intrinsic causes of asthma are inherited or acquired during life remains a matter of debate (Wenzel 2012, Kudo, Ishigatsubo et al. 2013). Asthma is considered a diffuse disease, as it affects the entirety of the lung. It is however heterogeneous, as different regions of the bronchial tree can be differently affected (Elliot, Jones et al. 2015).

Pathophysiologically, asthma is defined as an obstructive and inflammatory disease. Both airway remodeling and excessive narrowing contribute to the airflow obstruction observed in asthmatic patients. An increased smooth muscle mass and reactivity to normal stimuli (hyperresponsiveness) are the most important determinants of airflow obstruction (Lambert,

Wiggs et al. 1993, Oliver, Fabry et al. 2007). While the leading role of bronchospasm during asthma attacks has long been recognized, the implication of inflammation to asthma pathophysiology was only described few decades ago, and its contribution to disease development and/or maintenance is still ill-defined. Several structural and immune cells contribute to the asthmatic response within the bronchi. Interestingly, among these cells, ASM cells have marked immunomodulating properties, making them potentially significant contributors to the inflammatory features observed in asthmatic airways (Damera, Tliba et al. 2009).

The relationship existing between bronchospasm, remodeling, and inflammation in asthma is far from clear. It has been speculated for some time that inflammation represents the initiating insult, causing remodeling and airway responsiveness (Kudo, Ishigatsubo et al. 2013). However, recent evidence argues against this theory and suggests that bronchospasm and remodeling themselves might in turn induce or facilitate inflammation (Damera, Tliba et al. 2009, Grainge, Lau et al. 2011).

Airway hyperresponsiveness

Airway hyperresponsiveness (AHR) is defined as an excessive decline of lung function upon ASM activation. AHR is a well-recognized feature of the asthmatic disease, however the primary contributors to this phenomenon remain to be elucidated (Martin, Duguet et al. 2000). Whether and to which extent ASM cell hypercontractility (abnormally increased degree of contraction) and hyperreactivity (contractile response in the presence of normally non-harmful stimuli) contribute to the development of AHR is still unknown. While ASM seems intuitively to be the key of such pathological behavior of the airways, a large amount of evidence supports that other non-contractile components of the airway wall as well as of the pulmonary parenchyma can contribute to the pathogenesis of AHR (Bosse, Riesenfeld et al. 2010). In other words: while ASM contraction is likely to be the central point, the load against

which the muscle contracts as well as the environment in which the muscle is placed must be taken into account (**Figure 2**) [65].

Smooth muscle-related determinants of AHR

Both static and dynamic modeling of pulmonary mechanics identify ASM as the most important determinant of airway narrowing and AHR (Lambert, Wiggs et al. 1993, Oliver, Fabry et al. 2007). Airway smooth muscle mass is increased in asthmatics and such increase is associated with the severity of the disease (James, Bai et al. 2009). However, whether such increase coexists with phenotypic changes affecting ASM contractile, proliferative or secretory properties and to what extent they would contribute to AHR in asthma remains unclear (Hirota, Nguyen et al. 2009). The possibility that functional anomalies of the ASM cells represent a central determinant of AHR and asthma pathogenesis must be kept in mind.

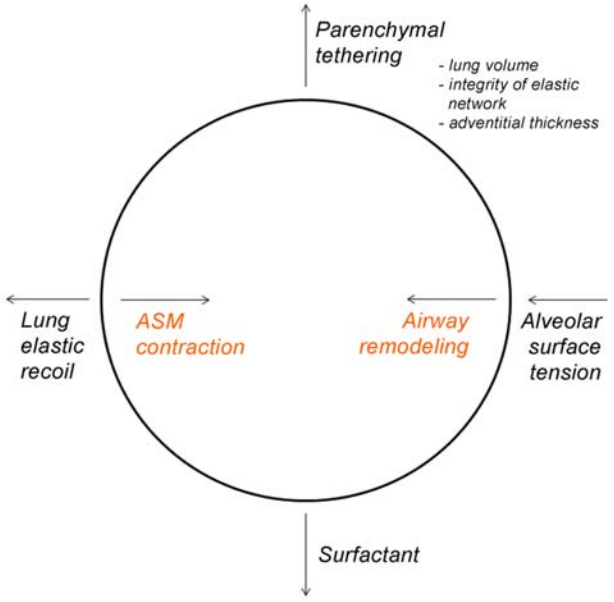

Figure 2. Determinants of airway patency and hyperresponsiveness. The arrows indicate forces (pressures) acting on the bronchial wall. In red are indicated alterations observed in asthma.

Anatomical factors. ASM is organized in bundles running along the bronchial wall in a spiral fashion (Bates and Martin 1990). In such spirals, ASM orientation seems to acquire a wide range of angles along the airways, following a distribution pattern likely conserved among species and among different airway generations (Smiley-Jewell, Tran et al. 2002). The magnitude of ASM obliquity along the bronchi has been judged as unlikely to result in physiologically important changes in airway length during bronchoconstriction (Lei, Ghezzo et al. 1997). Contrarily, airway caliber would be significantly affected (Bates and Martin 1990). It has been speculated that AHR might even result simply from a stiffening of the airway in the longitudinal direction (Bates and Martin 1990), a situation possibly reflecting the act of breathing at high lung volumes as during an asthma attack. Paradoxically, an *in silico* model of the bronchial tree suggested that bronchial malfunction related to asthma could be a « necessary consequence of the optimized efficiency of the structure of the human bronchial tree » (Mauroy, Filoche et al. 2004). In other words, an AHR could be the drawback of a bronchial tree anatomically optimized to be physiologically efficient.

Remodeling. The smooth muscle mass is increased in asthmatic airways (James, Elliot et al. 2012). As the force produced by the smooth muscle is proportional to its cross-section (Chin, Bosse et al. 2012), the asthmatic smooth muscle could cause AHR simply because a greater force would act against the mechanisms of airway patency. This could occur in the absence of mechanical alterations of the ASM in asthma, and is supported by mathematical models of the lung (Wiggs, Bosken et al. 1992, Lambert, Wiggs et al. 1993). However, the unchanged FEV_1 (forced expiratory volume in 1 second) reported after ASM reduction obtained by bronchial thermoplasty in severe asthmatics (Pavord, Thomson et al. 2013, Denner, Doeing et al. 2015) argues against the hypothesis that AHR in asthma is just the result of the increased smooth muscle mass. The mechanical properties of ASM cells are influenced by their curvature. Particularly, cell stiffness is maximal at curvature similar to those expected in terminal membranous bronchioles (diameter 1.5 mm) and decreases at smaller or greater curvatures. Cell contractility instead is thought to be inversely proportional to airway dimension, as it decreases consistently as the curvature increases (Montaudon, Desbarats et al. 2007, West 2008, Xu, Chen et al. 2011). It is possible that hypertrophy and hyperplasia of ASM might

alter the distribution of ASM cell orientation in asthmatic airways (Lei, Ghezzo et al. 1997), accentuating airflow heterogeneity during bronchospasm. *In vitro*, ASM cell alignment depends on the strain they are exposed to (Morioka, Parameswaran et al. 2011, Ghezzi, Risse et al. 2013). Airway smooth muscle orientation has the potential to influence the mechanics of airway narrowing by influencing the vector of forces acting on the airway wall during bronchoconstriction. That is, a portion of the forces developed by ASM contraction could potentially shorten the airway in addition to narrowing its lumen (Lei 1997).

Phenotype plasticity. Two different populations (phenotypes) of ASM cells are recognized *in vitro*, characterized by increased contractile or synthetic properties (Hirota, Nguyen et al. 2009), which were first described in 1997 (Ma, Li et al. 1997). Of note, the phenotype is not a constant property of the ASM cell. It can instead vary depending on the chemical and mechanical environment the cell is exposed to (Hirota, Nguyen et al. 2009). A large body of evidence supports the hypothesis that asthmatic ASM cells express a more proliferative/synthetic and less contractile phenotype compared to healthy ASM cells *in vitro* (Wright, Trian et al. 2013). Whether this is true also *in vivo* remains to be determined. The fact that hyperplasia is a recognized mechanism of increased ASM mass in asthmatic subjects, and that ASM cells are now recognized as immunomodulatory cells able to secrete a various array of pro-inflammatory molecules (James, Elliot et al. 2012, Wright, Trian et al. 2013) sustains this theory.

Length adaptation. Two very important and unique properties of the smooth muscle have been described that highlight its adaptable nature. Length adaptability was first proposed in 1995 to explain that the force generated by smooth muscle contraction is (relatively) independent of its length (Pratusevich, Seow et al. 1995). In fact, when the smooth muscle length is changed, a decline of its strength is initially observed but it gradually recovers over time. *In situ*, length adaptation may be initiated by a change in transmural pressure (causing bronchoconstriction or bronchodilation), which is a primary physiological determinant of ASM length. When healthy subjects are prevented from taking deep breaths for 20-30 minutes, they indeed develop AHR (Skloot, Permutt et al. 1995). The recognized

bronchoprotective and bronchodilatory effects of deep inspirations observed in healthy subjects have been ascribed to this mechanism as well. However, despite the fact that bronchi of asthmatic subjects can dilate as much as those of healthy patients *in vivo* (Brown, Scichilone et al. 2001), the bronchoprotective effect of deep inspirations is impaired in asthmatics, suggesting that length adaptation alone is not the sole explanation of AHR in asthma. Recent studies performed using intact airway sections instead of isolated smooth muscle strips further support the inability of smooth muscle length adaptation alone to cause AHR, especially at the central level, because of the coupling existing between airway wall and ASM layer (Ansell, McFawn et al. 2015, Dowie, Ansell et al. 2016).

Force adaptation. Force adaptation has been described more recently as the ability of the muscle to increase its force-generating capacity in response to stimulation (Bosse, Chin et al. 2009). Differently from length adaptation, force adaptation *in vitro* is not inhibited by oscillating maneuvers simulating breathing pattern (Pascoe, Jiao et al. 2012). Length and force adaptation can coexist and have additive effects (Bosse, Chin et al. 2010) . The significance of this finding in asthmatic airways, chronically exposed to bronchoconstrictive stimuli, appears straightforward but has not been proven yet.

Myosin isoforms. Smooth muscle myosin heavy chain (SMMHC) is a cytoplasmic structural protein that is a major component of the contractile apparatus in smooth muscle cells. It is expressed as four different isoforms, generated by alternative splicing of the myosin gene. In particular, two of these isoforms regulate the velocity of contraction of the myosin head once it is attached to the actin filament. At a molecular level, they differ by the presence [(+)insert, also called SM-B] or absence [(-)insert, also called SM-A] of a 7–amino acid insert within the protein structure. The (+)isoform induces a propulsion velocity of the actin filament twice as fast as the (-)isoform, and it is overexpressed in human and equine asthmatic bronchial tissue compared to healthy subjects (Leguillette, Laviolette et al. 2009, Boivin, Vargas et al. 2014). A more rapid smooth muscle contraction could contribute to the blunted response of asthmatic patients to deep inspirations.

Fluidity. Muscle contractile properties are equilibrated dynamically. *In vivo*, non-activated ASM is continuously exposed to force fluctuations (stress) that induce cell length variations (strains), which significantly inhibit its contractile capacity (Krishnan, Trepat et al. 2008). In asthmatics, ASM is activated by different stimuli likely reducing its strain and conferring quasi-static conditions. Using an immediate terminology (partly borrowed from the past), the group of Fredberg described the normal ASM as "fluid" and the asthmatic ASM as "frozen" (Krishnan, Trepat et al. 2008). One of the possible molecular explanations of this theory is that, during breathing, a number of cross-bridges between the myosin head and the actin filament are broken up mechanically and prematurely compared to the complete myosin duty cycle. This reduces the number of cross-bridges and the force-generating capacity of the muscle when it is in its fluid state (Fredberg, Inouye et al. 1999). In fact, when healthy individuals are restrained from taking deep breaths, their ASM "freeze" in a contracted position after only 15 minutes, becoming hyperresponsive to the same extent as asthmatic smooth muscle (Krishnan, Trepat et al. 2008).

The contractile properties of ASM have been studied in healthy subjects, in asthmatics and in animal models of asthma, in order to understand if any abnormality of the ASM could explain the exaggerated response to contractile stimuli observed in asthma. Main results of previous studies made on human tissues in static conditions have been variable and quite inconclusive, especially in light of the fact that ASM behavior *in vivo* is better represented by oscillatory conditions (Fredberg, Inouye et al. 1999). Furthermore, many studies included a too limited number of specimens to guarantee statistically sound comparisons between groups (de Jongste, Mons et al. 1987, Bramley, Thomson et al. 1994, Thomson, Bramley et al. 1996). Limitations of the early approaches used to study ASM mechanical properties are now recognized (Bai, Bates et al. 2004). Among others, as heterogeneity has been appreciated in ASM derived from different anatomical regions, most strikingly between trachea and bronchi (Bramley, Thomson et al. 1994, Ma, Li et al. 1997, Matusovsky, Kachmar et al. 2015), it seems inappropriate to compare specimens from different anatomical regions or to draw conclusions concerning the bronchial behavior from studies employing tracheal specimens. Having said that, comparing samples obtained from the same anatomical region between

asthmatics and non-asthmatics may however identify abnormalities in ASM behavior related to the disease. Two recent studies (Chin, Bossé et al. 2012, Ijpma, Kachmar et al. 2015) found no difference in the intrinsic properties of ASM in asthma, supporting the hypothesis that is it the environment where the muscle is located rather than the muscle itself, the main determinant of AHR in asthma.

Non-smooth muscle determinants of AHR

Lung volume and airway-parenchyma interdependence. Airways are intimately connected to the pulmonary parenchyma, so that increasing lung volume stretches the bronchi both longitudinally and transversally, decreasing airway resistance. Such airway wall tension is primarily determined by non-contractile airway wall components when ASM is relaxed, but it is progressively transferred to the ASM during its active contraction (Martin, Duguet et al. 2000, Bosse, Riesenfeld et al. 2010). Lung expansion provides an elastic force contrasting that induced by ASM contraction. However, parenchyma and ASM are not directly linked; between them lies the external bronchial layer made of loose connective tissue, lymphatics and bronchial microvessels in small airways, in addition to cartilage and submucosal glands in large airways. This layer may attenuate force transmission between the two compartments, especially when its morphological and mechanical features are altered such as in asthma (Martin, Duguet et al. 2000, Dolhnikoff, da Silva et al. 2009).

Airway wall morphology and remodeling. The spatial arrangement and volumes of the constituents of the airway wall are crucial in determining how the contraction of the ASM affects airway narrowing and airflow obstruction (Bosse, Riesenfeld et al. 2010). Specifically, the disposition and mechanical features of tissues lying around and within the ASM layer must be considered. Increasing the volume of tissues outside the ASM may lead to an uncoupling of the system, modifying the patterns of force transmission from lung parenchyma to the airway wall. On the other hand, the increased deposition of structures lying inside the ASM layer represents a higher load for the ASM to contract against (which theoretically

would be beneficial during bronchospasm), but also some space-occupying material protruding towards the airway lumen and reducing its caliber, facilitating airway obstruction (Lambert, Wiggs et al. 1993). Especially during ASM contraction, even small reductions of the airway lumen may increase dramatically airway resistance (Lambert, Wiggs et al. 1993). Remodeling of the ECM among and surrounding the ASM cells also deserves to be considered. Due to its relative stiffness, this ECM "cage" is thought to limit the contraction-induced cross-sectional widening of the smooth muscle bundle, and thus its excessive shortening. This theory is known as the radial constraint hypothesis (Meiss 1999), and it is supported by the reduced fraction of ECM proteins observed within the ASM layer of severe compared to moderate asthmatics (Pini, Hamid et al. 2007).

Heterogeneity. Structural and mechanical properties of the bronchial tree vary in time or among different bronchial generations. Pathological entities affecting such properties – such as asthma –further increase the heterogeneity of the lung (Bosse, Riesenfeld et al. 2010). Using a mathematical model, it has been shown that airway resistance can significantly increase in response of uneven bronchial tone along the bronchial tree (Bates 1993). Airway smooth muscle mechanical heterogeneity is also recognized at tissue and cellular level (Ma, Li et al. 1997) which, in addition to anatomical heterogeneity, can produce AHR (Bosse, Riesenfeld et al. 2010). Any factor contributing to lung heterogeneity can finally lead to increased airway resistance and amplify the effect of contractile stimuli on respiratory physiology.

Inflammation. Evidence exists linking the increased inflammatory infiltrate into the ASM layer and its increased proliferation (Ramos-Barbon, Fraga-Iriso et al. 2010). For this reason, inflammation could indirectly induce AHR by increasing the ASM mass in asthmatic airways. Furthermore, inflammatory cells may produce and secrete multiple bronchoconstrictor agents (Bossé 2014) that can contribute to overstimulate the asthmatic ASM cells.

Airway remodeling

Asthmatic airways are characterized by marked structural changes affecting most of their histologic components, and commonly referred to as airway remodeling. It significantly contributes to airflow obstruction in asthma, particularly during episodes of bronchoconstriction. Airway remodeling can affect small and/or large airways, possibly in relation with the asthma inflammatory phenotype (Elliot, Jones et al. 2015).

Remodeling has been considered for a long time as the consequence of chronic tissue inflammation and dysregulated repair. However, the similar degree of remodeling observed in recent compared to long-standing mild asthmatics (Boulet, Turcotte et al. 2000), the demonstration of bronchial remodeling in wheezing children (Saglani, Payne et al. 2007, O'Reilly, Ullmann et al. 2013), and the impressively quick remodeling response observed after antigen challenge in asthmatic patients (Kariyawasam, Aizen et al. 2007) argue against the fact that a chronic insult is necessary in order to develop airway remodeling. Different studies have shown that the degree of remodeling is related to the severity and not to the duration of the disease (Chetta, Foresi et al. 1997, James, Bai et al. 2009).

Due to the importance of the small airways in asthma pathogenesis and to the difficulty of sampling (Contoli, Bousquet et al. 2010, Burgel 2011), a lot of of attention has been focused on understanding whether the morphological changes observed in large and small airways are correlated (Postma, Brightling et al. 2015). To date, the information available about small airway remodeling comes from a limited number of studies performed on autopsy specimens which have provided precious insights into the contribution of the silent portion of the lung to the asthmatic disease.

Epithelium

The bronchial epithelium is the first physical barrier (complemented by the mucociliary escalator) to external airborne antigens and thus the structure in direct contact with all

inspired molecules that can reach the lungs. As such, it is a structure with important immunoregulatory roles, able to orchestrate complex innate and acquired responses to several triggers (Damera, Tliba et al. 2009). There is evidence supporting an impaired function of the airway epithelium in asthma (Swindle, Collins et al. 2009), in part mirrored by an altered structure. Increased susceptibility to injury and altered repair are considered to be important inducers of airway wall remodeling in asthma.

Bronchial epithelial fragility and detachment has been associated with asthma for long time, due to the high number of epithelial cells observed in sputum samples or within the bronchial lumen in histologic preparations. The evidence provided by *in vitro* studies of stressed and fragile airway epithelium in asthmatic airways (Swindle, Collins et al. 2009), incapable of adequate repair mechanisms (Puddicombe, Torres-Lozano et al. 2003), supports the fact that the macroscopic shedding of the bronchial epithelium often observed in endobronchial biopsy samples is now considered as being merely artefactual (Holgate 2011). The data obtained by Labonté and colleagues argue that the asthmatic epithelium could be macroscopically damaged only in the more proximal airways (Labonte, Laviolette et al. 2008), questioning whether the sampling site of endobronchial biopsy could have represented a confounder in previous studies.

Bronchial epithelial hyperplasia has been observed in severe compared to mild asthmatics and correlates with epithelial cell proliferation in some studies (Demoly, Simony-Lafontaine et al. 1994, Druilhe, Wallaert et al. 1998, Vignola, Chiappara et al. 2001, Cohen, E et al. 2007). However, two points need to be raised: first, the thickness of airway epithelium of severe asthmatics is not different from that of healthy subjects; second, severe asthmatics need corticosteroid treatment, which can profoundly modulate cell proliferation and apoptosis (Salmon, Koto et al. 1998, Dorscheid, Wojcik et al. 2001, Al-Wadei, Takahasi et al. 2005, Gallelli, Pelaia et al. 2010). In this perspective, whether epithelial hyperplasia does occur in severe asthma or it is the result of corticosteroid-induced alteration in epithelial cell cycle remains to be determined.

The observation of a thinner epithelium in mild asthmatics compared to healthy controls in the absence of increased cell apoptosis and occasionally in the presence of increased proliferation (Benayoun, Letuve et al. 2001, Vignola, Chiappara et al. 2001, Cohen, E et al. 2007) suggests that epithelial shedding and dysregulated repair could be a feature of the disease, successfully controlled by corticosteroid treatment. On the other hand, allergen challenge appears to increase epithelial proliferation in subjects with mild asthma without inducing epithelial shedding (Ricciardolo, Di Stefano et al. 2003), supporting a real thickening of airway mucosal layer in the more severe forms of the disease.

Mucostasis is an overlooked trait of asthmatic airways, certainly contributing to the obstructive nature of the disease. The amount of mucus within the airways results from the balance between the number of goblet cells and mucus glands, their synthetic and secretory activity, and the clearance ability of ciliated cells. Alterations in all of these mechanisms have been proposed as contributors to mucostasis in asthma. Because mucus is principally a mixture of mucin glycoproteins and plasma proteins, it is also possible that abnormalities at the vascular endothelial level could result in excess airway mucus (Fahy 2001). Even mild asthma is associated with goblet cell hyperplasia and increased (up to 3-fold) stored mucin in the bronchial epithelium, whereas in moderate asthmatics both stored and secreted pools are increased (Ordonez, Khashayar et al. 2001, Samitas, Zervas et al. 2011). These findings suggest that acute degranulation of hyperplastic goblet cells may represent a mechanism for exacerbations in mild and moderate asthma. This hypothesis however is not supported by a previous study showing a stable or increased quantity of mucus stored in the bronchial epithelium of mild asthmatics 1 or 48 hours after antigen inhalation (Hays, Woodruff et al. 2001). Chronic degranulation of goblet cells may contribute to chronic airway narrowing in moderate to severe asthma (Ordonez, Khashayar et al. 2001).

In response to acute or chronic airway injuries, the increase in percentage of goblet cells can occur by selective cellular proliferation, also called hyperplasia, or by cell transdifferentiation. The latter is defined as the conversion of a specific differentiated cell type into another type of differentiated cell. It belongs to a wider class of cell-type switches termed metaplasia

(Boucherat, Boczkowski et al. 2013). As goblet cells are normally found in bronchi but not in bronchioles, their increase in cartilaginous airways is termed goblet cell hyperplasia and their appearance in small membranous airways is defined as goblet cell metaplasia (Tesfaigzi 2008).

Extracellular matrix

The ECM is a dynamic and complex network of macromolecules whose primary aim is to form a scaffold for the airways. It acts as mechanical support but also plays a crucial role in the maintenance of airway function and structure, being involved in many cellular events such as adhesion, migration, synthesis, and proliferation (Hirst, Twort et al. 2000, Johnson, Burgess et al. 2004, Dekkers, Schaafsma et al. 2007, Araujo, Dolhnikoff et al. 2008, Liesker, Ten Hacken et al. 2009). The bronchial ECM is composed of collagen, proteoglycans, elastin, and other glycoproteins. They are mainly synthetized and secreted by fibroblasts within the submucosa. The content of ECM is regulated by several mediators, mainly metalloproteinases (MMPs) and their counterpart, tissue inhibitors of metalloproteinases (TIMPs). The ECM deposition is altered in asthmatic airways (Kuwano, Bosken et al. 1993) (**Figure 3**). Most of the studies published on this subject are focused on structural changes occurring within the lamina propria, while less is known concerning adventitial alterations, mainly because of the difficulty of sampling this outer region even in the large airways.

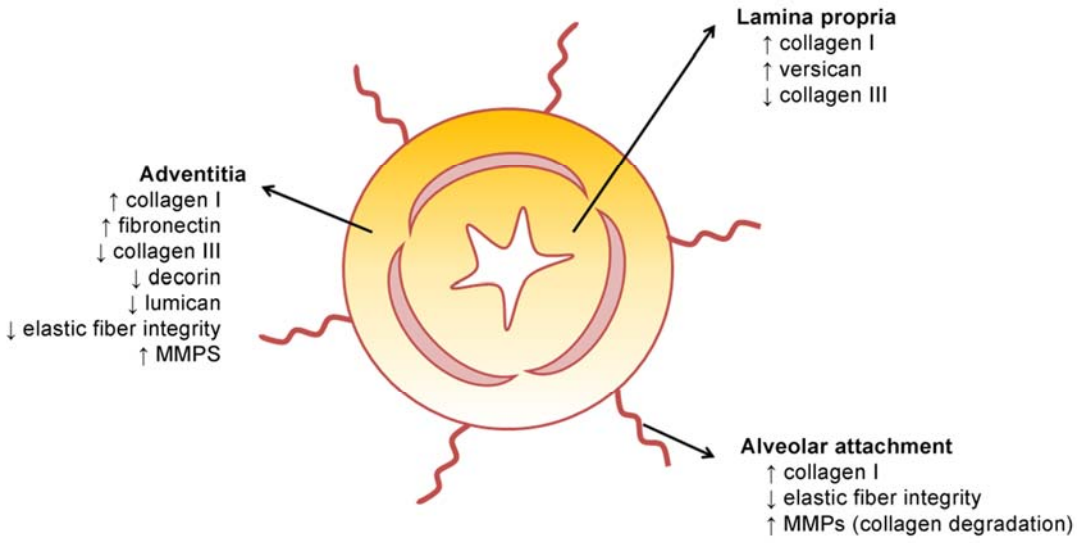

Figure 3. Summary of the alterations in ECM seen in asthma. ECM is altered at the level of the lamina propria, adventitia, and alveolar attachment in asthmatic patients. MMPs: metalloproteinases.

The earliest ECM change recognized in asthma was the increased basal membrane thickness. Specifically, it is not the real basal membrane that is increased but the underlying *lamina reticularis*, due to increased deposition of collagen III and tenascin in the presence of an increased number of subepithelial fibroblasts (Roche, Beasley et al. 1989, Laitinen, Altraja et al. 1997, Hoshino, Nakamura et al. 1998, Liesker, Ten Hacken et al. 2009). Whether reticular basement membrane thickness is related to asthma severity is not well established, as studies comparing mild and severe asthmatics have come to different conclusions (Chetta, Foresi et al. 1997, Benayoun, Druilhe et al. 2003, Bourdin, Neveu et al. 2007). Nevertheless, it has been reported to correlate with the degree of remodeling of smooth muscle and submucosal mucous gland in large cartilaginous airways, and with inner wall area and area of smooth muscle in small cartilaginous airways However, is was not related to airway wall remodeling

in membranous airways (James, Maxwell et al. 2002). Allergen challenge in patients with mild asthma induces activation of submucosal fibroblasts and increased tenascin deposition within the reticular basement membrane (Phipps, Benyahia et al. 2004). As tenascin is a glycoprotein markedly expressed during tissue repair processes (Laitinen, Altraja et al. 1997), it has been speculated that the increased reticular basement membrane thickness results from previous damage, possibly chronic.

Within the lamina propria of central airways, proteoglycan deposition is increased in asthmatics compared to control subjects (Huang, Olivenstein et al. 1999, de Kluijver, Schrumpf et al. 2005, Pini, Hamid et al. 2007). The lamina propria of asthmatic patients is characterized also by enhanced collagen I deposition (Minshall, Leung et al. 1997). Increased type I and type III collagen fibers have been detected in the large airways of patients with severe compared to milder form of the disease, but does not differ between mild asthmatics and healthy subjects (Chu, Halliday et al. 1998, Benayoun, Druilhe et al. 2003, Chakir, Shannon et al. 2003). Interestingly, although severe asthmatics having an increased deposition of both collagen and proteoglycans in the lamina reticularis, larger mucous glands (Benayoun, Druilhe et al. 2003), and increased vessel density compared to mild asthmatics, the thickness of their lamina propria is reduced compared to moderate asthmatics (Pepe, Foley et al. 2005). A similar counterintuitive result has been observed in patients with occupational asthma, whose bronchial lamina propria was thinner than those of healthy controls (Sumi, Foley et al. 2007). One possible explanation for this finding is the centripetal expansion of the markedly increased ASM layer observed in this group of asthmatics (Pepe, Foley et al. 2005). Alternatively (or as a consequence of this inward expansion of the smooth muscle layer), collagen fibers could be more densely packed in asthma (Roche, Beasley et al. 1989). Scarce information is available concerning morphologic changes at the adventitial level in large bronchi. However, an increased deposition of ECM (i.e. perichondral fibrosis) seems to occur in asthma (Carroll, Elliot et al. 1993, Haraguchi, Shimura et al. 1999), which is not explained by an increased deposition of collagen (Dolhnikoff, da Silva et al. 2009) and could possibly decrease airway-parenchymal interdependence and thereby allow excessive airway narrowing (Macklem 1996).

Small peripheral airways as well show extensive quantitative remodeling of their ECM, both at the level of the lamina propria and of the adventitia, and related to the degree of severity of the disease (Carroll, Elliot et al. 1993, Kuwano, Bosken et al. 1993). Specifically, collagen I is increased while collagen III was shown to be decreased at both levels, which is indicative of a profibrotic process (Dolhnikoff, da Silva et al. 2009). Collagen III is related to tissue distensibility while collagen I is the major matrix element that resists tensile stresses. The observed alterations argue in favour of the presence of stiffer and less distensible airways in asthma (Dolhnikoff, da Silva et al. 2009). Airway wall distensibility is indeed decreased in asthmatics, possibly in relation with bronchial ECM remodeling (Wilson, Li et al. 1993, Brown, Salome et al. 2007).

Conflicting data exist concerning alterations of elastin within the lamina propria of large and peripheral bronchi. In healthy subjects, elastic fibers are complexly intertwined with collagen and other ECM elements. They are initially organized in longitudinal bundles in the main bronchi and they gradually become oriented more circularly and form two layers: one beneath the basement membrane and perpendicular to it (superficial thin network) and one in the deeper submucosa, close to the smooth muscle (deep thick network) (Bousquet, Lacoste et al. 1996, Kamel, Beckert et al. 2009). Elastic fibers of asthmatic central bronchi were initially described as enlarged (or hypertrophic) and fragmented, but overall not increased in quantity compared to healthy subjects (Gabbrielli, Di Lollo et al. 1994, Godfrey, Lorimer et al. 1995, Bousquet, Lacoste et al. 1996). Also, the alterations observed in asthmatics were not linked to the severity or duration of asthma (Bousquet, Lacoste et al. 1996). Since then, few studies have addressed this question using quantitative approaches, showing a reduced or unchanged quantity of elastin in asthmatic airways most of the time (Reddel, Weiss et al. 2012).

Evidence of the increased and dysregulated deposition of ECM proteins observed *in vivo* is supported by *in vitro* studies. Proteoglycan synthesis is increased in asthmatic fibroblasts cultured and subjected to mechanical strain (Ludwig, Ftouhi-Paquin et al. 2004). Furthermore, their reduced proliferation rate linked to such increase in extracellular protein synthesis has

raised the hypothesis of different phenotypes of fibroblasts in the airways of asthmatics and controls (Nihlberg, Andersson-Sjoland et al. 2010).

Airway smooth muscle

The ASM mass can increase at every level of the bronchial tree in asthma (Huber and Koessler 1922, Ebina, Yaegashi et al. 1990, Carroll, Elliot et al. 1993, Girodet, Allard et al. 2015), and this is accompanied by an increased number and size of these cells, respectively referred to as myocyte hyperplasia and hypertrophy (Ebina, Takahashi et al. 1993, James, Elliot et al. 2012). An increase in myocyte mitochondria has also been reported in asthma (Girodet, Allard et al. 2015). Recently, the potential contribution of ECM deposition within the smooth muscle layer and of cellular (fibroblast/myofibroblast) migration have also been recognized (Pini, Hamid et al. 2007, Bentley and Hershenson 2008, James, Elliot et al. 2012, Yick, Ferreira et al. 2012). Although the crisscross arrangement of smooth muscle bundles within the bronchial wall could help prevent buckling in the airway during muscle shortening (Lei, Ghezzo et al. 1997), an increased smooth muscle mass facilitates airway closure during bronchospasm (Lambert, Wiggs et al. 1993, Oliver, Fabry et al. 2007) and it is indeed associated with the severity of the disease (Chetta, Foresi et al. 1997, James, Bai et al. 2009, Macedo, Hew et al. 2009). As previously discussed, not only the abundance of ASM but also its orientation and possibly the shape of its cells can influence the degree of airway obstruction in disease (Bates and Martin 1990, Lambert, Wiggs et al. 1993, Alford, Nesmith et al. 2011, Ye, Aratyn-Schaus et al. 2014).

The relative contribution of hyperplasia versus hypertrophy to the increase in smooth muscle mass is not well established as different studies have produced contrasting results (Woodruff, Dolganov et al. 2004, James, Elliot et al. 2012). Whether their respective contribution changes in relation to the severity of the disease is also ambiguous. It is worth mentioning that real hyperplasia (defined as an increased number of cells per basal membrane length or per airway tract) is difficult to assess in central airways *in vivo*. This is because the relationship between

basal membrane and smooth muscle may be strongly and unpredictably influenced by sampling in endobronchial biopsy samples. For this reason, myocyte hyperplasia is often indirectly assessed by measuring ASM cell proliferation rate or the balance between proliferation and apoptosis. On the other hand, hypertrophy is hard to assess directly as cell borders are not readily identifiable on histological preparations. For this reason, cell size is also often calculated indirectly by dividing the smooth muscle area or volume by the number of myocyte nuclei counted within that space. ASM hypertrophy in proximal airways was identified as a selective determinant of severe persistent asthma in one study (Benayoun, Druilhe et al. 2003). A possible pitfall of this approach is related to the presence of ECM elements within the muscle bundles (**Figure 4**) which can be altered in asthma (Pini, Hamid et al. 2007, James, Elliot et al. 2012) and, if not properly identified and excluded from the muscle volume, could bias the results.

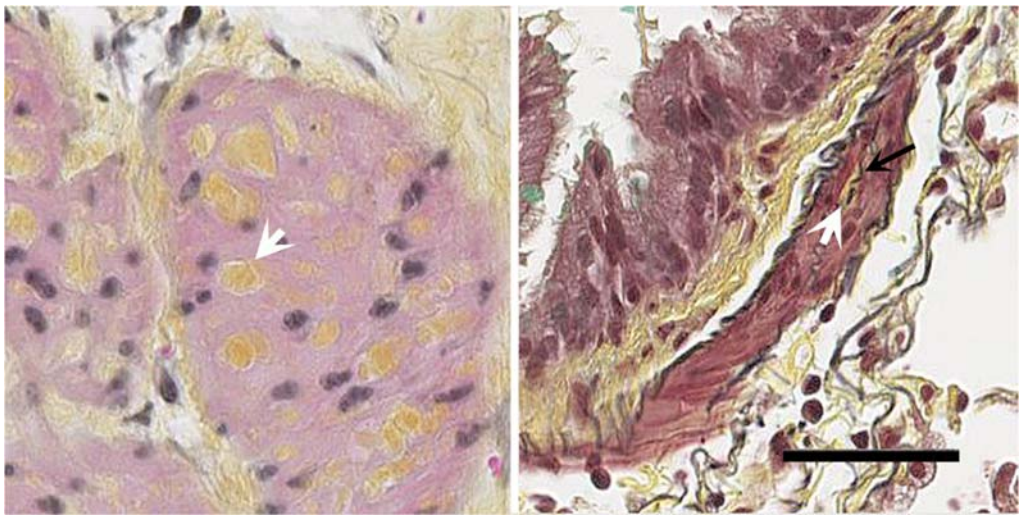

Figure 4. ECM fraction in the ASM layer. The smooth muscle layer of central (left panel) and peripheral airways (right panel) contains ECM proteins. Deposits of collagen (yellow, white arrows) and elastin (black, black arrows) can readily be visualized among smooth muscle fibers (pink). Staining: hematoxylin-eosin-phloxine-saffron (HEPS) in the left panel,

and Russell-Movat's pentachrome in the right panel. Scale is the same for both images (scale bar = 50 μm).

That said, true myocyte hyperplasia in large airways has been consistently identified in fatal asthma in studies in which stereology-based approaches were employed (Ebina, Takahashi et al. 1993, James, Elliot et al. 2012). Also, one study investigating myocyte hyperplasia in endobronchial biopsy samples of mild asthmatics has reported an increased number of myocytes per surface of basal membrane (Woodruff, Dolganov et al. 2004). Increased myocyte proliferation has been reported in large ASM of moderate-to-severe asthmatics as an indirect index of hyperplasia (Hassan, Jo et al. 2010, Ramos-Barbon, Fraga-Iriso et al. 2010). The increased proliferation rate of ASM cells has been associated with the switching of these cells from a physiological contractile to a "synthetic/proliferative" phenotype (Hirota, Nguyen et al. 2009). Further i*n vitro* studies have shown in more detail that the synthesis of structural or inflammatory molecules and proliferation can be differentially regulated by distinct stimuli (Ward, Harris et al. 2008, Alexander, Murgai et al. 2012). This finding, joined with the existence of a "hypercontractile phenotype" mediated by the expression of the fast-contracting isoform of the myosin (Leguillette, Laviolette et al. 2009), implies that up to four myocyte phenotypes with virtually different physiological roles may coexist in the airways of asthmatic patients (**Table 2**). That said, it is important to stress that when we talk about ASM cells with different phenotypes, for example with a contractile vs. a proliferative phenotype, we refer to ASM cells that are likely to maintain both contractile and proliferative ability, but in which one function slightly or markedly prevails over the other. For this reason, even if we simplistically refer to one or another cell phenotype, it is more likely that an imponderable number of intermediate phenotypes of ASM cells exist simultaneously.

An increased deposition of proteoglycans (versican and hyaluronan) between and around the smooth muscle bundles was observed by Roberts in 1995, who described their distribution within the airways as «in bordering areas that appear to be "space" following routine formalin fixation and paraffin embedding», concluding that «These "spaces" are hydrated

proteoglycan-rich domains in life» (Roberts 1995). The ECM surrounding the smooth muscle layer is overall decreased in the large airways of fatal and severe asthmatics compared to the less severe forms of the disease (Pini, Hamid et al. 2007, James, Elliot et al. 2012). However, no differences were observed when the deposition of specific components of the ECM was assessed separately (Yick, Ferreira et al. 2012).

Table 2. Myocyte phenotypes.

ASM cell phenotype	Description
Contractile	In normal conditions, most myocytes are contractile cells. They express α-SMA, myosin, and desmin.
Hyperproliferative*	Myocytes with a decreased contractile function (reduced expression of α-SMA, myosin, and desmin) undergoing exaggerated proliferation (increased expression of proliferation markers such as Ki67 or PCNA).
Synthetic*	Myocytes with a decreased contractile function synthetizing increased quantities of immunoregulatory or structural proteins (cytokines, chemokines, collagen).
Hypercontractile	Myocytes characterized by an increased expression of the (+)insert isoform of the SMMHC, which promotes a faster contraction.

α-SMA: alpha-smooth muscle actin, PCNA: proliferating cell nuclear antigen, SMMHC: smooth muscle myosin heavy chain. *: possibly different features of a single phenotype.

Cell migration has not been definitely demonstrated in asthmatics. However, the finding of increased myofibroblasts in the submucosa of asthmatics (Brewster, Howarth et al. 1990, Gabbrielli, Di Lollo et al. 1994), which is even more marked after allergen exposure (Gizycki,

Adelroth et al. 1997, Kelly, Chakir et al. 2006, Kelly, O'Connor et al. 2010), supports this theory. **Figure 5** summarizes the alterations sustained by ASM cells in asthma.

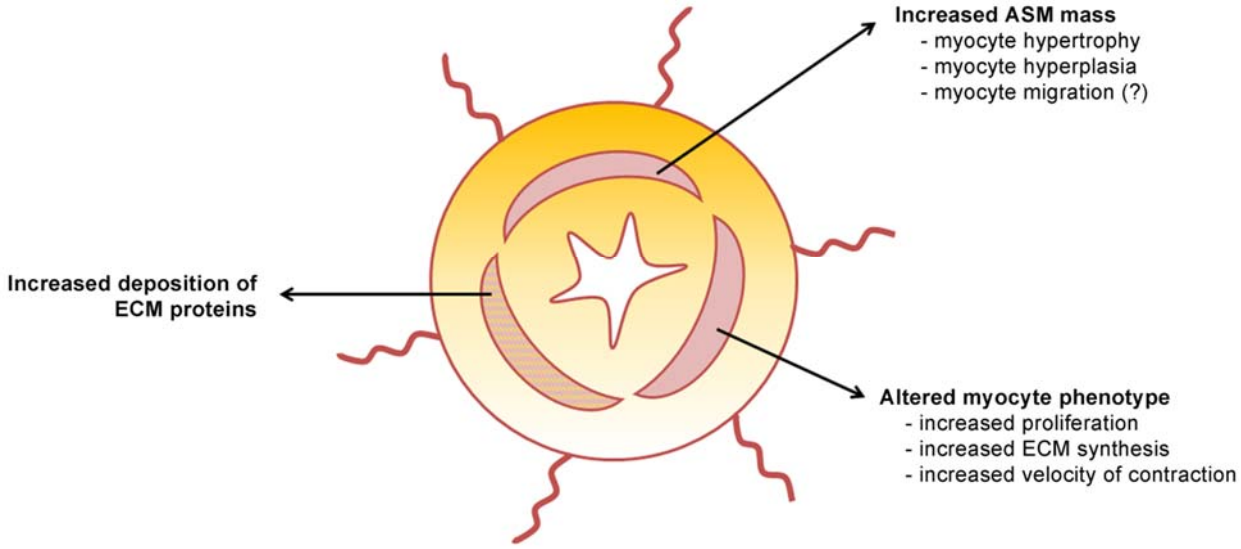

Figure 5. Summary of the alterations in ASM seen in asthma.

Angiogenesis

Compelling evidence suggests that angiogenesis occurs in asthma (Dunnill 1960, Carroll, Cooke et al. 1997, Li and Wilson 1997, Tanaka, Yamada et al. 2003), most likely as a consequence of chronic inflammation and tissue damage (Rydell-Tormanen, Johnson et al. 2008, Rydell-Tormanen, Uller et al. 2009, Yahaya, Baker et al. 2011, Van der Velden, Barker et al. 2012). Angiogenesis is the growth of new blood vessels from existing ones, whereas microvascular remodeling involves the presence of structural alterations (usually enlargement) without the formation of new vessels. Vascularity is increased in bronchial

biopsy samples from asthmatics compared to healthy subjects, both in terms of number and dimensions of the vessels (Li and Wilson 1997, Hoshino, Nakamura et al. 2001), which causes an increased submucosal blood flow in the bronchi (Lazaar and Panettieri 2003). An increased vascularity of the submucosal plexus has been reported in medium and small size airways of asthmatics, which correlated with the degree of airway obstruction (Hashimoto, Tanaka et al. 2005). Furthermore, the presence of immature newly formed vessels contributes to the increased vascular permeability, increased vascular leakage and edema commonly reported in asthma (Harkness, Kanabar et al. 2014). The percentage of the lamina propria occupied by vessels is similar in mild asthmatics and healthy subjects (Orsida, Ward et al. 2001, Hashimoto, Tanaka et al. 2005). It is worth mentioning that vessels are mainly located in close proximity (<150-200 μm) to the epithelium, and a thicker lamina propria as that occasionally observed in asthmatics could "dilute" the vascular density in this group, minimizing the difference existing between controls and asthmatics (Orsida, Ward et al. 2001).

The role of lymphangiogenesis in asthma has also been gaining attention in the last years (Baluk, Tammela et al. 2005, Zgraggen, Ochsenbein et al. 2013). As angiogenesis is present during chronic inflammation, hyperpermeable and activated new vessels lead to continuous extravasation of inflammatory cells and fluid into the inflamed tissue, which accumulate when the draining capacity of local lymphatics is overwhelmed. The lack of studies on lymphatic remodeling in the lung compared to other organs is due to the fact that in this organ the commonly used lymphatic marker LYVE-1 (lymphatic vessel endothelial hyaluronan receptor-1) stains both lymphatic and blood vascular endothelial cells, preventing an adequate differentiation of these two structures. However, recent study using the specific lymphatic marker podoplanin has revealed that fatal asthmatics have decreased lymphatics in their airways compared to aged-matched controls, suggesting an impaired drainage of airway edema as a potential mechanism contributing to the severe airway obstruction observed in these patients (Ebina 2008). The ability of Th-2 cytokines to inhibit lymphangiogenesis *in vitro* and *in vivo* in animal models further strengthens this finding (Savetsky, Ghanta et al. 2015, Shin, Kataru et al. 2015).

Cartilage

Alterations in bronchial cartilage have been sporadically reported, consistent with cartilage proteoglycan degradation in distal airways (Roberts 1995) or increased cartilage area in medium size bronchi of severe asthmatics compared to mild patients and controls (Carroll, Elliot et al. 1993). In endobronchial biopsies, the volume fraction occupied by the cartilage is similar between asthmatics and healthy patients, and seems to be strictly dependent on the sampling site (Labonte, Laviolette et al. 2008). Evidence of cartilage degeneration consistent with loss of cellular or pericellular metachromasia (territorial matrix) and pyknotic or absent chondrocytes has also been observed in asthmatics, correlating with the number of neutrophils infiltrating the airway wall (Haraguchi, Shimura et al. 1999).

Lung parenchyma

Remodeling of the lung parenchyma in asthma has been often disregarded in the past but very recent studies have showed that significant changes are already present even in patients with mild asthma (Nihlberg, Andersson-Sjoland et al. 2010). Specifically, increased collagen deposition and decreased decorin and biglycan have been reported in asthmatics with well-controlled disease, while an increase in decorin has been reported in uncontrolled asthma (Nihlberg, Andersson-Sjoland et al. 2010, Weitoft, Andersson et al. 2014). Lung granulomata have also been repeatedly found in severe asthmatics (Wenzel, Vitari et al. 2012).

Airway inflammation

The concept of asthma as a chronic inflammatory condition is relatively recent, and an exaggerated inflammatory response to normally non-harmful stimuli has been so far recognized as the underlying force preceding (and possibly producing) asthma exacerbations (Maneechotesuwan, Essilfie-Quaye et al. 2007). The role of inflammation is asthma is

multiple. A large amount of inflammatory mediators is liberated within the bronchial wall and lumen during or before asthma exacerbations, which may act at three levels:

- they may precipitate bronchospasm by directly inducing ASM cell contraction (i.e.: cysteinyl leukotrienes, histamine, thromboxane)
- they may foster remodeling by causing structural damages or alteration to the bronchial structures (i.e.: metalloproteinases, elastases, TGF-β-induced fibrosis, IL-13-induced goblet cell metaplasia (Boucherat, Boczkowski et al. 2013))
- they may act as a positive stimulus for the recruitment of other inflammatory cells (i.e.: IL-8, TNF-α).

Although inflammation is commonly considered the *primum movens* (first cause) of asthma, there are exceptions to this thought that deserve to be mentioned. As stated by Holgate: «while asthma is an inflammatory disorder of the conducting airways, inflammation itself does not explain the origin(s) of this disease nor why the airways are so susceptible to a range of different environmental factors» (Holgate 2011). Asthma was unanimously considered an eosinophilic, Th-2 biased inflammatory disease. It is now recognized that only half of asthmatics display a Th2-biased inflammatory response (Fahy 2015) and that a subset of patients exists (phenotype) in which neutrophils rather than eosinophils represent the most abundant inflammatory cell type in bronchial secretions. Neutrophilic asthmatics are commonly, but not always, affected by more severe forms of asthma and less responsive to corticosteroid treatment compared to eosinophilic asthmatics (Macedo, Hew et al. 2009, Moore, Hastie et al. 2013, Chung, Wenzel et al. 2014). This phenotypic heterogeneity, joined with the relative ineffectiveness of anti-Th2 monotherapies for asthma treatment (Fahy 2015), has brought awareness that the Th2-biased immune process alone is inadequate to explain the pathogenesis and persistence of asthma in adults, a concept also known as the «Th-2 paradigm insufficiency» (Chanez, Wenzel et al. 2007). The mechanisms behind these diverse inflammatory profiles are likely complex and currently incompletely understood. While a Th2-immune response is involved in the eosinophilic severe asthma phenotype, the immune mechanisms underlying the neutrophilic phenotype are not well described. Th1 and Th17-

biased inflammation could be involved, as well as alterations of the innate immunity. Neutrophilic moderate-to-severe asthma is characterized by increased expression of IL-8 and IL-17 compared to mild asthma and healthy subjects (Park, Jung et al. 1998, Ordonez, Shaughnessy et al. 2000, Chakir, Shannon et al. 2003). The persistence of inflammation in neutrophilic asthma in spite of treatment may also result from deficiencies in endogenous homeostatic processes that promote resolution of inflammation (Chung, Wenzel et al. 2014). In this regard, a recently developed mathematical model has shown how the inflammation resolution speed after a severe exacerbation can significantly affect airway remodeling (Chernyavsky, Croisier et al. 2014).

Asthmatics experiencing a rapid decline in lung function and ultimately a loss of disease control are those who have experienced the most severe exacerbations (Matsunaga, Hirano et al. 2015). This finding supports the hypothesis that asthma exacerbations are associated with the progression of airway functional changes that lead to persistent airflow limitation, although the causality between the occurrence of asthma exacerbations and loss of lung function remains to be demonstrated.

Importantly, while emphasis has been placed on assessing inflammation by analysis of bronchoalveolar lavage fluid (BALF) or sputum samples, its relationship to cellular profiles within airway or lung tissues is poor (Lemiere, Ernst et al. 2006, Macedo, Hew et al. 2009). Possibly, it is because many structural cells may operate incognito, playing important pro-inflammatory roles that remain undetected in morphological studies of inflammatory infiltrate. In fact, while a multitude of *in vitro* studies have investigated the inflammatory potential of fibroblasts, myofibroblasts, and ASM cells (Damera, Tliba et al. 2009, Alkhouri, Poppinga et al. 2014), their role *in vivo* is still commonly overlooked. Central and peripheral airways, as well as the lung parenchyma of asthmatics have increased inflammatory infiltrate compared to control subjects (Laitinen, Laitinen et al. 1993, Kraft, Djukanovic et al. 1996, Laitinen, Laitinen et al. 1996, Hamid, Song et al. 1997). Tissue inflammation appears to be similar in large airway biopsy samples obtained from asthmatics experiencing disease of different severity and with significantly different values of lung function and methacholine

responsiveness (Lemiere, Ernst et al. 2006, Macedo, Hew et al. 2009), which would argue against a direct effect of airway tissue inflammation in AHR and remodeling. However, severe asthma is characterized by an increased number of total inflammatory cells within the small airways compared to other pulmonary compartments (Balzar, Wenzel et al. 2002), which is limited to the eosinophils in mild-to-moderate asthmatics (Hamid, Song et al. 1997). These data strongly support a central role for small airways in asthma pathophysiology. Given the absence of differences in the distribution of inflammatory cell types infiltrating large and small airways, the assumption was made that large airway inflammation is qualitatively representative of small airway inflammation (Carroll, Lehmann et al. 1996). Significant correlations have not been reported however, and the growing interest in small airway structure and function (Postma, Brightling et al. 2015) hints at the inadequacy of large airway findings alone to explain the clinical aspects of the disease.

Functional consequences

It is hard to define what the causes are in asthma pathophysiology. The disease is characterized by severe airflow obstruction caused by bronchoconstriction and excessive mucus secretion within the airway lumen. Airway caliber results from the balance of opposite forces acting on the bronchial wall, which are severely altered in asthma due to hyperresponsiveness, remodeling, and inflammation. Airway hyperresponsiveness can induce remodeling and an inflammatory response by mean of the stress exerted on structural cells by exaggerated strain (Manuyakorn 2014, Gosens and Grainge 2015, Park, Fredberg et al. 2015). Airway inflammation itself may accentuate bronchial responsiveness as several bronchoconstrictive mediators are secreted by inflammatory cells (Bjornsdottir and Cypcar 1999) that can prevent complete airway expansion fostering excessive airway narrowing (Bosse, Chapman et al. 2011). Several products of inflammation can influence tissue remodeling as well (Fahy, Corry et al. 2000, Ramos-Barbon, Presley et al. 2005). Meanwhile, remodeling can profoundly impact AHR by altering force generation and distribution among

the different compartments of the bronchial walls and lung parenchyma (Bosse, Riesenfeld et al. 2010, Bosse, Solomon et al. 2010) through several mechanisms:

- an increased ASM mass produces greater forces upon constriction, which overwhelm the mechanisms that maintain airway patency;
- abnormal quantities or composition of the ECM may affect airway mechanics in asthma, both through geometric effects and through direct changes in tissue biomechanics (i.e. versican may increase lung stiffness by inhibiting elastin-binding proteins and interfering with the assembly of elastic fibers (Merrilees, Ching et al. 2008); on the other hand, increased ECM degradation can accentuate/facilitate ASM contractility (Roberts 1995));
- the loss of integrity of elastic fibers could prevent uniform intrapulmonary tension distribution, accentuating airflow heterogeneity and hyperresponsiveness (Bousquet, Lacoste et al. 1996).

The role of small vs large airways

The whole bronchial tree displays pathological alterations in asthma. There is no doubt, however, that large and small airways differ in many physiological aspects (**Table 3**), which would explain their variable contribution to several pathological traits. This dichotomy is maintained in asthma, but cellular function was found to be altered in disease, even in mildly affected subjects with normal lung function (Nihlberg, Andersson-Sjoland et al. 2010).

Whether central or peripheral bronchospasm is the major determinant of obstruction during acute exacerbations remains a matter of debate (Thompson, Douglass et al. 2013). During periods of disease control, central airway remodeling significantly correlates with the degree of airflow obstruction measured by spirometry (Kaminska, Foley et al. 2009). On the other hand, the more severe forms of the disease – that is, patients needing higher doses of treatment to be controlled, or still incompletely controlled despite high dose treatments – are

characterized by a greater degree of remodeling and inflammation in the distal bronchi and lung parenchyma (Carroll, Elliot et al. 1993, Weitoft, Andersson et al. 2014). These observations suggest that large airway pathology better reflects disease clinical expression, while small airway pathology long-term disease control.

Table 3. Differences between small and large airways.

Small (peripheral) airways	Large (central) airways
Absence of cartilage	Presence of cartilage
Absence of goblet cells and mucus glands	Presence of goblet cells and mucus glands
Non-ciliated epithelium	Ciliated epithelium
Low gas velocity and true laminar flow	High gas velocity and mixed flow (turbulent close to the airway wall and laminar at the center of the airway lumen)
Surfactant within the epithelial lining fluid	-

Highlights and unanswered questions

Airway inflammation, remodeling, and hyperresponsiveness are intimately interconnected and the precise pathophysiology of these contributors to asthma is incompletely described. The implication of small airways in asthma, especially in its severe form, is increasingly recognized, but tools for studying the progression of the disease at this level are not readily available. On the other hand, central airways can be easily accessed and sampled, but whether changes observed at the central level are representative of peripheral airway pathology remains to be established.

Means of assessment of airway remodeling in asthma

In vivo studies of airway structure and function in asthma have been hampered somewhat by the limited availability of tissue specimens (Lazaar and Panettieri 2003). Provided that histology still remains the gold standard for the evaluation of tissue remodeling and inflammation, biopsy samples are very small and they supply limited (if any) information on the functional and mechanical properties of the airways. In order to gather this kind of information, large tissue samples are required, which cannot be obtained *in vivo* in man. For this reason, a series of indirect tools have been employed in the last years to evaluate bronchial function, remodeling, and inflammation in a non-invasive way. They are briefly introduced hereafter, with a special emphasis on those employed for the assessment of ASM.

Histology

Histology is considered the gold standard technique for detection and quantification of airway remodeling and inflammation along the bronchial tree (Jeffery, Holgate et al. 2003). Representative tissue samples can be obtained *in vivo* by means of biopsy techniques. Endobronchial biopsy yields central airway samples obtained with forceps during bronchoscopy (Jeffery, Holgate et al. 2003). In regard of sample size and integrity, best results are reported to be obtained when larger instruments are used. Accumulated evidence suggests that reusable forceps should not be used for more than 5 bronchoscopies in human medicine (Jeffery, Holgate et al. 2003). It is a safe technique, which provides satisfying samples in 60% of cases on average (Van Vyve, Chanez et al. 1992, Labonte, Laviolette et al. 2008). Samples obtained by transbronchial biopsies or lung resections (performed for other reasons) have been used to study small airways *in vivo*. These techniques are characterized by a low safety profile compared to endobronchial biopsies, with complications such as

pneumothorax occasionally reported. Satisfactory samples are obtained in 28% of cases (Balzar, Wenzel et al. 2002). All biopsy-based techniques however are characterized by two major disadvantages which limit their usefulness in asthma: first, they yield non-repeatable samples, which partly precludes the possibility of longitudinal studies to be performed on the same bronchial region, and second, they provide only partial samples of the airway wall. Airway smooth muscle mass normalization by the length of the basal membrane or of the internal perimeter is commonly performed in histomorphometry (James, Hogg et al. 1988, McParland, Pare et al. 2004, James, Green et al. 2008, Elliot, Budgeon et al. 2015), which cannot be done when endobronchial biopsy samples are used. Also, only the carinae are sampled and the full thickness of the central bronchial wall is rarely sampled using endobronchial biopsy forceps, which prevents random sampling and further questions their reliability for the quantitative assessment of the most abaxial structures of the airways, such as the ASM (Woodruff and Innes 2006).

For research purposes, tissues should be obtained based on stereology-based sampling, that is: randomly and systematically. This method minimizes variability associated to subject, sample site, and sample sections. However, *in vivo* sampling is inevitably biased. Also, insufficient, imprecise, or inadequate tissue sampling does not guarantee an adequate study power and, even worse, could introduce a bias in the results. Considering intra- and inter-subject variation, it has been calculated that >15 cases are needed to detect a 100% difference in ASM remodeling in large cartilaginous airways between asthmatics and controls using autopsy lungs (James and Carroll 2000). A similar number of subjects allow the demonstration of differences of inflammatory cells in endobronchial biopsy samples (Sont, Willems et al. 1997). No such data are available for small membranous airways. To obtain random endobronchial biopsy samples, it has been recommended that a list of 10 to 15 potential biopsy sites located in the second, third and fourth airway generations of the right and left lung be generated before starting the procedure. Three to eight sites from this list must then be randomly selected and biopsies performed. Ideally, both lungs would be sampled equally, but due to practical issues and potential complications, it is accepted to take all eight biopsies from the same lung. Variability between different histological sections of the same biopsy

specimen has been reported to be 5%, suggesting that the evaluation of a single histological section for each endobronchial biopsy provides reliable results. A valuable sample should ideally have a subepithelial area, excluding artifact, cartilage, or blood clots, of at least 0.3 to 0.5 mm^2. Samples of blood and mucus, scrapings of epithelium, and fragments of glands are unacceptable (Jeffery, Holgate et al. 2003, Woodruff and Innes 2006). Although not described, the same protocol could be used to determine sites of transbronchial biopsy sampling, being careful to reduce to a minimum the number of biopsies harvested due to the high complication rate. Two to four biopsies per subject are considered to provide reliable data (Balzar, Wenzel et al. 2002).

In order to guarantee optimal staining, the biopsy samples must be processed rapidly and using standardized protocols. A rigorous approach maximizes the possibilities to discriminate subtle yet clinically important differences between groups (Jeffery, Holgate et al. 2003). For example, different tissue fixation techniques may result in variable degrees of tissue shrinkage. Formalin fixation and paraffin embedding induce tissue shrinking (10% on average, but can be as much as 50% depending on the temperature and the organic solvents used during the procedure). As variation in shrinkage is probably random and occurs in all tissues, it is not likely that it represents a significant problem when comparing groups where similar processing protocols have been used. Standard staining techniques are generally used successfully for quantitative analysis of ASM mass or total collagen deposition (Araujo, Dolhnikoff et al. 2008, James, Elliot et al. 2012), whereas immunohistochemistry is required to differentiate ECM components (Bergeron, Hauber et al. 2005, Araujo, Dolhnikoff et al. 2008) or for assessing the expression of specific proteins involved in disease pathophysiology. Some components of the bronchial submucosa other than smooth muscle could be mistaken for smooth muscle cells using a histological stain such as hematoxylin and eosin, which differentiates poorly between muscle and non-muscle tissue elements. This may lead to an incorrect estimation of ASM mass, especially at lower magnifications, at which individual smooth muscle fibers are less clearly identified. Other staining techniques such as Masson (trichrome) or Movat (pentachrome) can adequately identify and differentiate muscle from connective tissue and are thus preferable (Jeffery, Holgate et al. 2003).

Quantitative evaluation of bronchial structures has often been performed bi-dimensionally. This implies measuring the number or the area occupied by the structure of interest within a transversely-cut airway. As mentioned before, in order to compare airways of different size and degree of contraction, a measuring unit independent of bronchial dimension and constriction was developed. It was validated initially as a normalizing method for the assessment of smooth muscle mass, and it consisted in dividing the area occupied by ASM around the airway by the length of the basal membrane or of the internal perimeter of the bronchus (James, Hogg et al. 1988, Herszberg, Ramos-Barbon et al. 2006). It has to be noted that methods of quantitation of ASM mass in endobronchial biopsy samples are still not validated or standardized. This means that measuring units of ASM mass vary among the different studies, which makes it virtually impossible to compare them and to draw general conclusions. The use of stereological approaches is now strongly suggested, as they provide unbiased estimation of tridimensional structure distribution (Muhlfeld, Knudsen et al. 2013, Schneider and Ochs 2013). An official ATS/ERS Research Policy Statement has recently been published together with an extensive review providing general recommendations on which stereological methods are to be used in quantitative microscopy of the lung in respiratory research (Hsia, Hyde et al. 2010, Muhlfeld and Ochs 2013, Ochs and Muhlfeld 2013).

Diagnostic imaging

Imaging techniques currently used to investigate airway wall thickness include high-resolution computed tomography (HRCT), endobronchial ultrasound (EBUS), and optical coherence tomography (OCT). Noble gases (mainly helium and xenon) magnetic resonance imaging (MRI), single photon emission computed tomography (SPECT), positron-emission tomography (PET) are other research tools that can provide indirect measurements of bronchial remodeling and activation based on the distribution of inhaled particles or airflow

heterogeneity assessment (Laurent and Tunon de Lara 2011). Unfortunately, none of them can differentiate and specifically allow the assessment of the smooth muscle layer.

High-resolution computed tomography has been used in several studies and different techniques have been developed for assessment of bronchial wall thickness both in small and in large airways. Despite several algorithms developed to optimize airway wall measurement, the technique is still imprecise, especially when used to evaluate small airways. Indeed, the resolution of common CT scanners does not allow precise measurements of small airways thickness (Coxson 2008, Walker, Gupta et al. 2012) and no consensus on the appropriate method for measuring airways has been reached among researchers (Coxson and Lam 2009). High-resolution computed tomography measurements overestimate airway wall area and underestimate airway lumen area (de Jong, Muller et al. 2005). Nevertheless, a correlation between airway wall measurements obtained using CT and those obtained using histology has been shown (Nakano, Wong et al. 2005).

Endobronchial ultrasound and OCT are techniques with comparable outcomes but based on different principles. Image acquisition is based on reflectance and return of ultrasound waves for EBUS and on infrared light for OCT (Kurimoto, Murayama et al. 1999, Coxson and Lam 2009). The advantages for these techniques are that no radiation is required thus they have a better safety profile than HRCT and they have a good resolution to allow accurate measurements of small airway walls. Although they both require bronchoscopy, OCT overcomes a problem strictly related to EBUS, such as the need of a coupling between the probe and the airway wall. It cannot however clearly distinguish between different airway wall layers as EBUS does.

Brief introduction to the endobronchial ultrasonography

History

Endoscopic ultrasound was first used in human gastroenterology to investigate intestinal wall involvement in neoplasms and infiltrative diseases (Kim and Telford 2009). The application of endoscopic ultrasound (EUS) to the respiratory tract was first described by Hürter and Hanrath in 1992 (Hurter and Hanrath 1992), and it was named EBUS. The first transducer consisted of a single-element, rotating transducer placed at the tip of a metal shaft. The rotation of the transducer allowed obtaining 360° images of the surrounding tissues, producing cross-sectional images of the bronchi. The ultrasound frequency of the first EBUS transducer was 20 MHz, the maximum field of view was reported to be 5 cm in diameter under optimal conditions, and the limit of the radial resolution in the order of 200 μm. Since 1999, EBUS is commercially available (Burgers, Herth et al. 2001) and its use in human respiratory medicine has progressively increased.

Technique and instrumentation

The instruments used to perform EBUS consist of a flexible videoendoscope with a working channel of 2.2-3.6 mm in diameter, a high frequency echo-transducer, and software for image analysis.

There are currently two main types of probes available which can be used to perform EBUS: radial probes and curvilinear probes (Medford 2010). Radial probes can be further divided into ultra-miniature probes and radial balloon probes (Haas, Vachani et al. 2010). Radial probes are high-frequency, circulating probes. The transducer rotates on its own axis at high speed and generates 360° (cross-sectional) images of the bronchial walls and their surrounding tissues (**Figure 6A**). Ultra-miniature probes differ from radial balloon probes because of the absence or presence, respectively, of an inflatable balloon on their surface. Because physical contact is required, ultraminiature probes are generally used while placed

inside a sheath provided with a water-inflatable balloon. The balloon can be water-inflated up to a diameter of 1.5-2 cm. A pressure-dependent safety mechanism might be present preventing an excessive inflation of the balloon (**Figure 7**).

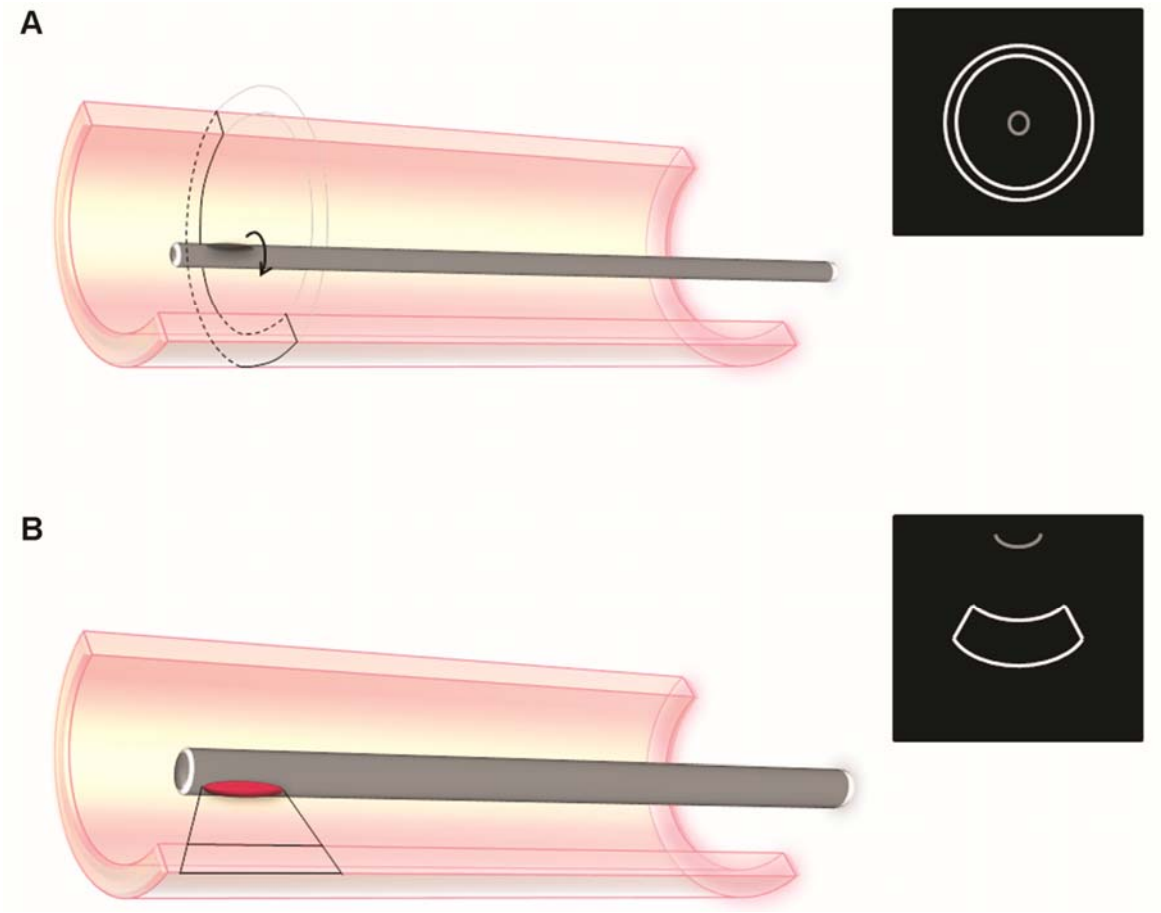

Figure 6. EBUS probes. A) Radial probes consist of a small disc-shaped ultrasound transducer oriented perpendicularly to the airway longitudinal axis and attached to a motor drive. Images are acquired during high-speed rotation of the transducer and depict the bronchial wall cross-sectionally (360°). B) Curvilinear probes have a fixed electronic transducer oriented so as to produce a sector-shaped (50° angle) image parallel to the long axis of the endoscope

Radial probes have been available at frequencies ranging from 12 to 30 MHz, with 20 MHz being the frequency more commonly used for clinical purposes (Sheski and Mathur 2008). The 30 MHz probe is no longer available on the market. A single probe, if carefully handled, is reported to approximately last 50 to 75 examinations (Falcone, Fois et al. 2003, Sheski and Mathur 2008).

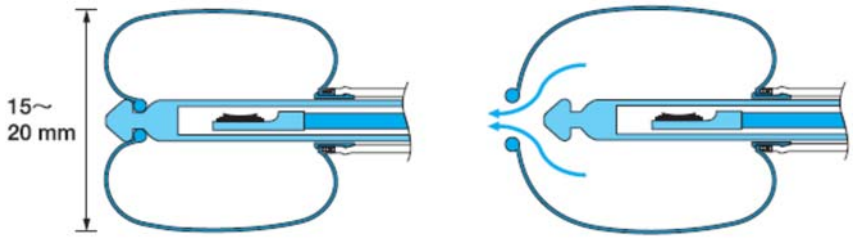

Figure 7. Pressure-dependent safety mechanism of the inflatable balloon. The distal extremity of the balloon is provided with an elastic ring which fits into a circular depression on the tip of the EBUS probe. Once the probe is in the airway, water is injected into the balloon to inflate it. If the pressure inside the balloon is too high, the elastic ring is released and water flows out. Image obtained from http://www.olympus-europa.com/medical/en/medical_systems/mediacentre/_media_detail_6476.jsp, accessed on 02/06/2016.

Few studies have compared the image quality of the bronchial wall obtained using EBUS probes of different frequencies (Dong, Takahashi et al. 2001, Kurimoto, Murayama et al. 2002, Nakamura, Endo et al. 2004). Data suggest that images obtained with 30 MHz probes are characterized by better quality and resolution but limited penetration compared to those obtained with 20 MHz probes. Average times reported to perform a radial EBUS examination vary between 8 and 20 minutes (Falcone, Fois et al. 2003). The learning curve associated to the EBUS technique is quite slow. The European Respiratory Society and the American Thoracic Society recommend 40 supervised procedures followed by 25 procedures per year

for radial EBUS to maintain skills, while the American College of Chest Physicians guidelines advise a minimum of 50 supervised procedures followed by 5 to 10 procedures per year (Bolliger, Mathur et al. 2002, Ernst, Silvestri et al. 2003). Linear EBUS is likely to require 40-50 supervised procedures as well in order to acquire the necessary skills (Medford 2010).

Sonographic airway anatomy

Bronchial and tracheal walls are imaged ultrasonographically as multilayered structures. Hypo- and hyperechoic layers alternate regularly, with the innermost layer being hyperechoic (**Figure 8**).

In the most recent human studies, ultrasonographic imaging of the cartilaginous airways detects a maximum of five layers (Soja, Grzanka et al. 2009, Kita, Fujimura et al. 2010). However, there are reports in which authors were able to detect only three layers (Hurter and Hanrath 1992), while others identified six (Baba, Sekine et al. 2002) or even seven distinct layers (Becker 1996, Herth, Ernst et al. 2003, Nakamura, Endo et al. 2004). Of note, studies in which seven layers were detected ultrasonographically lacked a validation step identifying the corresponding microscopic structure. The stratigraphy of the airway wall varies depending on the bronchial site imaged. Specifically, the presence of a cartilage plate in the bronchial wall is associated with an increased number of echoic layers. This also means that it is virtually impossible to have a cross-sectional image of an airway in which the same layer disposition is observed all around, as the airways are not encircled by complete cartilaginous rings. Instead, is it expected that the number of layers and their thickness vary around the airway wall.

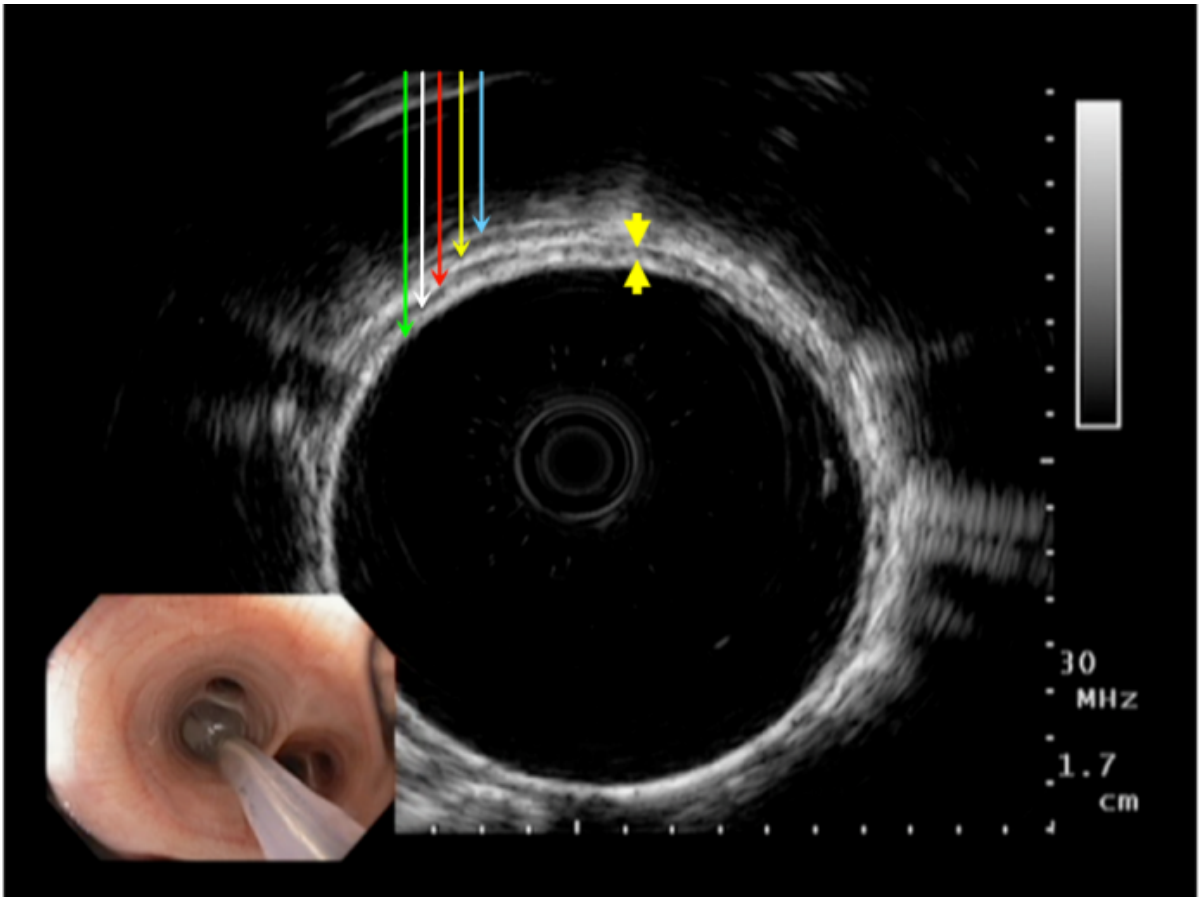

Figure 8. Multilayered structure of the bronchial wall at EBUS. The five colored arrows on the left indicate the five layers of the bronchial wall (green: first layer [L1], white: second layer [L2], red: third layer [L3], yellow: fourth layer [L4], and blue: fifth layer [L5]). The yellow thick arrows on the right indicate the thickness of L2. In the left bottom panel, the probe is in the bronchial lumen with the balloon inflated to allow coupling with the airway wall.

Marginal echoes are artifacts produced by ultrasound wave reverberation. They are identified as hyperechoic bands propagating from hyperechoic structures toward the travelling direction of the waves (that is, centrifugally). The presence of marginal echoes must be kept in mind when evaluating an endobronchial sonogram, as they actually "cover" a portion of the

underlying hypoechoic layer (**Figure 9**). They cannot be clearly identified and removed or subtracted. In fact, the first, third and fifth layer themselves are marginal echoes. Kurimoto and colleagues (Kurimoto, Murayama et al. 1999) investigated the localization and magnitude of marginal echoes in airway sonograms. They can be visualized as echoes propagating outwards and starting at the epithelial layer, the inner border of cartilaginous plates, and the adventitia. The marginal echo propagating from the epithelial layer has been recognized as the more prominent in human bronchi.

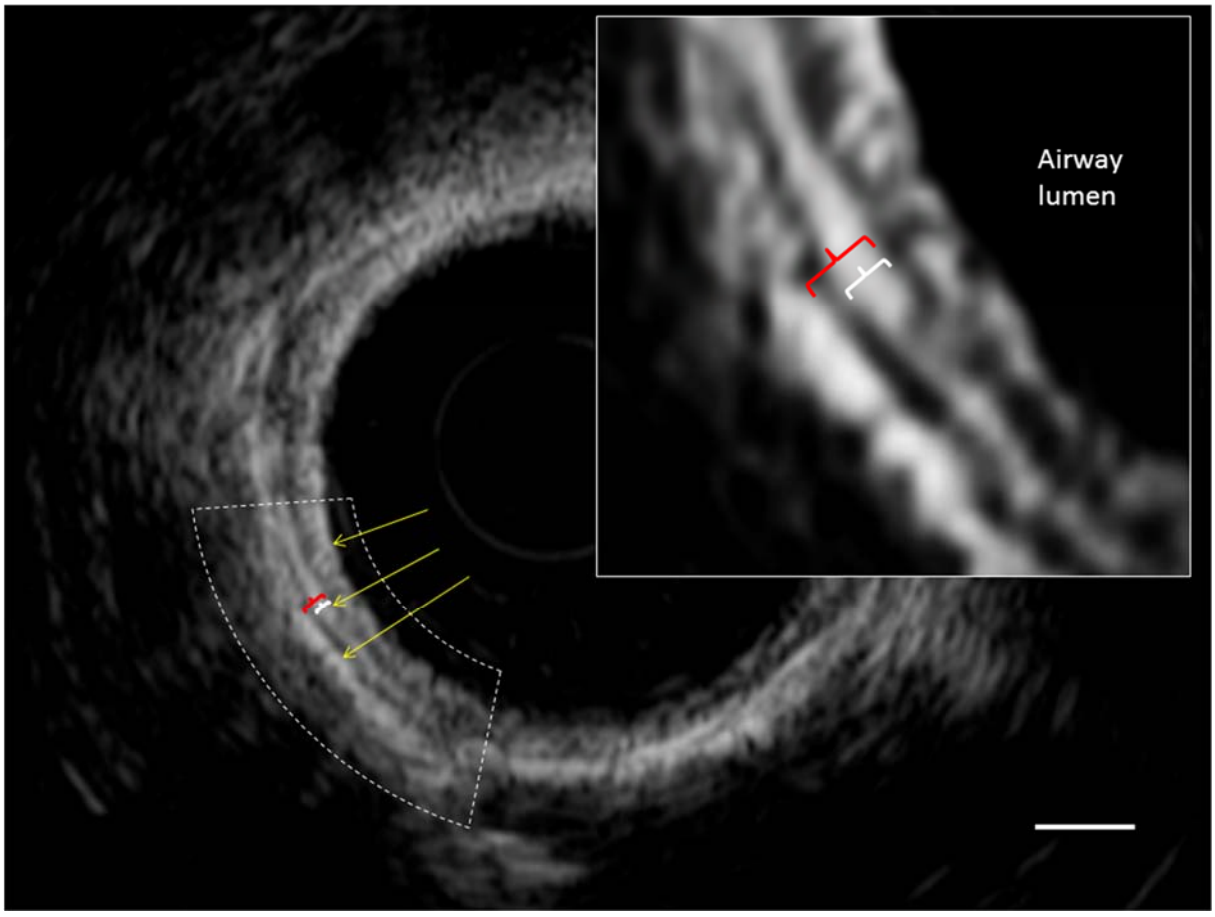

Figure 9. Marginal echoes in EBUS images. Yellow arrows indicate marginal echoes. They can be identified between an inner hyperechoic and an outer hypoechoic layer, and they play a role in the thickness of both layers: the hyperechoic inner layer will appear on the sonogram

thicker than it is in reality because its marginal echo will be included in the total thickness. As a consequence, the following external hypoechoic layer will be displayed in the sonogram as thinner than it is in reality. White brace: marginal echo of the inner border of the cartilage. Red brace: thickness of the bronchial cartilage. Scale bar: 1 mm.

Although discrepancies exist concerning the number of echographic layers detectable in cartilaginous portions of the airways, there is general agreement that in membranous portions of the airways three layers are identifiable. The only exception is the first report of EBUS by Hurter and Hanrath (1992), in which they described the membranous airway wall as a monolayered structure. Baba and colleagues (2002) have successively speculated that the low contrast and high gains used for EBUS in that initial report (justified by the aim to describe not only airway structures but also adjacent vessels and lymph nodes) could have been the reason why the bronchial wall appeared as monolayered.

Based on the most recent observations, the first layer at the luminal side is a hyperechoic layer, which has been described to represent the surface of the latex balloon (when present), the epithelium, and a variable portion of the submucosa. The second layer is hypoechoic and there is evidence that it represents the remaining part of the submucosa and the smooth muscle. Vessels, nerves and glands are also present within the second layer. The marginal echo of the first layer extends from the inner margin of the epithelium (or from the inner surface of the balloon) to a variable part of the inner submucosal tissue (Kurimoto, Murayama et al. 1999). The first two sonographic layers represent the same structures both in cartilaginous and membranous portions of the airways. The second layer may be occasionally compressed by and mixed with the balloon echo (Baba, Sekine et al. 2002) and thus differentiation between the first and the second layer may be challenging. On the contrary, boundary lines between the second and the third layer (cartilaginous portions) on the sonogram are generally well demarcated and represent a reliable image of the real tissue organization in that area (Miyazu, Miyazawa et al. 2002).

In membranous portions of the airways, the third layer is described as the last layer detectable by most of the authors, representing the adventitia. Using a 20 MHz probe, Baba (2002) reported a six-layered structure of the membranous portions of the extra-pulmonary airways where the third and fourth layers, hyper- and hypoechoic, respectively, represented the longitudinal muscle and the bronchial glands. The fifth hyperechoic layer was considered the sum of marginal echo and adventitia, and the sixth as the adventitia. These findings were confirmed microscopically.

In cartilaginous portions of the airways, the third, fourth and fifth layers identify the cartilaginous plates. The third layer is hyperechoic and represents the inner border of the bronchial cartilage (endochondrium). The fourth layer is hypoechoic and represents the body of the bronchial cartilage. The fifth layer is highly hyperechoic and it represents the outer border of the cartilage (pericondrium) and the adventitia. The marginal echo of the third layer starts at the luminal side of the bronchial cartilage and it might extend up to half of the cartilage body, whilst the marginal echo of the fifth layer starts at the outer margin of the bronchial cartilage and extends to the adventitia (Kurimoto, Murayama et al. 1999). Authors that recognized a sixth layer described it as hypoechoic (Becker 1996, Miyazu, Miyazawa et al. 2002) or slightly hyperechoic (Baba, Sekine et al. 2002) and suggested it may represent supporting connective tissue or the adventitia itself, respectively. The seventh hyperechoic layer was described as an image of adventitia, when identifiable (**Table 4**).

At present, all but one study reported have been performed by means of subjective interpretation of the ultrasonographic images. In these cases, the layers were detected by independent, experienced observers. Layer differentiation can also be accomplished by means of dedicated software, which allows more accurate and objective interpretation of the images. Using NIH Image (National Institutes of Health, Bethesda, MD) different tonalities of the grayscale characterizing each pixel of the sonogram representing the bronchial wall are analyzed. When only the pixels disposed along a radius of the circle formed by the bronchial wall are considered, a “W-shaped” curve is obtained as a result, where every change in direction of the curve represents the change from a layer to the subsequent one in the EBUS

image. A maximum of seven layers could be detected in the cartilaginous portions of intrapulmonary airways using 30 MHz radial microprobe-obtained sonograms and this method of analysis (Nakamura, Endo et al. 2004).

Anatomical orientation in the tracheobronchial tree is difficult, and a good knowledge of thoracic anatomy and physiology is necessary to perform the EBUS technique. Both respiration and cardiac pulsation may lead to artifacts in the images collected (Baba, Sekine et al. 2002, Falcone, Fois et al. 2003). Generally, esophagus, main arteries and veins are used as anatomic landmarks in human medicine. Contrasts have been used to better visualize pulmonary vessels during both trans-cutaneous (Gorg 2007) and bronchoscopic (Hurter and Hanrath 1992) ultrasound examination.

Table 4. US findings of the tracheobronchial wall.

Layer	Echodensity	US findings								
		Becker et al.	Herth et al.	Nakamura et al.	Kurimoto et al./Miyazu et al.		Baba et al.		Hurter et al.	
		Cartilaginous airways (trachea)		Cartilaginous airways	Cartilaginous airways	Membranous airways	Cartilaginous airways	Membranous airways	Cartilaginous airways	Non-cartilaginous portions of cartilaginous
1st	hyperechoic	bright echo enhanced by adjacent balloon	mucosa	balloon sheath and bronchial epithelium	marginal echo containing the epitelium and epithelium and submucosal tissue	marginal echo containing the epitelium and epithelium and submucosal tissue	interface echo containing epithelium and lamina propria	interface echo containing epithelium and lamina propria	endo-chondrium	UNILAMINAR STRUCTURE
2nd	hypoechoic	submucosa	submucosa	submucosal layer and bronchial smooth muscle layer	submucosal tissue	smooth muscle	submucosa with lamina propria, smooth muscle and extramuscular layers; layer occasionally compressed and mixed with the balloon echo	submucosa with lamina propria, smooth muscle and extramuscular layers	cartilage	
3rd	hyperechoic	strong echo of inner cartilage and endochondrium	endochondrium	marginal echo of the luminal aspect of the bronchial cartilaginous layer	marginal echo of the inner side of the bronchial cartilage	adventitia	interface echo, cartilage	longitudinal muscle	peri-chondrium	
4th	hypoechoic	connective tissue of inner cartilage	cartilage	bronchial cartilaginous layer	bronchial cartilage		cartilage	bronchial glands		

5th	hyperechoic	strong echo of outer cartilage, perichondrium	perichondral layer	marginal echo of the outer cartilaginous layer	marginal echo starting from the outer side of the bronchial cartilage and containing adventitia	interface echo, adventitia	interface echo, adventitia
6th	hypoechoic	supporting connective tissue	connective tissue	*	in some cases in tracheal wall	adventitia	adventitia
7th	hyperechoic	adventitia	adventitia	*	in some cases in tracheal wall		

*: not described in the article, but can be detected in the article's figures.

Current and future applications

Current indications for the use of EBUS in human respiratory medicine are staging and diagnosing lung cancer, evaluating mediastinal lymphadenopathy, as well as assessing airway wall infiltration and peripheral nodules involvement in neoplastic diseases (Burgers, Herth et al. 2001, Kurimoto, Murayama et al. 2002, Anantham, Koh et al. 2009). Most of the clinical experiences and published papers employ EBUS for ultrasound-guided transbronchial needle aspiration (US-TBNA) (Herth, Becker et al. 2004, De Leyn, Lardinois et al. 2007, Medford, Bennett et al. 2010), for which 7.5 MHz curvilinear probes are used. Endobronchial ultrasonography is employed also for locating mediastinal and bronchogenic cysts, for diagnosing relapsing polychondritis and sarcoidosis (Miyazu, Miyazawa et al. 2003, Garwood, Judson et al. 2007), as well as for detecting thrombi of the pulmonary artery and central pulmonary emboli (Kennedy, McWilliams et al. 2007, Aumiller, Herth et al. 2009).

Current research is focused on the assessment of pulmonary vascular disease (Medford 2010) and airway remodeling associated with asthma and chronic obstructive pulmonary disease (Yamasaki, Tomita et al. 2003, Shaw, Wakely et al. 2004, Soja, Grzanka et al. 2009, Soja, Loboda et al. 2015), which may represent further future clinical applications for EBUS. Differently from EUS (endoscopic ultrasound) probes, EBUS transducers do not allow visualization of contrast agents at present (Reddy, Ioncica et al. 2011, Saftoiu 2011). A model of 3D-EBUS has been developed as well, but its application *in vivo* has been difficult, leading to poor results (Andreassen, Ellingsen et al. 2005). Until now, the use of EBUS has not been described in veterinary medicine.

Advantages and limitations

As every other technique, EBUS offers several advantages but is not free of limitations. Main advantages are represented by:

- minimal invasiveness;
- repeatability over time;

- direct and real-time visualization of the airway wall at specific locations of interest;
- ability to assess small airways and peripheral areas of the lungs;
- high sensitivity as a prognostic tool in human respiratory medicine (mainly oncology), further increased when coupled with other complementary diagnostic techniques as HRCT (High Resolution Computed Tomography);
- low radiation exposure.

On the other hand, the following points have been considered as limitations of the EBUS technique:

- technically speaking, the resolution of 20 MHz probes is not sufficient to differentiate the several histological layers constituting the airway wall (Miyazu, Miyazawa et al. 2002, Wakamatsu, Tsushima et al. 2006), to visualize the basement membrane (Baba, Sekine et al. 2002), or to differentiate the smooth muscle from the lamina propria (Kurimoto, Murayama et al. 1999, Soja, Grzanka et al. 2009). However, 20 MHz probes were used in the majority of the studies performed and satisfactory results could generally be obtained in terms of submucosal thickness (second layer, L2).
- Specific regions of the bronchial tree cannot be reached with this technique, such as the upper lobes of the lungs. In general, the limiting factor is represented by the bending ability of the bronchoscope.
- Only a small area of the lung can be visualized at a time (Omori, Takiguchi et al. 2002).
- The latex balloon itself represents a source of different problems: first, when inflated, the balloon might alter the appearance of the inner bronchial layers, causing a misinterpretation of the sonograms. The balloon itself appears as a hyperechoic structure on ultrasound images and its thickness is generally added to the thickness of the first layer (L1) which represents the bronchial mucosa. Furthermore, by overdistending the airways, the balloon could alter bronchial morphology and prevent an adequate differentiation of echographic layers. EBUS measurements are indeed

characterized by different degrees of accuracy for different layers, with the outermost being the most accurate (Kurimoto, Murayama et al. 1999, Baba, Sekine et al. 2002). Secondly, the maximum diameter of the balloon is 2 cm, which is not enough to allow an optimal coupling between the bronchial walls and the probe in larger airways. However, in human medicine, this problem is encountered only when trying to image the tracheal wall. As inflating the balloon in the trachea also prevents normal breathing, the procedure must be completed in a short period of time (Herth, Ernst et al. 2003, Wakamatsu, Tsushima et al. 2006).

- Training and learning curve for this technique are time-consuming and a trained staff is required (Bolliger, Mathur et al. 2002, Miyazu, Miyazawa et al. 2002, Ernst, Silvestri et al. 2003, Sheski and Mathur 2008, Anantham, Koh et al. 2009, Haas, Vachani et al. 2010, Medford 2010).
- EBUS alone is not diagnostic for any lung disease (Kurimoto, Murayama et al. 2002).

Lung function

Lung function is often evaluated in human studies to indirectly assess pulmonary remodeling. Spirometry is the most used technique for lung function assessment in man; it is based on air volume and flow generated during maximal expiration (from total lung capacity to residual volume). The forced expiratory volume in 1 second (FEV_1) corresponds to the amount of air that can be expelled during the first second of maximal expiration. It is a commonly employed parameter for evaluating airway obstruction. A consistent correlation of FEV_1 with airway wall thickness was shown (Niimi, Matsumoto et al. 2000, Hoshino, Matsuoka et al. 2010). A correlation has been also demonstrated between residual volume and airway wall thickness or lumen area, suggesting that airway wall thickening and subsequent lumen obstruction result in air trapping in asthmatic subjects (Kosciuch, Krenke et al. 2009). Lung function also correlates with AHR and airway inflammation in some but not all studies (de Jong, Muller et al. 2005). These contrasting results may be partly due to the fact that pulmonary remodeling affects to a greater extent smaller airways, while spirometry mainly reflects large airways impaired function (Johnson and Hamid 2012, van den Berge, ten Hacken et al. 2013). Impulse

oscillometry has been suggested as a better tool to investigate small airway function (Pisi, Tzani et al. 2013). Lastly, the measure of airway distensibility (the change in anatomic dead space with lung volume) has been proposed as a physiological variable correlated to airway remodeling in asthmatic subjects (Ward, Johns et al. 2001).

Highlights and unanswered questions

Histology is the gold-standard technique to assess airway remodeling. Among the several structures involved in asthma pathophysiology, the ASM is of particular interest due to its crucial role in bronchoconstriction. Measuring the ASM mass in central and peripheral airway would provide data of paramount importance concerning disease pathophysiology or prognosis (James, Bai et al. 2009, Elliot, Jones et al. 2015, Girodet, Allard et al. 2015). However, tissue sample harvesting *in vivo*, especially from the peripheral lung regions, represents a real challenge in respiratory research and it is rarely performed. On the other hand, endobronchial biopsies produce partial and non-full-thickness samples of the bronchial wall, which lack a standardized and validated method for ASM quantification. Lung function and imaging techniques have been used to estimate peripheral remodeling, but they cannot specifically differentiate the several components of the bronchial wall. Endobronchial ultrasound may provide interesting information on central bronchial remodeling, but the studies performed until now on human patients have limits. Further work is required to establish the validity of this technique for the estimation of ASM remodeling.

Reversibility of asthma and available treatments

Several epidemiological studies have demonstrated that asthma remission may occur in affected children. Specifically, in about two thirds of children diagnosed with asthma, the disease "quietens down" by puberty, and only one third of these patients who were asthma-free at puberty have asthma symptoms in their mid-twenties (Brewczynski and Brodziak 2015). On the contrary, adult-onset asthma is unanimously considered a chronic disease, "incurable" in most cases, although clinical symptoms of airway obstruction can be well managed by means of appropriate pharmacological treatments in most cases (Chanez, Wenzel et al. 2007). Asthma remission rates of 0.6% to 2% per year have been reported (Ronmark, Jonsson et al. 1999, Holm, Omenaas et al. 2007), but residual airway obstruction and hyperresponsiveness are maintained even after years of clinical remission (Boulet, Turcotte et al. 1994), probably mirroring the incomplete reversibility of airway remodeling and inflammation (Broekema, Volbeda et al. 2010, Broekema, Timens et al. 2011).

Current GINA (Global Initiative for Asthma) guidelines state that asthma treatments should be administered based on the clinical needs of the patients (GINA, 2015). Short-term asthma control has two recognized goals: to control the clinical symptoms and to decrease the risk factors for adverse outcomes; both of them aiming at reducing the exacerbation rate in the long-term. Of note, the assessed outcomes in the short-term are limited to patient perception of the disease and supported by lung function data, with lung inflammation and remodeling being rarely evaluated, if at all. At present, indeed, neither sputum nor FeNO-guided treatment is recommended for the general asthma population (Chung, Wenzel et al. 2014, 2015).

Asthma treatments are thus effective in treating acute airway narrowing and possibly reducing inflammation. However, whether they can reverse bronchial remodeling or not – or to which extent – is often disregarded or considered a secondary outcome. Structural changes at the bronchial level are likely to be early events in the pathogenesis of asthma, as supported by the increased ASM mass observed in newly diagnosed asthmatic children (Regamey, Ochs et al. 2008). It has also been postulated that ASM remodeling might precede asthma presentation.

This concept is known as “premodeling” (Holgate, Holloway et al. 2004), and has been strengthened by the finding that preschool wheezers who developed asthma later in life had increased ASM mass compared to those who did not (O'Reilly, Ullmann et al. 2013). The early nature of bronchial remodeling in asthma suggests that most structural changes are already well-established when the treatment is started, which might prevent a thorough reversal. The ability of currently employed asthma treatments to reverse airway remodeling is largely unknown. Identifying therapeutic strategies able to maximize remodeling reversal could bring important prognostic advantages. For this reason, it is expected that modulation of airway remodeling will become an important endpoint for long-term asthma control in the near future (Sandstrom 2010).

Two key issues need to be considered when assessing the influence of therapeutic interventions on airway remodeling: first, whether the process of interest is considered to be reversible at all (i.e.: in severe asthma, disruption of alveolar walls attaching to bronchi may occur, which is irreversible and which irreversibly alters the mechanical balance between bronchial dimension and lung volume), and second, whether the processes are overall beneficial or adverse for the patient (taking into account the fact that the same process, a reduction of collagen deposition for instance, might have different outcomes when it occurs within the lamina propria, within the smooth muscle layer, or even in the bronchial adventitia). Furthermore, as hard as it may be, pros and cons of any intervention aiming at specific airway remodeling reversal should also possibly be considered in term of both their short- and long-term effects.

Airway smooth muscle excessive contraction has long been recognized as the most important determinant of airway obstruction in asthma (Huber and Koessler 1922, Lambert, Wiggs et al. 1993, Oliver, Fabry et al. 2007), and most of the initial treatments for asthmatic patients were intended to target this abnormal contractile response. In parallel to the discovery of airway inflammation as a hallmark of asthma, antinflammatory treatments began to be used, allowing an adequate control of the disease even in the absence of bronchodilators. This finding shifted the attention from the ASM to the immune response as the major trigger/initiator of asthma. A new «era» began during which the asthmatic inflammatory response has been extensively

studied, as well as the environmental causes associated with it, and several treatments have been developed to specifically antagonize the effect of the main inflammatory mediators in asthma. Despite the promising results obtained in rodent animal models, most of these treatments proved to be ineffective in asthmatic patients (summarized in **Table 5**), and corticosteroids remain the most efficacious and safest class of drug to be used for asthma control. Although clinical improvement of the disease usually begins within days of initiating treatment, the full benefit may only be evident after 3 or 4 months. It is recognized that in severe and chronically under-treated disease, it may take even longer (GINA, 2015).

Table 5. Efficacy of asthma treatments on rodent animal models and asthmatic patients.

	Rodents	Man
Antinflammatory agents		
Inhaled corticosteroids	✓	✓
Systemic corticosteroids	✓	✓
Anti-leukotrienes	✓	(✓),*
Cromones	✓	(✓),*
Non-steroidal antinflammatory drugs		-
Bronchodilators		
β-adrenergic agents	(✓)	*
Anticholinergic agents	✓	(✓),*
Phosphodiesterase (PDE) inhibitors		
Methylxanthine inhibitors	✓	(✓),*
Selective PDE inhibitors	✓	-
Antioxidants	✓	(✓),*
Expectorant, mucolytic and mucokinetic agents	(✓)	-
Immunomodulators	✓	(✓)
Antibiotics		
Macrolides	✓	(✓),*
Cellular therapy		
Adoptive transfer of stem cells	✓	?

✓: treatment with demonstrated efficacy in that species. (✓): treatment whose efficacy in that species is supported by a low level of scientific evidence. - : treatment with no demonstrated efficacy in that species. ? : treatment with unknown efficacy in that species. * : treatment suggested as possible add-on therapy in selected cases. Modified from Bullone and Lavoie (2015).

NON-PHARMACOLOGICAL TREATMENTS

In the context of this thesis, it is appropriate to mention that some asthma phenotypes largely benefit also from non-pharmacological treatment. Non-pharmacological treatments available for asthmatics are of two types: one consisting in environmental changes, commonly referred to as antigen or allergen avoidance, and one intended to surgically resolve bronchospasm by ASM ablation using bronchial thermoplasty, a recently developed technique.

Antigen avoidance

The level of evidence supporting antigen avoidance strategies for asthma control varies according to the level of evidence linking specific antigens to the asthmatic response. That is, antigen avoidance is presently accepted as the cornerstone of disease control only in cases of work-related asthma (GINA, 2015). As a matter of fact, antigen avoidance can hardly be accomplished in everyday life, possibly explaining the low success rate of this approach (Platts-Mills 2008). Few studies adequately exploring this treatment strategy in non-work-related asthma have been performed. Among them, the most comprehensive studied a group of asthmatic children housed for 3 months at high altitude (1750 m) where environmental antigens, particularly dust mite, are reduced to a minimum for most of the year. They showed a significant reduction of AHR, eosinophilic sputum inflammation, and fractional exhaled NO in allergic asthmatic children (Boner, Peroni et al. 1993, Sensi, Piacentini et al. 1994, Piacentini, Martinati et al. 1996). A very limited number of studies have involved adult asthmatic patients, demonstrating overall a significant effect of antigen avoidance on bronchial hyperresponsiveness (Platts-Mills, Tovey et al. 1982, Walshaw and Evans 1986, Dorward, Colloff et al. 1988, Platts-Mills 2008). There is scarce information concerning the severity of airway inflammation and remodeling during non-pharmacologically induced clinical remission of asthma. A significant increase in reticular basement membrane thickness was reported in a group of young adults in clinical remission of asthma compared with control subjects (van den Toorn, Overbeek et al. 2001). However, that group of patients had only been symptom-free and off medication for 1 year at the time of assessment and still had significant ongoing

airway inflammation. When remodeling and inflammation were assessed in a group of patients with occupational asthma 14 years (on average) after cessation of the exposure, the only residual differences found in control patients were the thickness of the basal membrane and of the ECM layer between the bronchial epithelium and smooth muscle (Sumi, Foley et al. 2007). Of note, although no difference was observed for the ASM mass between the two groups, the mean ratio of ASM area to total biopsy area was twice as high in OA as in control subjects. In these studies, corticosteroid treatment was administered at the time of diagnosis but then discontinued. In this regard, their findings are supported by the results of another study comparing inflammation and remodeling in asthmatic patients (on and off corticosteroids) and asthmatics in complete or clinical remission (based on absence or presence of bronchial hyperresponsiveness). It was observed that the thickness of the basal membrane was reduced only in the group of asthmatics on inhaled corticosteroid treatment (Broekema, Timens et al. 2011), despite similar levels of sputum and tissue inflammation. Overall, these studies suggest that bronchial remodeling in adult asthma is incompletely reversible, and that some aspects are strictly linked to pharmacological treatment. As for ASM remodeling, few studies have investigated the effect of treatments on ASM mass and there is limited information on its reversibility in human asthma.

Work-related asthma provides an ideal model to study antigen avoidance (Sumi, Foley et al. 2007). It also underlines the complex and heterogeneous nature of this disease. In fact, even if in these cases the sensitizing agent is generally recognized, the immune response varies depending on antigen size, with only high molecular weight antigens producing an allergic response. It is still uncertain whether low molecular weight irritants also act via IgE-mediated mechanisms, despite a Th-2 biased inflammatory response (Malo, Tarlo et al. 2015). Overall, antigen avoidance significantly improves the control of work-related asthma cases (GINA, 2015). A remission rate (defined as the absence of residual AHR) up to 72% has been reported after 5 years of antigen avoidance in work-related asthma cases (Pisati, Baruffini et al. 2007), which is astonishingly higher compared to that reported in non-work related asthma (Ronmark, Jonsson et al. 1999, Holm, Omenaas et al. 2007). Furthermore, the prevalence of residual AHR in work-related asthmatics in remission seems to diminish over time after antigen exposure is stopped (Mapp, Corona et al. 1988, Paggiaro, Vagaggini et al. 1993,

Lemiere, Cartier et al. 1996, Pisati, Baruffini et al. 2007), underlining the central role of antigen exposure in disease pathophysiology. Nevertheless, displaying mild instead of severe asthma symptoms at presentation seems to be the only clinical predictor of disease remission in these subjects (Bronnimann and Burrows 1986, Panhuysen, Vonk et al. 1997, Ronmark, Jonsson et al. 1999, Holm, Omenaas et al. 2007, Pisati, Baruffini et al. 2007). A window of a few months (<6) of exposure after disease appearance is considered the maximum exposure time still allowing complete disease remission to be attained (Pisati, Baruffini et al. 2007), suggesting that the presence of chronic inflammatory and remodeling processes lead to irreversible airway changes. Indeed, despite airway remodeling and lumen inflammation being reduced in asthmatic subjects after long-term antigen avoidance, their bronchial wall inflammation is unchanged and bronchial remodeling is still increased compared to healthy controls (Saetta, Maestrelli et al. 1992, Saetta, Maestrelli et al. 1995, Sumi, Foley et al. 2007).

Bronchial thermoplasty

Bronchial thermoplasty refers to the endoscopic-guided application of heat (65°C, in form of radiofrequency energy) directly on the central airways of affected patients. Only airways >3 mm in diameter can be treated (Cox, Miller et al. 2004). Briefly, a catheter made of an expandable basket with four electrode arms is passed through the working channel of the endoscope and, once in the bronchi, it is opened to make contact with the airway wall circumferentially and 10 second-cycles of heat are applied throughout the central bronchial tree (Duhamel and Hales 2010).

Primum non nocere, secundum purgare. The observation that smooth muscle is the only structure of the airway wall displaying long-term heat-sensitivity (Danek, Lombard et al. 2004, Miller, Cox et al. 2005, Pavord, Cox et al. 2007) provided the first evidence of the very specific action of bronchial thermoplasty on the ASM, whose role in the airways, if any, remains unknown (Cox, Miller et al. 2004). Then, the acknowledgment that central airways contribute to airflow obstruction during disease exacerbations provided the rationale for attempting this treatment strategy in asthma. The recent observation that large (>4 mm in

diameter) but not small ASM mass is increased in cases of fatal compared to non-fatal asthma (James, Elliot et al. 2012) further supports this approach.

After promising clinical trials, bronchial thermoplasty was approved by the U.S. Food and Drug Administration in 2010 as a treatment for selected severe asthmatic patients. Since then, there has been accumulating evidence supporting a positive effect of bronchial thermoplasty in term of disease control (decreased number of exacerbations (Castro, Rubin et al. 2010, Chakir, Haj-Salem et al. 2015)). Nevertheless, the dose of pharmacological treatment needed by the patients to control the clinical signs of asthma remains unchanged after bronchial thermoplasty (Pavord, Thomson et al. 2013). In pathophysiological terms, a significant decrease of AHR after bronchial thermoplasty has been documented only in dogs (Brown, Wizeman et al. 2005, Brown, Wizeman et al. 2007). Independently of hyperresponsiveness, the FEV_1 (forced expiratory volume in 1 second) remains often unchanged after bronchial thermoplasty in severe asthmatics (Pavord, Thomson et al. 2013, Denner, Doeing et al. 2015), questioning the overall effect on airflow obstruction of this technique. Very few data are currently available also concerning the effect of bronchial thermoplasty on airway remodeling and inflammation. Evidence exists supporting the ability of bronchial thermoplasty at reversing airway lumen inflammation (Denner, Doeing et al. 2015) up to six weeks after the procedure. As for remodeling, the first 2 studies showing a decreased ASM mass after bronchial thermoplasty sampled exactly the same bronchial carinas on multiple occasions (Pretolani, Dombret et al. 2014, Denner, Doeing et al. 2015), and one of them reported a great ASM reduction even at the non-treated sites (Pretolani, Dombret et al. 2014), raising concerns on whether the observed effect was real rather than a technical artifact (Bonta, d'Hooghe et al. 2015). However, a significant but smaller effect was also observed in a recent study sampling different carinae before and after the procedure, confirming the effectiveness of bronchial thermoplasty at decreasing ASM and further reporting a reduction of subepithelial ECM deposition (Chakir, Haj-Salem et al. 2015) lasting for up to 8 weeks.

PHARMACOLOGICAL TREATMENTS

Pharmacological treatment of asthma is based on a 5-step protocol starting with the occasional use of inhaled β_2-agonists and going up to moderate to high doses of inhaled corticosteroids and long-acting β_2-agonists, possibly in conjunction with supplementary treatments such as oral corticosteroids or other targeted therapies such as anti-IgE, anti-leukotrienes, and long-acting muscarinic agents (GINA, 2015).

Drug delivery

Systemic administration

Systemic administration of a drug can be performed in several ways. The easiest and commonest is by far the oral route of administration, with intramuscular and intravenous injection being less common and often restricted to hospital use or to drugs that would be inactivated into the gastrointestinal tract. Oral administration of β_2-agonists has been abandoned years ago, and oral corticosteroids are strictly reserved to severely affected patients in order to reduce to a minimum the incidence of the side-effects associated with their chronic use (Treadwell, Sever et al. 1964, McAllen 1970, Ryall, Sillence et al. 2006).

Inhalation

Delivery of drugs through inhalation (oral or nasal) is an attractive alternative to systemic drug delivery, and it is considered the best option for treating lung diseases. Inhalation therapy has multiple advantages over systemic drug administration:

- requires smaller doses than systemic treatment;
- drug delivery is targeted to the respiratory system for local pulmonary effect;
- systemic side effects are fewer and less severe.

Inhalation therapy seems however to be characterized by a reduced patient adherence to treatment compared to oral administration (Cohn 2003), but the lower dosing frequency often associated with the oral therapy could have introduced a bias (Rand, Bilderback et al. 2007).

The effectiveness of an inhalation therapy is dependent on the amount of the medication that reaches the intended site of deposition, which, in turn, depends on the physical and chemical properties of the drug particles, on the characteristics of the delivery device used, as well as on the breathing pattern of the patient. In particular, particle size is the major determinant of the site of deposition of the drug in the respiratory tract. Particles larger than 5 μm, also called droplets, deposit by impaction in the oropharynx or upper airways, not reaching the lung. Particles of 0.5-5 μm in size deposit by sedimentation or diffusion in the most caudal portions of the bronchial tree and alveoli, while particles smaller than 0.5 μm are generally exhaled (CCADD, 2000). Inhaled particles with an aerodynamic particle size <2 μm are considered optimal, especially if in monodispersity (Ibrahim, Verma et al. 2015), although an increased risk of phagocytosis by alveolar macrophages has been described for particles of this size with particular chemical properties (Muralidharan, Malapit et al. 2015).

Corticosteroids

Corticosteroids are the only drug with proven efficacy at decreasing asthma mortality (Suissa, Ernst et al. 2000). Different pathological aspects of asthma, namely AHR, inflammation and remodeling, have shown different time courses for improvement as well as different outcomes depending on the lung region assessed (central vs peripheral).

Mechanism of action

Corticosteroids are a class of anti-inflammatory drugs with a very broad spectrum of targets. As such, their effect is mediated mainly by their repressive action on transcription factors regulating the expression of pro-inflammatory genes, such as NF-Kappa β or AP-1. Activation

of anti-inflammatory genes and non-transcriptional effects of corticosteroids are also recognized as minor mechanisms of action (Barnes 2006). Of particular interest for asthma, corticosteroids can exert non-genomic effects regulating both inflammatory cell activation and smooth muscle contractile properties (Sun, Miao et al. 2006, Zhou, Liu et al. 2008, Mendes, Rebolledo et al. 2014, Mendes, Cadet et al. 2015).

Side effects

Despite the safer profile of ICS compared to systemic corticosteroids, adverse effects have been reported to occur with long-term administration (Hanania, Chapman et al. 1995, Dahl 2006, Barnes 2007), particularly in the elderly (Mattishent, Thavarajah et al. 2014).

Effect on inflammation

Soon after the observation that inflammation was present even in the airway of mild asthmatics during periods of disease remission (Djukanovic, Roche et al. 1990), the effect of corticosteroid at reducing the number and activation of inflammatory cells was described both at the level of central airway walls and conducting airways down to the alveoli, by means of endobronchial biopsies and bronchoalveolar lavage, respectively (Djukanovic, Wilson et al. 1992, Janson-Bjerklie, Fahy et al. 1993, Ward, Pais et al. 2002, de Kluijver, Schrumpf et al. 2005). Since then, sputum has been validated as an indicator of bronchoalveolar inflammation in asthma (Nocker, Out et al. 2000), and studies have confirmed the antinflammatory effect of corticosteroids in most, but not all, patients. Many bronchial structural cells have recognized immunomodulatory properties. While corticosteroids significantly reduce the expression of inflammatory mediators in epithelial cells, fibroblasts, and ASM cells *in vitro*, their role *in vivo* remains unclearly defined (Yick, Zwinderman et al. 2013). Recently, the rapid-onset inhibition of blood flow (vasoconstriction) induced by corticosteroids in the airways of asthmatics has also been proposed as a mechanism contributing to the antinflammatory action of these drugs (Mendes, Rebolledo et al. 2014).

Several studies have demonstrated a reduction of lymphocyte, eosinophils, and neutrophils in the epithelium and subepithelial layer in the central airways of asthmatics treated with both inhaled and systemic corticosteroids (Chanez, Bourdin et al. 2004). Nevertheless, whether they exert significant antinflammatory effects also in the distal lung (small airways and parenchyma) is not clear (Martin 2002). One study specifically investigating the effect of a 6-week treatment with inhaled corticosteroids on peripheral lung inflammation in asthmatics reported a significant improvement only in the number of lung eosinophils, while neutrophils increased, and the other inflammatory cells remained unvaried (Hauber, Gotfried et al. 2003).

Effect on hyperresponsiveness

Long-term corticosteroid treatment decreases AHR in asthma (Ward, Pais et al. 2002). Nevertheless, whether the asthma-associated decline in lung function is prevented by this class of drug remains unsettled (Ernst 2006, Haahtela 2006, Lange, Scharling et al. 2006, Chanoine, Dumas et al. 2015), and even well-controlled asthmatics still show airway reactivity to abnormally low methacholine concentrations (Yick, Zwinderman et al. 2013). In this regard, a 2-week period of inhaled fluticasone discontinued 24 h before allergen challenge did not offer any additional protection against the asthmatic response in terms of airway responsiveness (or sputum eosinophilia) compared with a single dose of the same drug administered immediately before allergen challenge (Parameswaran, Inman et al. 2000). The mechanisms by which corticosteroids impact AHR are not fully elucidated. Although corticosteroids can reduce inflammation, which contributes to AHR, they may also produce direct effects on ASM cells. On the other hand, the long-term effect observed of corticosteroids on AHR could be ascribed to their ability (demonstrated only in animal models) at reversing, although only partially, airway remodeling (Leclere, Lavoie-Lamoureux et al. 2012).

As mentioned above, corticosteroids may directly affect ASM gene expression *in vitro*. However, whether the clinical benefits provided by corticosteroid therapy in the vast majority of asthma patients could result, at least in part, from their direct action on ASM remains an open issue that needs to be clarified. The only evidence that this could occur *in vivo* has been

provided by a recent study investigating the effect of a 2-week corticosteroid treatment on quantitative gene expression in laser-microdissected smooth muscle from endobronchial biopsy samples of mild asthmatics (Yick, Zwinderman et al. 2013). In their study, Yick and colleagues have shown for the first time in man that after 2 weeks of oral corticosteroid treatment, the expression of 15 genes was altered in central ASM from asthmatic patients. They also found that 2 of these genes significantly correlated with AHR. However, the fact that both treated and untreated groups were analyzed together, the absence of a healthy control group, and of any statistical correction for multiple comparisons slightly reduces the impact of this observation. In the same study, myocardin expression was increased after treatment (Yick, Zwinderman et al. 2013). Myocardin, a protein involved in the activation of the transcription factor SRF (serum response factor), has been associated with the contractile (differentiated) phenotype of smooth muscle cells of other tissues (i.e. vessels and intestine). Its overexpression in treated asthma patients suggests that corticosteroids could possibly modulate ASM phenotype switching *in vivo*.

Effect on remodeling

Based on *in vitro* studies, the effects of corticosteroids on airway remodeling could extend to all compartments of the bronchial wall. Nevertheless, *in vivo* evidence of airway remodeling reversal in steroid-treated asthmatic patients is limited by the fact that obtaining endobronchial biopsy samples is an invasive procedure whose interpretation is also complicated by the unavailability of pre-treatment data in most cases.

As previously mentioned, whether epithelial hyperplasia and detachment are features of asthma or technical artifacts has not been definitely clarified. Nevertheless, epithelial fragility and dysfunction is a unanimously recognized trait of asthma (Knight and Holgate 2003). Few studies have investigated the effect of corticosteroid treatment on human bronchial epithelial structure or function *in vivo*, reporting an increased proliferation and a restitution of the epithelial thickness to normal values (Vignola, Chiappara et al. 2001), joined with a reduction of epithelial cells in BALF (Ward, Pais et al. 2002). Also, a normalization of the ciliated to

goblet cell ratio has been reported following inhaled corticosteroid treatment (Laitinen and Laitinen 1995).

Most studies investigating the effect of corticosteroid treatment on airway remodeling have focused on changes of the bronchial ECM in endobronchial biopsy samples. It is now recognized that long-term high-dose corticosteroids treatment can decrease the basal membrane thickness in central asthmatic airways (Trigg, Manolitsas et al. 1994, Laitinen, Altraja et al. 1997, Olivieri, Chetta et al. 1997). However, this reversal is not observed in patients receiving low-dose treatment for the same amount of time and showing a similar clinical improvement (Chetta, Zanini et al. 2003). Whether corticosteroids can also decrease the amount of ECM deposition within the lamina propria and to which extent in central and peripheral airways is far less settled. Endothelin, a protein involved in the process of lung repair and fibrosis, is increased in corticosteroid-naïve asthmatics compared to ICS-treated asthmatics and controls (Redington, Springall et al. 1997). It has been reported that 2 weeks of oral corticosteroid did not reverse collagen deposition (Chakir, Shannon et al. 2003), while a significant decrease was observed after 6 months of inhaled budesonide given at high dosage, modulated by tissue MMPs and TIMPs (Hoshino, Takahashi et al. 1999). On the other hand, a 2-year treatment with low dose corticosteroids was not successful at decreasing collagen deposition within the lamina propria (Chakir, Loubaki et al. 2010). Another study reported that neither short-term fixed dose nor long-term (< 6 months) treatment with variable doses of inhaled steroids significantly altered the collagen content of the tissue (Godfrey, Lorimer et al. 1995). The synthesis of ECM proteins is stimulated by TGF-β, which is increased in asthmatic airways and blood, and respiratory secretions (Halwani, Al-Muhsen et al. 2011). Corticosteroid treatment can partly decrease serum and sputum TGF-β expression in the long but not in the short-term (Chakir, Shannon et al. 2003, Kai, Nomura et al. 2007, Kawayama, Kinoshita et al. 2008, Manuyakorn, Kamchaisatian et al. 2008). Nevertheless, TGF-β levels remain higher in asthmatic patients compared to healthy subjects (Manuyakorn, Kamchaisatian et al. 2008, Yamaguchi, Niimi et al. 2008). Transforming growth factor-β expression within the bronchial wall is not affected by corticosteroid treatment at all (Tomkowicz, Kraus-Filarska et al. 2008). Elastin is another ECM component undergoing remodeling in asthma. However, corticosteroid treatment does not affect elastin content of the

tissue in large airways (Godfrey, Lorimer et al. 1995, Bousquet, Lacoste et al. 1996). Overall, these findings suggest that airway fibrosis and ECM deposition can be reversed by long-term corticosteroid treatment, but this effect is not mediated by a decrease of TGF-β expression in the airways.

A single study has investigated the efficacy of corticosteroids at reversing established ASM remodeling in asthma (Bergeron, Hauber et al. 2005), showing that 6 weeks of treatment reduced the density of smooth muscle alpha-actin (SMA-α) peripherally but not centrally. No studies are currently available investigating by which mechanism this could have occurred *in vivo*. Corticosteroids appear to inhibit human ASM proliferation *in vitro* (Stewart, Harris et al. 1999). However, this response can be blunted when ASM cells are seeded on collagen (Bonacci, Schuliga et al. 2006). Asthmatic smooth muscle is hyperproliferative and lies in an inflamed, fibrotic and collagen-rich microenvironment, which could inhibit corticosteroid responsiveness in terms of remodeling reversal. Whether the synthetic response of ASM cells is regulated by corticosteroids in asthmatics is not well established. Dexamethasone and fluticasone inhibit SMA-α expression in primary bronchial smooth muscle cells (Goldsmith, Hershenson et al. 2007). However, the synthesis of pro-inflammatory ECM proteins by asthmatic ASM cells is only partially controlled by corticosteroid therapy *in vitro*, and whether the same responses are attained *in vivo* is still unclear (Ammit, Burgess et al. 2009). It should be noted that ASM cells appear to be characterized by a species-specific response to several synthetic stimuli and treatments (Ammit, Burgess et al. 2009), implying that caution must be taken when translating results obtained in animal models to human asthma. No data are currently available on the effect of corticosteroids on ASM hypertrophy in human asthma. Studies in animal models of asthma have shown that corticosteroid therapy only partially reverses established ASM hypertrophy (Leclere, Lavoie-Lamoureux et al. 2012, Alrifai, Marsh et al. 2014). Corticosteroids could also be effective at inhibiting ASM cell migration in asthmatic airways, although currently available data are conflicting (Hoshino, Takahashi et al. 1999, Kelly, O'Connor et al. 2010).

Vasodilatation, increased vascular permeability, and angiogenesis are the three aspects of vascular remodeling in asthma. They all appear to be reversible with corticosteroid treatment,

particularly when administered at high doses (Chetta, Zanini et al. 2007, Feltis, Wignarajah et al. 2007).

β_2-adrenergic bronchodilators

Adrenergic bronchodilators were first used for asthma more than 100 years ago (Tattersfield 2006). They rapidly reverse bronchospasm, but they also are advocated some antinflammatory and antiremodeling properties, even when administered as monotherapy (Orsida, Ward et al. 2001).

Mechanism of action

Many structural and inflammatory cells express β_2-adrenergic receptors, suggesting that the physiologic role of catecholamines extends far beyond the regulation of the acute stress response, and might exert relevant immunomodulatory actions. In asthma, β_2-adrenergic bronchodilators act mainly by promoting ASM relaxation, which dilates the airways and reverses airflow obstruction. In addition, they can inhibit cholinergic transmission, reduce vascular permeability, and increase mucociliary clearance, further contributing to airway patency (Tattersfield 2006).

Side effects

The administration of β_2-adrenergic agonists might cause rapid and delayed side effects, due to different mechanisms. The rapidly occurring side effects appear concomitantly with the pharmacological effect of the drug, and are driven by the same mechanism of action; they are mainly represented by tachycardia and increased sweating. However, recently synthetized drugs administered by inhalation are characterized by an improved safety profile. The

continuous administration of β_2-adrenergic agonists can also induce tachyphylaxis, or desensitization, which decreases the clinical efficacy of these drugs.

Based on the results of a meta-analysis including more than 100 trials, regular beta agonists use is now discouraged (GINA, 2015) as it worsens asthma control and increases mortality (Levenson 2008). These drugs induce vasodilation as well as bronchodilation, which could facilitate the influx of inflammatory cells into the airways or participate in airway wall thickening. Two unresolved and intriguing questions are whether all long-acting β_2-agonists confer the same risk and whether they have a dose-related safety profile (Dissanayake 2015).

Effect on hyperresponsiveness

Despite effectively bronchodilating the airways even when administered for extended periods of time (especially partial agonists such as salmeterol), β_2-agonists do not improve airway responsiveness, which instead appears to increase (Cheung, Timmers et al. 1992, Boulet, Cartier et al. 1998).

Effect on inflammation

While effective at suppressing symptoms, β_2-agonists given as a monotherapy could mask underlying deterioration of airway inflammation (Knight, Mak et al. 2015). Although *in vitro* studies have shown antinflammatory effects of β_2-agonists, *in vivo* evidence is scarce and appears to be limited to the neutrophilic component of inflammation in some studies (Jeffery, Venge et al. 2002, Wallin, Pourazar et al. 2004, Maris, de Vos et al. 2005) but not in all studies (Kraft, Wenzel et al. 1997, Altraja, Laitinen et al. 1999, Roberts, Bradding et al. 1999, Tagaya, Kondo et al. 2015). While not reducing their number, β_2-agonists are effective stabilizers of mast cells. Their effect is however limited in terms of time as tolerance occurs more readily in these cells compared to what is reported for smooth muscle relaxation. This could lead to a situation in which β_2-agonists fail to prevent the release of inflammatory mediators from mast cells while still effectively controlling bronchospasm (Peachell 2006).

Effect on remodeling

Limited *in vivo* data are available on the effect of β_2-agonists alone on airway remodeling in human asthma. Their unsafe therapeutic profile when used as monotherapies now prevents such information from being obtained. Although restricted to a few studies, there is evidence that β_2-agonists could modulate remodeling of the bronchial ECM in asthma when administered for extended period of time. Twelve weeks of treatment with salbutamol reduced the tenascin but not the collagen content of the bronchial wall in biopsies from mild asthmatic patients (Altraja, Laitinen et al. 1999), while only six weeks of salmeterol treatment had no effect on the underlying remodeling processes despite improving the clinical indices of the disease (Roberts, Bradding et al. 1999). β_2-agonist administration improves the mucociliary clearance in asthmatics (Daviskas, Anderson et al. 2005). The recent and somehow surprising finding that bronchoconstriction itself can induce remodeling in asthmatic patients (Grainge, Lau et al. 2011) raises legitimate questions as to whether the opposite occurs as well, that is, whether bronchodilation *per se* could reverse airway remodeling.

Data obtained from animal models of asthma suggest a possible implication of β_2-agonists in airway remodeling regulation at multiple sites of the bronchial wall. In non-challenged mice, salbutamol stimulated epithelial cell proliferation and thickening in a dose and time dependent manner (Tamaoki, Tagaya et al. 2004), and appeared to induce goblet cell hyperplasia (Kamachi, Munakata et al. 2001, Riesenfeld, Sullivan et al. 2010). A large body of evidence obtained *in vitro* indicates that β_2-agonists do inhibit ASM migration and proliferation, two processes strongly linked to airway remodeling, while their effect on ASM synthetic activity is still debated (Hirst, Martin et al. 2004, Ammit, Burgess et al. 2009). The synthesis of pro-inflammatory mediators and of ECM proteins by ASM cells can be regulated by different mechanisms. Also, the composition of the ECM where ASM cells lie is thought to affect their proliferative and synthetic properties (Altraja, Laitinen et al. 1999, Hirst, Twort et al. 2000, Lambers, Qi et al. 2014). Whether β_2-agonists are able to modulate ASM hypertrophy or phenotype switching is not established at this time.

Combinations

Asthma treatment guidelines propose combination therapy with inhaled corticosteroids (ICS) and bronchodilators (long acting β_2-agonists, LABA) as the treatment of choice for moderate to severe disease when patients do not respond to ICS treatment alone at low-to-medium dose (GINA, 2015). Several trials have demonstrated that adding a LABA is more effective in terms of exacerbation rate and lung function improvement compared to doubling the dose of ICS in adult asthmatics (Greening, Ind et al. 1994, Woolcock, Lundback et al. 1996), although the same does not appear to be true for 4-fold increases in ICS dose (Pauwels, Lofdahl et al. 1997). The rationale of adding a LABA instead of increasing ICS dose in asthma treatment is based on the following observations (reviewed by (Barnes 2002)):

- ICS are characterized by a relatively flat dose/response curve, indicating that most of the effect of these drugs is obtained at lower doses;
- inhaled β_2-agonists are the most effective bronchodilators, and asthma is a disease characterized by the presence of bronchospasm;
- β_2-agonists exert some non-bronchodilator effects particularly interesting for asthma treatment, such as their ability to stabilize mast cell and inhibit plasma exudation;
- a synergistic interaction existing between β_2-agonists and corticosteroids at the receptor and transcriptional level, with corticosteroids preventing the loss of function (tachyphylaxis, desensitization) of β_2-agonists with chronic use, whereas β_2-agonists may potentiate the local anti-inflammatory action of corticosteroids.

Over the years, β_2-agonists have been shown to be more effective than muscarinic agents as add-on treatment to ICS in asthma (Chauhan and Ducharme 2014). As briefly summarized above, β_2-agonists affect different aspects of the pathophysiology of asthma than corticosteroids and for most patients both treatments are needed to fully control symptoms.

Effect on hyperresponsiveness and inflammation

The combination of ICS/LABA provides better control of lung function and AHR compared to either ICS or LABA alone (Lundback, Ronmark et al. 2006). Clinical studies also suggest a better control of inflammation with combination therapy compared to single ICS or LABA treatment (Li, Ward et al. 1999, Walters, Bjermer et al. 2000, Wallin, Sue-Chu et al. 2003, Tagaya, Kondo et al. 2015), supported by *in vitro* data. The combinations of ICS/LABA also show a more rapid onset of action compared to ICS alone in term of control of asthma symptoms, airflow limitation, airway inflammation, and AHR (Matsunaga, Kawabata et al. 2013). Nevertheless, the paucity of studies comparing the same dose of ICS in the presence or absence of LABA does not permit to draw definitive conclusions on the specific advantage of adding a LABA to the ICS therapy. New, extra-fine formulations of several ICS/LABA combination therapies appear to significantly improve peripheral airway function and inflammation in asthmatics (Lipworth 2013, Scichilone, Spatafora et al. 2013).

Effect on remodeling

Very little information is available concerning the effect of ICS/LABA combination on airway remodeling in asthma, especially in comparison to ICS monotherapy. Given their superiority at controlling asthma symptoms and exacerbation rate compared to ICS treatment alone, it is legitimate to wonder whether it is caused solely by a better control of the underlying inflammation or whether an enhanced degree of remodeling reversal is involved. It would be of interest to determine whether such improved remodeling reversal, if any, occurs before, concurrently, or after the observed decrease of inflammation in the central airways and whether the same effect is also observable in small peripheral airways.

When used as add-on treatment to moderate doses of ICS in severe asthmatics, salmeterol significantly reduced the submucosal vascularization compared to increased doses of ICS or to placebo in central airways (Orsida, Ward et al. 2001). Another study has reported a decreased

expression of vascular endothelial growth factor (VEGF) and its receptor VEGFR1 in endobronchial biopsy samples of asthmatics after 6 months of budesonide/formoterol treatment (Wang, Liu et al. 2008). In the same study, asthmatic patients showed as well reduced ASM mass and thickness, quantity of submucosal gland, fibrosis, and vascularization of their bronchial wall. In term of ASM mass, another study reported a protective role for the combination of budesonide/formoterol during antigen challenge in asthmatics. The combination prevented ASM remodeling and myofibroblast accumulation within the submucosa compared to both placebo and budesonide pretreatment alone (Kelly, O'Connor et al. 2010). Briefly, the *in vitro* data support an additive/synergistic role for ICS/LABA combinations at reversing asthma-associated inflammation and remodeling, both at the ECM and ASM level (Howarth, Knox et al. 2004, Todorova, Gurcan et al. 2006, Dekkers, Pehlic et al. 2012).

Highlights and unanswered questions

Pharmacological treatment is the best therapeutic option in asthma. The combinations of ICS/LABA represent the most advantageous treatment choice for patients not responding to ICS monotherapy in term of disease control (exacerbation rate and patient clinical perception of the disease), lung function improvement, and safety. Whether the enhanced efficacy of ICS/LABA compared to ICS alone is related to a better control of airway inflammation, remodeling, or to the ability to reach more peripheral bronchi remains to be established.

Intuitively, adding a bronchodilator to ICS treatment would promote ICS deposition more distally within the bronchial tree. However, recent data point out that small airway dysfunction occurs in these patients despite treatment up to step 4 (medium-to-high dose of ICS/LABA) of current asthma guidelines (Anderson, Zajda et al. 2012), questioning whether these formulations can effectively reach the distal lung. Also, bronchial thermoplasty reduces exacerbation rate as well as remodeling and inflammation of the central airways but the same dose of pharmacological treatment is needed to maintain asthma control afterwards, suggesting that the bull's eye of the disease lies in the "silent" zone of the lung.

In this perspective, new extra-fine drug formulations have been serendipitously discovered and found to improve penetration to the most peripheral portion of the lung (Zeidler and Corren 2004). The main challenge is now to test their efficacy (more precisely: their superiority) at that level in terms of hyperresponsiveness, inflammation, and/or remodeling. As small peripheral airways cannot be sampled easily, a large multi-centric study has recently started with the aim of identifying specific and sensible markers of peripheral airway function (Postma, Brightling et al. 2015). It is however hard to believe that peripheral airway wall inflammation and remodeling can readily be assessed indirectly. There is an unmet need of reliable models for studying asthma remodeling and its reversal in response to treatment.

Equine asthma

Approximately 10 to 20% of adult horses living in temperate climates develop a respiratory obstructive asthma-like condition (Hotchkiss, Reid et al. 2007) known under different names, the most recent being equine asthma (Lavoie 2015).Previously, the same disease was known as heaves, COPD (chronic airway obstruction), or RAO (recurrent airway obstruction). SPAOPD (summer-pasture associated obstructive airway disease) is the summer variant of heaves, while IAD (inflammatory airway disease) is its milder form. **Figure 10** provides an exhaustive scheme of the current classification of these conditions. The severe form of the disease is typically associated with recurrent episodes of respiratory distress at rest, bronchoalveolar lavage neutrophilia and peripheral airway remodeling, and it is an accepted model for human asthma (Leclere, Lavoie-Lamoureux et al. 2011, Boivin, Vargas et al. 2014, Matusovsky, Kachmar et al. 2015).

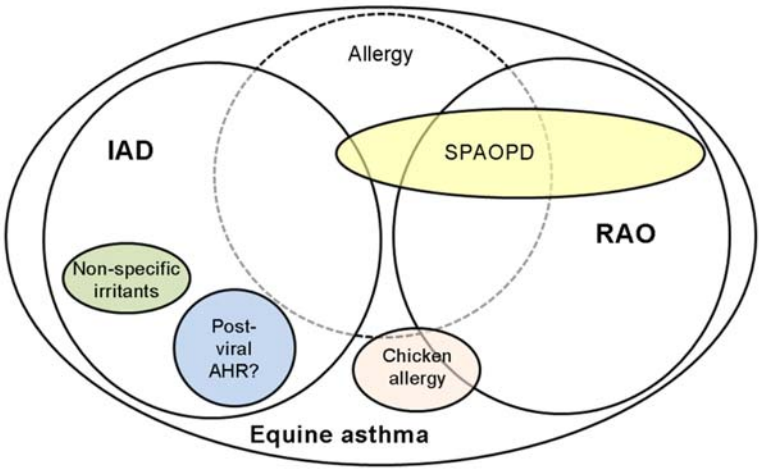

Figure 10. Equine asthma. IAD: inflammatory airway disease, RAO: recurrent airway obstruction, SPAOPD: summer-pasture associated obstructive airway disease.

Rationale for using the equine model

The need of animal models of asthma is undiscussed. Rodent models have played, and still play, a crucial role for discovering genetic variations or cellular pathways involved in disease development and regulation. They are an irreplaceable tool in mechanistic molecular studies. Nevertheless, rodent models have proven unreliable when assessing the efficacy of asthma therapies, possibly because of the non-spontaneous and acute (days to months) nature of inflammation and remodeling compared to humans. In this perspective, equine asthma (particularly its severe form, named *heaves*) represents a unique translational model for the disease.

Similarities between equine and human asthma

Severe equine and human asthma share many pathophysiological and clinical similarities. Both diseases are characterized by the presence of bronchospasm, inflammation and remodeling of the lung (Leclere, Lavoie-Lamoureux et al. 2011).

Bronchospasm –The main determinant of airflow obstruction in asthma is ASM contraction (Lambert, Wiggs et al. 1993, Oliver, Fabry et al. 2007). Severely asthmatic horses experience moderate to severe episodes of airflow obstruction (disease exacerbations) when exposed to barn dust, hay, and straw, during which pulmonary resistance and elastance are increased (Leclere, Lavoie-Lamoureux et al. 2011). The rapid improvement in lung function with bronchodilators demonstrates the involvement of an exaggerated ASM contraction in equine asthma (Derksen, Olszewski et al. 1999, de Lagarde, Rodrigues et al. 2014). Episodes of bronchospasm are reversible by treatment both in human and equine asthma. Bronchodilators are however short-acting and effective only for immediate relief. Whether asthmatic smooth muscle is hyperresponsive or not remains a matter of debate (Chin, Bosse et al. 2012). Tissues isolated from horses with heaves are not hyperresponsive (Yu, Wang et al. 1994, Olszewski, Robinson et al. 1999, Matusovsky, Kachmar et al. 2015). Nevertheless, smooth muscle isolated from intrapulmonary airways (3 to 7 mm in diameter) of asthmatic horses was shown to be hypercontractile compared to ASM isolated from healthy animals, at least in term of

maximal velocity of shortening (Matusovsky, Kachmar et al. 2015). A recent study of isolated bronchial smooth muscle strips supports the equine model as being mechanically representative of human asthma (Matusovsky, Kachmar et al. 2015).

Corticosteroid therapy is the cornerstone of asthma treatment in both species, which underlines the inflammatory nature of both diseases. Antigen avoidance strategies are also an effective way of controlling both diseases (Leclere, Lavoie-Lamoureux et al. 2012, Malo, Tarlo et al. 2015). It is implemented more easily in horses by limiting/avoiding the exposure to hay and environmental dust associated with stabling. In human asthma however this approach is generally limited to those phenotypes with known environmental triggers, such as work-related asthma. There is increasing awareness that housing conditions may strongly influence the natural history of the disease (Krieger, Jacobs et al. 2010, Keall, Crane et al. 2012).

Inflammation – Human asthma is a chronic inflammatory disease. Although generally described as an allergic and eosinophilic disease, there is growing awareness of the role of neutrophil-mediated mechanisms in asthma pathogenesis, particularly in the more severe forms of the disease (Chanez, Wenzel et al. 2007). Only about half of asthmatic adults are atopic (Anderson 2008) and a Th2-biased response is detectable in only 50% of individuals with asthma (Lambrecht and Hammad 2012). Severe equine asthma is also a chronic inflammatory condition, characterized by BALF neutrophilia in most cases. Despite the absence of eosinophilic inflammation in BALF and bronchial tissues of severely affected horses (Leclere, Lavoie-Lamoureux et al. 2011, Dubuc and Lavoie 2014), allergic and IgE-mediated mechanisms are likely to participate into disease pathogenesis (Kunzle, Gerber et al. 2007, Tahon, Baselgia et al. 2009), and a Th2-biased response is observed in some but not all cases of severe equine asthma (Lavoie, Maghni et al. 2001, Ainsworth, Grunig et al. 2003).

In man, the neutrophilic asthma phenotype is more frequently associated with the severe or work-related forms of the disease, but is observed also in mild asthmatics, and it appears to predict a poor response to corticosteroid treatment (Fahy 2009, Monteseirin 2009). It has been associated with a Th17-biased response, with a concomitant high expression of the chemoattractants IL-8 and TNF-α (Wenzel 2012). Severely asthmatic horses typically present with BALF neutrophilia. Also a significant proportion of mild asthmatic horses have a

neutrophilic inflammatory phenotype in their BALF, characterized clinically by the presence of persistent cough and increased mucus secretions in the airways (Bedenice, Mazan et al. 2008, Ryhner, Muller et al. 2008, Koblinger, Nicol et al. 2011). Severe equine asthma is associated with increased expression of IL-8, IL-4, IL-17, TNF-α, and IL-1β in BALF cells (Franchini, Gill et al. 2000, Giguere, Viel et al. 2002, Debrue, Hamilton et al. 2005). Bronchoalveolar lavage or sputum neutrophilia has been shown to precede disease recrudescence after corticosteroids withdrawal and it is frequently observed during acute attacks of asthma (Fahy, Kim et al. 1995, Lamblin, Gosset et al. 1998, Maneechotesuwan, Essilfie-Quaye et al. 2007). Neutrophilic asthma is also associated with systemic inflammation, both in the human and equine form of the disease (Lavoie-Lamoureux, Beauchamp et al. 2012, Lavoie-Lamoureux, Leclere et al. 2012, Wood, Baines et al. 2012).

Remodeling – As observed in human asthmatic patients, horses affected by severe asthma experience repeated episodes of airway obstruction occurring over periods of years or sometimes even decades. The first structural abnormality noticed in isolated asthmatic lungs was the increased quantity of bronchial smooth muscle (Huber and Koessler 1922). Similarly, almost a century later, the first quantitative demonstration of remodeling in severe equine asthma concerned bronchial smooth muscle (Herszberg, Ramos-Barbon et al. 2006). Beside the smooth muscle mass, many other aspects of remodeling have been studied and there seems to be marked overlap between structural alterations observed in human and equine asthma. It has to be noted that, until now, most of the studies performed investigating remodeling in asthmatic horses have focused on peripheral alterations. Contrarily, endobronchial biopsies from central airways have been the samples most commonly studied in human asthma, from which has arisen the notion that an increased thickness of the *lamina reticularis* (basal membrane) is a feature of asthma (Roche, Beasley et al. 1989). Neutrophilic asthma is however not characterized by thickening of the basement membrane (Fahy 2009).

Comparative anatomy and physiology of the lung

Equine bronchial branching is monopodial: each main bronchus continues almost directly towards the lung periphery giving off a series of smaller branches (Nakakuki 1993). Human bronchial tree is characterized by a dichotomous branching pattern. That is, each bronchus divides in two distal bronchi whose diameter is bigger than half the diameter of the original bronchus (Weibel and Gomez 1962). From the trachea to terminal bronchioles, the airway branches up to 25 times in man (McLaughlin, Williamson et al. 2008), while the exact number of branches of the equine tracheobronchial tree has not been determined (Robinson and Furlow 2007). Subjective variations in the pattern of bronchial branching in horses seem to be greater than described in other species (O'Callaghan and Sanderson 1982, Olszewski, Robinson et al. 1999), possibly also related to the marked anatomical differences among several breeds. Horses differ from men and other mammals as they lack respiratory bronchioles (Tyler 1983, Robinson and Furlow 2007). Nevertheless, anatomically, equine lungs closely resemble human lungs for the adjacent disposition of airways and vessels (McLaughlin 1983, Magno 1990, Chen, Klein et al. 1992, Nakakuki 1993), which is not observed in the lungs of other animal species used as models for asthma. Indeed, similarly to humans but differently from rodents, horses have a double lung circulation (the bronchial one, supplying nutrients and oxygen to the cells that constitute the lungs, and the pulmonary one, which permits blood oxygenation and carbon dioxide removal). Furthermore, men and horses have cartilage in their conducting airways down to 3 to 5 mm in diameter, while rodents lack cartilage in their intrapulmonary airways, with the exception of their main-stem bronchi (Kennedy, Desrosiers et al. 1978).

Physiologically, horses differ from men as they have a biphasic breathing strategy, with active involvement of the respiratory muscles. The respiratory cycle of the horse occurs around rather than above the relaxation volume of their respiratory system, defined as the volume at which passive lung recoiling forces (inward) equal passive chest wall expanding forces (outward). For this reason, end inspiration and end expiration are active processes (Koterba, Kosch et al. 1988), the latter requiring abdominal muscle contraction. In severe asthmatic horses, chronically experiencing an increased expiratory effort, the external abdominal oblique muscle may become markedly hypertrophic (**Figure 11**), producing a *heave line*.

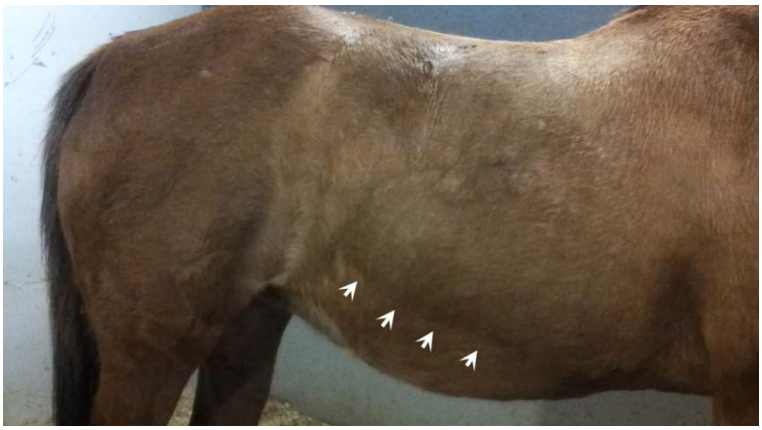

Figure 11. Heave line in an asthmatic horse. White arrows point to the heave line on the right side of a horse with asthma.

Advantages and disadvantages of the equine model

Using equine asthma as an animal model for the human disease offers both technical and translational advantages.

The environmental triggers of equine asthma exacerbations are well-known (barn dust, hay, and straw antigens). Disease status of asthmatic horses may thus be modulated as required by specific research needs, avoiding the use of compounds or antigens not naturally involved in disease development and possibly compromising the translational reliability of many animal models to human asthma. Thanks to their size, horses are especially well suited for prospective studies requiring multiple analyses repeated over time and on the same subjects. Venipuncture and blood analysis, but also bronchoscopy and BALF or tissue sample withdrawal can be performed repeatedly. Bronchoscopic procedures are performed in standing sedated animals, during which mucus, tracheal wash (TW) aspirates, BAL and/or bronchial epithelial brushing can be easily obtained. The equine tracheobronchial tree offers more than 40 reachable carinae for endobronchial biopsy collection (Robinson and Furlow 2007). Equine lungs also permit harvesting large peripheral lung biopsy samples by means of thoracoscopic surgery (Lugo, Stick et al. 2002, Relave, David et al. 2008, Relave, David et al. 2010), offering an ideal

approach for the study of small airway remodeling and its reversal over time. Furthermore, techniques such as spirometry and impulse oscillometry have been validated in horses, which permit to study lung function in this species (Couetil, Rosenthal et al. 2000, Van Erck, Votion et al. 2006).

The equine model has unique similarities with human asthma from a pathophysiological point of view. First, as equine asthma is a chronic naturally-occurring disease, its pathogenesis is more likely to represent what occurs in human asthma compared rodents, in which the disease is experimentally induced and spontaneously reversible when chronic challenges are stopped (Johnson, Wiley et al. 2004). Second, from a physiological point of view, horses' lifespan (≈ 30 years) is undoubtedly more similar to that of man than those of other animal models of asthma such as rodents, cats or dogs. Lastly, as previously mentioned, equine and human asthma respond to the same pharmacological therapies. Several molecules targeting specific intracellular pathways or mediators with a proven role in asthma were effective when tested in mice, but not in horses or men, which confirms its suitability as a translational or preclinical model. For instance, p38 MAPK inhibitors and PDE4 inhibitors were poorly effective when administered as monotherapy in asthmatic horses (Kolm, Zappe et al. 2003, Lavoie, Thompson et al. 2008) and men (Lavoie, Pasloske et al. 2006, Chopra, Kanoje et al. 2008, Bhavsar, Khorasani et al. 2010), despite showing promising outcomes in rodent models (Ma, Medicherla et al. 2008, Kim, Kim et al. 2016).

Using horses as an asthma model is however not free of drawbacks. A direct consequence of their large size is limited accessibility and higher cost for drugs, breeding facilities and equipment, procedure materials, and ordinary care. An equine tissue bank has been developed by our group for respiratory research (http://www.ertb.ca), which makes this model available to researchers lacking the facilities or the technical expertise required for handling these animals. Also, few antibodies have been validated for this species (Schnabel et al., 2013) and newly-discovered immune system cells may not yet be characterized in horses. The equine genome has been entirely sequenced (Wade et al., 2009), which facilitates the identification of homologous sequences among different species in order to improve cross-reactivity. Studying subjects of different breed, size, age and origin increases intra-group variability and further

complicates data analysis and interpretation. However, it provides a heterogeneous population similar the human one.

Severe equine asthma model: state of the art

Few studies have been published in which the asthmatic horse has been used as a model for human asthma in order to respond to specific questions concerning disease pathophysiology. New data emerging from these studies are however remarkable: the first evidence that peripheral ASM and ECM remodeling is at least partly reversible (even when naturally occurring and established for many years) by corticosteroid or antigen avoidance treatment was obtained in this species (Leclere, Lavoie-Lamoureux et al. 2012, Setlakwe, Lemos et al. 2014), together with the convincing demonstration of a mechanical difference existing between tracheal and bronchial smooth muscle of asthmatic lungs (Matusovsky, Kachmar et al. 2015). The inhibitory effect produced by ICS on the expression of the (+)insert isoform of SMMHC in the central airways was first demonstrated in asthmatic horses (Boivin, Vargas et al. 2014). Evidence of the involvement of SRF and its cofactor myocardin in peripheral ASM remodeling has also been reported in this model (Chevigny, Guerin-Montpetit et al. 2015). However, asthma exacerbations protracted up to 30 days do not worsen peripheral ASM remodeling in asthmatic horses. These findings seem to contradict the enhanced decline in lung function observed in asthmatic patients experiencing multiple exacerbations (Bai, Vonk et al. 2007, Matsunaga, Hirano et al. 2015). However, the consequences of multiple episodes of asthma exacerbation may be greater on ASM remodeling compared to a single protracted episode (Chernyavsky, Croisier et al. 2014). The significant correlation observed in severe asthmatic horses in remission between the quantity of ECM lying between the epithelium and the smooth muscle and pulmonary resistance (Setlakwe, Lemos et al. 2014) suggests that an excessive collagen deposition could be the cause of the observed decline in lung function in these subjects. There are currently no precise data concerning the presence and reversibility of central airway remodeling in severe equine asthma.

Histopathologically, equine asthma was described decades ago as a chronic bronchiolitic process, eosinophilic in nature early in the course of the disease and successively causing peribronchiolar and parenchymal remodeling (Thurlbeck and Lowell 1964). However, at that time, the two different phenotypes of equine asthma now recognized (namely mild and severe, also known as IAD and RAO) were probably considered as a unique pathological entity. This would explain why recent studies focusing solely on the severe form do not recognize the presence of eosinophilic infiltrate (Dubuc and Lavoie 2014). Unfortunately, histopathological reports describing specific pulmonary findings of mild forms of equine asthma are not currently available to validate our explanation.

Hypothesis and objectives

Small airway dysfunction is now considered a crucial component of asthma pathophysiology. Due to small airway inaccessibility, large airways are commonly sampled to monitor the disease response to several interventions. Structural changes of large and small airways in asthma are now recognized thanks to post-mortem studies systematically investigating remodeling and inflammation along the bronchial tree (Pini, Hamid et al. 2007, Araujo, Dolhnikoff et al. 2008, James, Elliot et al. 2012, Elliot, Jones et al. 2015). Nonetheless, it is not known whether remodeling and inflammation respond similarly to treatment or not, both in term of magnitude and rapidity of the effect. The preferential deposition of inhaled therapies in the oropharynx and large airways (Leach, Kuehl et al. 2012, Leach, Kuehl et al. 2015) argues for a greater effect centrally than peripherally. In this thesis, the severe equine asthma model has been used to investigate this aspect of the disease.

General hypotheses

- Central airways develop structural remodeling in severe equine asthma.
- Inhaled corticosteroid monotherapy reverses central airway remodeling in a shorter period of time compared to peripheral remodeling.
- The combination of inhaled corticosteroid and long-acting β_2-agonist enhances peripheral airway remodeling reversal via a greater control of inflammation.

General objectives

- To develop and validate a protocol that can reliably assess central airway remodeling in equine asthma, with a particular interest in ASM mass.

- To assess and compare the dynamics of central and peripheral airway remodeling reversal following long-term treatment with inhaled corticosteroids in the presence or absence of add-on bronchodilator administration (long acting β_2-agonist).

Methods

Experimental design

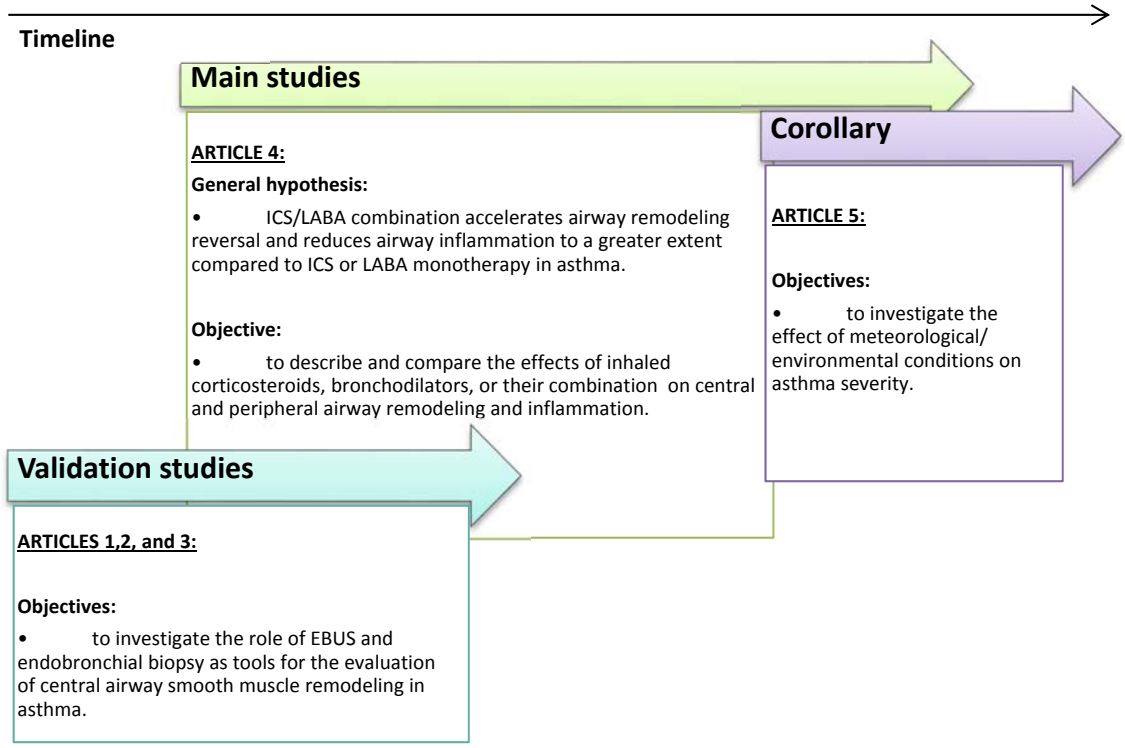

Figure 12. Overall experimental design. ICS: inhaled corticosteroids; LABA: long acting β_2-agonists; EBUS: endobronchial ultrasound.

In order to satisfy the objectives of this thesis, a series of studies was initially performed to determine the most appropriate approach to assess central airway remodeling in the equine model of asthma. Specifically, we investigated the reliability of endobronchial biopsies and endobronchial ultrasound (EBUS) for the estimation of ASM mass and evaluating any disease-induced quantitative alteration. A histological score for endobronchial biopsy samples based on morphological and inflammatory parameters was developed and validated, as well as an optimized protocol for endobronchial ultrasonography of the equine bronchi *in vivo*. With these tools implemented and available, the main studies were undertaken. They aimed at characterizing qualitative and quantitative aspects, as well as the kinetics, of airway

remodeling and inflammation reversibility in response to drugs in equine asthma. Two longitudinal studies were performed during which the effects of 3-month of fluticasone monotherapy, salmeterol monotherapy, fluticasone/salmeterol combination, or antigen avoidance protocol were compared. A further study was then performed as a corollary, in order to investigate whether environmental heat could negatively affect lung function in severe asthmatic horses. This hypothesis stemmed from the observation made during the main studies that several animals experienced a worsening of their clinical signs on days of heatwave.

Results

Article 1

Technical and physiological determinants of airway smooth muscle mass in endobronchial biopsy samples of asthmatic horses

Summary

This article aims at investigating the exploitability and reliability of endobronchial biopsy samples in the assessment of central airway remodeling in equine asthma, with particular attention to the ASM component. The data obtained suggest that endobronchial sampling in horses is a safe procedure producing good quality samples of bronchial tissue. Endobronchial biopsies permit quantitative morphological analysis of the airway epithelium and ECM deposition and/or composition. However, the smooth muscle layer can be evaluated only qualitatively and not quantitatively in equine endobronchial biopsies.

Contribution

I participated in study design (70%), experimental procedures and data collection (management of horses and treatment administration, 100%; impulse oscillometry function testing, 80%; bronchoscopy procedures, 60%; endobronchial biopsy processing, 100%; morphometric analysis, 100%; statistical analysis, 90%), and preparation of the manuscript (90%).

Article published

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TECHNICAL AND PHYSIOLOGICAL DETERMINANTS OF AIRWAY SMOOTH MUSCLE MASS IN ENDOBRONCHIAL BIOPSY SAMPLES OF ASTHMATIC HORSES

Michela Bullone[1], Mylène Chevigny[1], Marion Allano[1], James G. Martin[2] and Jean-Pierre Lavoie[1]

[1] Department of Clinical Sciences, Faculty of Veterinary Medicine, Université de Montréal, QC, Canada.

[2] Meakins-Christie Laboratories, Department of Medicine, McGill University, QC, Canada.

Running head: ASM mass determinants in endobronchial biopsies

Correspondence should be addressed to: Jean-Pierre Lavoie, Department of Clinical Sciences, Faculty of Veterinary Medicine, Université de Montréal, 3200 rue Sicotte, St-Hyacinthe J2S6C7, QC, Canada ; email : jean-pierre.lavoie@umontreal.ca.

Abstract

Morphometric analyses of endobronchial biopsies are commonly performed in asthma research but little is known concerning the technical and physiological parameters contributing to measurement variability. We investigated factors potentially affecting biopsy size, quality, and airway smooth muscle (ASM) content in heaves, an asthma-like disease of horses.

Horses with heaves in clinical exacerbation (n=6) or remission (n=6) from the disease and 6 controls were studied using a cross-over design. The effect of disease status, age, bronchodilation, biopsy forceps type and carina size on total biopsy area (A_{tot}), ASM area (A_{ASM}), ASM% (A_{ASM}/A_{tot}) and histologic quality were assessed. Concordance among different measuring techniques was also assessed.

Compared to other groups, horses with heaves in exacerbation yielded larger biopsies ($p<0.05$). Better quality biopsies were obtained from carinae of small sizes compared to large ones ($p=0.02$), and carina size and forceps type significantly affected the ASM content of the biopsy (interaction, $p<0.05$). A_{ASM} increased with age only in heaves-affected horses ($r=0.9$, $p<0.05$), and ASM% was negatively correlated with pulmonary resistance at 5 Hz in heaves-affected horses ($r=-0.74$, $p=0.01$), likely because of the increased thickness of the extracellular matrix layer in this group ($p=0.01$).

In conclusion, disease status, carina thickness, and the forceps used may significantly affect biopsy size, quality, and ASM content. Endobronchial biopsies are not appropriate samples for ASM quantification in heaves, and studies measuring ASM mass should not be compared when measuring techniques differ.

Keywords: endobronchial biopsies, asthma, airway smooth muscle, remodeling, horse.

Introduction

Endobronchial biopsies are important diagnostic and research tools in respiratory medicine. At present, they are the only means allowing histologic evaluation of central bronchial architecture, inflammation, and remodeling in patients with diseases such as asthma, COPD and cystic fibrosis. Nevertheless, few studies have systematically investigated factors likely affecting biopsy quality, and the measurement of their airway smooth muscle (ASM) content. Also, the effect of lung diseases on biopsy morphological parameters has not been addressed, although endobronchial biopsies are anecdotally known to be easier to obtain in asthmatic patients than in healthy individuals (10). Endobronchial biopsy in man is routinely performed under local anesthesia, following patient premedication with bronchodilating agents, commonly a β2-selective agonist followed or not by atropine (6). However, biopsy collection in the absence of inhaled β-adrenergic agonists has been performed in healthy and asthmatic patients without obvious adverse effect (30). No data are available evaluating the effect of prior bronchodilator treatment on biopsy size and quality. Also, little information is available on the contribution of the biopsy site or the type of forceps used to the quality of the biopsies (15).

Endobronchial biopsies allow the study of ASM quantity and composition, which are considered central to airway obstruction in human asthma (5, 18, 19). Indeed, ASM mass is increased in small and large airways in asthmatics, likely induced by both cellular hyperplasia and hypertrophy (2). However, the lack of correlation between ASM mass in endobronchial biopsies and lung function in mild asthmatics (22) and in an asthma-like disease of horses (heaves) has been reported (16). Whether this is due to the variable makeup of contractile protein in ASM or technical bias due to the biopsy collection or to the measurement method used has not been studied. Interestingly, when ASM mass was evaluated in bronchial biopsies of asthmatics as ASM percentage, a significant decrease was reported after antigen challenge. This finding was completely reversed by pretreating patients with a β2-adrenergic agonist for one week (12). The same reduction in ASM mass (%) was observed in heaves after antigen challenge in which bronchodilator pretreatment was not employed (16).

We hypothesized that the administration of a bronchodilator prior to the biopsy collection ameliorates the specimen quality by inhibiting bronchospasm in asthma. The primary objectives were to evaluate I) the effect of bronchodilation, forceps type, and the size of the carina on histomorphometric analysis of endobronchial biopsies in healthy and diseased airways; II) the influence of different analysis methods on ASM mass; and III) the relationship between endobronchial biopsy ASM content and lung function. This study was performed in horses with heaves, as remodeling of the airways in this natural disease has many similarities with that of human asthma (17). This model allows easy and relatively non-invasive bronchial tissue collection from central airways *in vivo*.

Material and Methods

Animals

Eighteen horses of mixed breed and age (mean±SD: 15.1±5.1 years) from the equine research and teaching herds of the Faculty of Veterinary Medicine (Université de Montréal) were studied. Twelve horses had a documented history of heaves, while the 6 control horses were considered free of respiratory disease on the basis of history, physical examination, and negative response to hay challenge. This study was approved by the Animal Care Committee of the Université de Montréal (Protocol Rech-1324) and conducted in compliance with guidelines of the Canadian Council on Animal Care.

Biopsy specimen collection and processing

A videoendoscope (13mm Ø, CF-H180AL, Olympus, ON, Canada) was passed through a nostril down to the lower airways in horses sedated with detomidine (0.012 mg/kg IV) and butorphanol (0.01 mg/kg IV). Airways were locally anesthetized with lidocaine solution (0.5%). Only one lung was sampled during each experiment. The right lung was biopsied during the first day of the study and the left lung during the second day. Biopsies were collected starting from the most caudal carina available [2.9 or 1.9, as described by Smith (27)] and then moving cranially, until reaching the main carina, following the scheme illustrated in **Figure 1**. Importantly, carinae were paired right-left based on their dimensions and position along the bronchial tree in order to reproduce as much as possible the same conditions during the two experiments. Six biopsy specimens were obtained during each experiment. Biopsy sites were classified as small or large depending on carina size. Three small biopsies from small carinae and three from large carinae were collected during each experiment. The main carina of each horse was biopsied twice, at different sites. Biopsy specimens were formalin-fixed and then enclosed in agar cylinders (**Figure 2**). Two 5 µm-thick histologic slides were obtained from each biopsy at 100-300 µm distances and stained with hematoxylin phloxine saffron (HPS).

Histologic analysis

Slides were digitized at 20x magnification with NanoZoomer 2.0-HT system (Hamamatsu Photonics, SZK, Japan). Biopsies were scored 1 (very poor) to 5 (optimal) for histological quality (**Table I**). Total biopsy area (A_{tot}), airway smooth muscle area (A_{ASM}), basal membrane length (BM) and the distance between epithelium and smooth muscle bundles were measured using ImageJ software (NIH, Bethesda, MD, USA)(**Figure 3A, 3B and 3C**). The A_{ASM}/A_{tot} ratio (or ASM%), the A_{ASM}/BM and the A_{ASM}/BM^2 ratios were calculated for each biopsy. Two further morphological parameters were calculated with newCast software version 4.5.1.324 (Visiopharm, Denmark) using a stereology-based approach (**Figure 3D**). ASM volume fraction ($V_{V(ASM)}$) was measured by point counting and expressed as the fraction of the total number of points falling on smooth muscle (P_{ASM}) over the total number of points falling on the biopsy (P_{ref}):

$$Vv_{ASM} = \frac{\sum P_{ASM}}{\sum P_{ref}}$$

The volume to surface ratio of smooth muscle per length of basal membrane ($V_{V(ASM)}/S_{V(BM)}$) was calculated by counting points falling on smooth muscle (P_{ASM}) and line intersections with the basal membrane (I_{BM}), as follow:

$$\frac{Vv_{ASM}}{Sv_{BM}} = \frac{P_{ASM} \cdot l(p)}{2\sum I_{BM}}$$

Where *l(p)* was a constant determined by the density of line probes and corresponded to 0.02421. A minimum of 200 points was counted for ASM from at least two biopsies per horse. Coefficient of error of the measurements (CE) was calculated accepted only if <0.1. All measurements were performed blindly by the same investigator.

Respiratory mechanics

Impulse oscillometry (IOS) was performed as described by van Erck (28) with the Equine MasterScreen IOS system (Jaeger GmbH, Würzburg, Germany). In brief, horses breathed through a mask connected to a loudspeaker generating multi-frequency impulses. The pressure-flow signal response of the respiratory system superimposed on the animal tidal breathing was measured by a pressure transducer connected to a pneumotachograph and placed directly in front of the face mask. Data were acquired for a minimum of 30 seconds and analyzed (LabManager, version 4.53, Jaeger GmbH, Würzburg, Germany and FAMOS, imc Mess-Systeme GmbH, Berlin, Germany) using Fast-Fourier transform to compute the resistance (R) and reactance (X) of the respiratory system at frequencies down to 1 Hz (25). Only values at frequencies ≤10 Hz were analyzed as they have been shown to reliably represent lung function in horses (13, 32). Recordings with a low coherence function were excluded from analyses (13).

Study design

A cross-over case-control study was performed. Six horses with heaves in clinical exacerbation of the disease (HE) and six control horses (C) were stabled and exposed to hay and dust starting two weeks before the study period. Six additional horses with heaves were kept at pasture and administered dexamethasone (0.06 mg/kg PO q24h) starting one week before the first biopsy collection and for the duration of the study in order to induce clinical remission of the disease (HR).

Horses were studied twice, at a 4-day interval, with and without the administration of a bronchodilator agent (N-butylscopolammonium bromide, 0.3 mg/kg, Buscopan®, Boehringer Ingelheim, Germany)(4). The order of treatment (bronchodilator vs placebo) was randomly determined for each subject. Lung function was evaluated using IOS before (baseline) and 15 minutes after sedation, independently of whether or not they received or not the bronchodilator 5 minutes after sedation. During the first series of experiments, three of the six biopsies randomly chosen for each horse were obtained with a smooth oval disposable biopsy forceps

(2.85 mm in diameter, FB-234U, Olympus, ON, Canada), while the remaining three were taken with an alligator jaw disposable forceps (2.85 mm in diameter, FB-214U, Olympus, ON, Canada). During the second series, forceps selection was simply inverted.

Statistics

Statistical analysis was performed with SAS v.9.3 (Cary, NC, USA) and Prism 5 (GraphPad Software Inc, La Jolla, CA, USA). The effect of technical parameters (bronchodilator administration, forceps type, carina size and disease status) on morphological variables (A_{tot}, A_{ASM} and ASM%) was studied with a repeated-measures linear model. The effect of technical parameters on biopsy quality was assessed with a generalized estimating equation model for ordinal variables. Technical parameters were always considered as intra-subject factors. The effect of multiple uses of the forceps on biopsy quality was assessed with the Cochran-Mantel-Haenszel test, considering repeated measures observed for every subject. Re-biopsy parameters were analyzed with two-way ANOVA and Bonferroni post-test. One-way ANOVA or χ^2 test was used for comparison of continuous variables or proportions between the 3 groups. Paired t-tests were used for comparison of lung function parameters before and after bronchodilator administration within the same group. Correlations were assessed with Pearson test when $N>8$ and data were distributed normally (Kolmogorov-Smirnov test) or Spearmen test when $N<8$ or data were not normally distributed. For all analysis, a p-value<0.05 was considered as significant.

Results

Biopsy specimens

The procedure was well tolerated by all horses, with only mild bleeding occasionally noticed at the biopsy site. A total of 216 biopsies were collected during the study period, all of which were considered of appropriate size. Two biopsies were lost during the embedding process; 214 biopsies were finally analyzed.

Overall, 39.2% of biopsies were considered as being of good to optimal quality (score 4 and 5), 20.6% as partially suitable for assessment (score of 3), and 40.2% were of poor or very poor quality (score of 2 and 1) and not suitable for analysis. Smooth muscle bundles were identified in 84.6% of biopsies, with no significant differences between groups (p=0.98).

I. Technical variables

Disease status had a strong effect on A_{tot} (p=0.003), with the size of the biopsies collected from heaves-affected horses during exacerbations being significantly larger than biopsies obtained from both horses with heaves in remission and control horses ($p<0.05$). No significant difference was found between A_{tot} of heaves asymptomatic and control horses (**Figure 4A**). Biopsy A_{ASM} (p=0.38), ASM% (p=0.87), and the quality score of the biopsies (p=0.49) were not affected by disease status (**Figure 4B, 4C** and **4D,** respectively). Bronchodilation did not affect A_{tot} (p=0.16), A_{ASM} (p=0.2), ASM% (p=0.64) or quality score of the biopsies (p=0.11).

The size of the carina and the forceps type did not significantly affect A_{tot} (p=0.73 and p=0.3, respectively), A_{ASM} (p=0.46 and p=0.85) or ASM% (p=0.49 and p=0.5). However, significant interactions were found between the size of the carina and the forceps used, both for A_{ASM} and ASM% (p=0.046 and p=0.04, respectively). This was due to a trend for A_{ASM} (p=0.06) and ASM% (p=0.052) to be lower in large carinae compared to small ones with smooth oval forceps, but not with alligator jaw forceps (p=0.47 and p=0.36, respectively). There was also a

trend (p=0.058) for ASM% to be higher in biopsies obtained from small carinae with smooth oval forceps compared to those obtained with jaw alligator forceps. No difference was found between mean ASM% of biopsies obtained from large carinae with smooth oval forceps compared to those obtained with jaw alligator forceps (p=0.36).

The quality of the biopsies was not influenced by the forceps used (p=0.08). However, the size of the carina had a significant effect on histologic quality of the biopsies (p=0.02). There was a 2.2 times increased probability of obtaining an optimal quality biopsy when the sampling site was a small carina compared to a large one. Reutilizing the same forceps for collecting biopsies from 6 to 8 horses did not result in significant changes detectable on biopsy quality (p=0.61) or biopsy size (p=0.41).

Only the main carina was biopsied twice at a 4-day interval in each horse, and at a distance allowing the avoidance of a biopsy being obtained that overlapped the site of the first biopsy taken. Re-biopsy did not significantly affect A_{tot} (p=0.19). However a significant effect of the group was observed for A_{tot} (p=0.009). Interestingly, A_{tot} was increased in re-biopsies of 67% horses with heaves in exacerbation and controls, but not in horses with heaves in clinical remission. ASM% was significantly decreased in re-biopsies (p=0.005) and a similar trend was observed for A_{ASM} (p=0.05). Biopsy quality was similar in the first and second biopsies (p=0.65).

II. Analysis methods

When only good quality biopsies (grades 3 to 5) were analyzed, smooth muscle content increased significantly compared to when all biopsies were analyzed together, independently of the parameter used for its quantification (from [mean±SD] 0.54±0.31 to 0.68±0.33 mm^2 for A_{ASM}, p=0.0003; from 25±7% to 32±6% for ASM%, p=0.005). A_{ASM} and ASM% increased on average 26% and 23%, respectively, after exclusion of poor quality biopsies. Also, intra-subject variability expressed as the coefficient of variation decreased significantly when only good quality biopsies were taken into account (p=0.002 with an average 25% reduction in variability for A_{ASM}, and p=0.0001 with an average 35% reduction in variability for ASM%).

Comparison between all and only good quality biopsies was not performed for A_{ASM}/MB, A_{ASM}/MB^2, $Vv_{(ASM)}$ and $Vv_{(ASM)}/Sv_{(MB)}$ as these parameters were calculated only for good quality biopsies. Considering only good quality biopsies, intra-subject variability of smooth muscle quantification varied significantly depending on the analysis method used (p=0.02), and it was higher when expressed as A_{ASM} compared to ASM% or $Vv_{(ASM)}$ for the same biopsy ($p<0.05$).

In good quality biopsies, correlations between ASM% and its stereological equivalent $Vv_{(ASM)}$, and between ASM/BM and its stereology equivalent were significant in all groups, confirming the appropriateness of our counts. However, correlations among different measuring techniques are poor and vary significantly among groups. Also, the measuring technique can significantly affect the outcome of the study (see **Table II and Figure 5**).

III. Structure-function relationships and other physiological variables

There was a significant difference in total lung resistance (R_3, p=0.02), reactance (X_3, p=0.009), and R_5:R_{10} ratio (p=0.01) among the 3 groups of horses at baseline (**Table III)**. Higher values of R_3, X_3 and R_5:R_{10} were observed in horses with heaves in exacerbation compared to controls ($p<0.05$). Values of airway function (Z_5, R_3, X_3) were not significantly different during the two days of experimentation for each group ($p>0.05$). The sedation with detomidine and butorphanol caused a significant increase of inspiratory resistance values, suggesting upper airway obstruction. Buscopan administration, but not placebo treatment, caused a bronchodilation in horses with heaves whether in exacerbation or in remission of the disease, as shown by the significant decrease in the R_5:R_{10} ratio (29) (**Table III** and **Figure 6**).

When only good quality biopsies were included in the analysis, baseline R_5 values were positively correlated with ASM% (r=0.84, p=0.03) in control horses, but only when bronchodilation was induced (**Figure 7A**). Interestingly, a negative correlation was observed between baseline R_5 and ASM% in heaves-affected horses after bronchodilation (horses with heaves in exacerbation and remission of the disease pooled together, p=0.01, r=-0.74) (**Figure 7B**). The negative correlation could be explained by an increased volume of the extracellular

matrix in heaves affected horses, as the distance between epithelium and smooth muscle bundles was increased compared to controls ([mean±SD] 0.143±0.049 mm in horses with heaves and 0.082±0.031 mm in controls, p=0.01, **Figure 8**) and the external border of the smooth muscle bundle was less frequently identifiable in biopsies from these animals (32% and 13% of biopsies from controls and horses in exacerbation, respectively).

When only good quality biopsies were considered, A_{ASM} was strongly correlated with age in both groups of horses with heaves (exacerbation: p=0.04, r=0.90; remission p=0.03, r=0.91) but not in control horses (p=0.85, r=0.10). Moreover, the regression lines derived from these data were significantly different between horses with heaves in exacerbation and remission of the disease (similar slopes, p=0.09; but different intercepts, p=0.02), as well as between horses with heaves in exacerbation and controls (different slopes, p=0.049). No significant differences were observed between horses with heaves in remission and controls, possibly because of the absence of very old horses in the control group (**Figure 9A**). The same trend was observed for A_{tot}, but correlation coefficients did not reach significance (r=0.82, p=0.09 for horses with heaves in exacerbation; r=0.63, p=0.17 for horses with heaves in remission and r=0.08, p=0.9 for controls). However, a significant difference was found between the regression lines constructed for the three groups from A_{tot} values plotted versus age (similar slopes, p=0.6; different intercepts, p=0.0009; **Figure 9B**).

Discussion

Previous studies reported a decrease in the ASM mass in endobronchial biopsies of asthmatic patients and horses with heaves after antigen exposure (12, 16). We postulated that methodological factors contributed to these findings, and therefore we investigated the effect of technical and physiological variables possibly affecting endobronchial biopsy quality and morphology. Contrary to what we had hypothesized, bronchodilation did not improve biopsy morphology or quality; however it allowed normalization of the structure-function relationships in our study. Indeed, when analyzing only biopsies obtained after bronchodilation, we detected significant negative correlations between ASM content of the biopsies (ASM%) and lung function in horses with heaves and controls, clearly linked to the remodeling features of the bronchial extracellular matrix. Also, biopsies obtained during heaves exacerbation (comparable to asthmatic attacks) were significantly larger (increased A_{tot}) than those obtained during the remission phase of the disease (comparable to the controlled asthmatic state) or from control horses, indicating that disease status is a critical determinant of commonly employed histomorphometric parameters. Biopsies from medium and small carinae increase the probability of obtaining good quality biopsies with greater quantity of smooth muscle, especially when forceps with smooth cutting surfaces are employed. Nevertheless, quantification of ASM mass is not reliable when performed on equine endobronchial biopsies, independently on the sampling techniques and analysis methods. Indeed, as even larger forceps rarely sample the full depth of the smooth muscle layer, ASM mass assessment can be biased if growth of smooth muscle cells goes towards the adventitial layer.

Effect of technical variables on biopsy size and quality

The size and quality of the biopsy specimens can greatly affect the assessment of morphological and histopathological features (10). We used the largest biopsy forceps instrument passing through the working channel of the endoscope in order to maximize biopsy size (10, 23), which explains why we obtained larger biopsies than those generally obtained in

men (on average, A_{tot} was 2 mm^2 in horses and 1mm^2 in man (15)). However, we likely sampled to a similar depth the bronchial tissues, as the central equine airways are also larger than human ones. Indeed, approximately 60% of our attempts provided samples suitable for histologic assessment, which is a similar result to that observed in adult asthmatics (15). A_{tot} was significantly affected by disease status, with horses during disease exacerbations (~asthma attacks) yielding larger biopsies than control horses or horses in clinical remission of the disease. At first sight, these results contrast with findings in human in which no significant differences were found in A_{tot} of biopsies from asthmatic and control patients (15, 23), or from severe and moderate asthmatic patients (11, 22). However, in these studies, asthmatic patients were under treatment or their disease was stable for months prior to sampling. Our results are thus in agreement with these findings, as no significant differences were shown between horses with heaves in remission of the disease and controls concerning the size of their biopsies. We attributed the increased A_{tot} of biopsies of subjects with heaves in exacerbation to the increased tissue inflammation during the active phase of the disease, leading to edema and tissue fragility. Very little is known on tissue morphology during spontaneous asthmatic attacks in man due to ethical, management and safety concerns and equine heaves is perhaps the only animal disease that allows studying this aspect of the disease prospectively.

Biopsy quality was significantly affected only by carina size. Smaller carinae were 2.2 times more likely to provide good quality biopsies than larger ones. Despite large forceps being used, main carina and carinae of the first generation of equine bronchi are often too thick to allow optimal forceps gripping, especially in symptomatic horses (14). On the other hand, contrarily to what is reported in man (15), almost all of the accessible carina sites in the horse are large enough to provide good quality endobronchial biopsies of the airway wall. This finding may explain why targeting smaller carinae improves the histological quality of the biopsy and the possibility to sample the ASM layer entirely in horses.

Effect of technical variables on ASM content

In our study, endobronchial biopsy ASM mass was not significantly affected by disease status when all biopsies were analyzed together. Despite an obvious trend was observable for A_{ASM} in our data, the difference did not reach statistical values, probably because of three reasons: the relatively small number of animals employed, the fact of having three groups instead of two, and the high intra-group variability of data. However, excluding poor quality biopsies, we found a significant effect of disease status only when ASM was quantified as A_{ASM}. To the same extent, differences were not statistically significant between A_{ASM} of horses with heaves in exacerbation and in remission because of lack of statistical power. We are aware of a single previous study in which a significant difference was found between A_{ASM} of moderate and severe asthmatics (22). More often ASM remodeling is reported as ASM%, and differences between asthmatics and controls are observed in most (1, 23, 26) but not all (15, 20, 26) studies investigating this aspect. ASM% did not change significantly among groups in our study. Importantly, biopsies were collected from the same sites in all subjects in order to avoid differences arising only as a consequence of the different proportion of small versus large carinae sampled. Indeed, we have shown that the carina size significantly affects the ASM content of the biopsy, especially when forceps with smooth cutting surfaces are employed.

Re-biopsy findings

The same carina may be biopsied twice or more in prospective human studies, due to limited number of sites for endobronchial biopsies in man (15). Because the effects of re-biopsying the same site on biopsy morphology or size are not known (10), we sampled the main carina on two occasions 4 days apart. Re-biopsy was associated with a generalized increase in A_{tot} which we hypothesized was the consequence of the inflammation induced by the first biopsy, as this effect was prevented by the administration of dexamethasone (group in remission of the disease). Also the ASM mass decreased in the re-biopsies, indicating that inflammation-induced submucosal edema possibly increased the distance between epithelium and ASM, as previously shown in asthmatics (22). As a consequence, short-term re-biopsy of the same site

should be avoided when investigating ASM mass or ECM content, even when performed at sites visually appearing to lack inflammation. Further studies may clarify how much time should elapse before a re-biopsy could be obtained without significant effect on tissue morphology in the absence of anti-inflammatory therapy.

Comparison among different measuring techniques

Endobronchial biopsy is the "gold standard" for the study of central airway remodeling (3). However, the high inter-subjects (up to 70%) and intra-subject (20%) biological variability (10), and the lack of standardization of the methods employed for tissue structure analysis (9) limit the usefulness of these measurements. Remarkably, identification of external ASM boundaries, which is required to ensure that the full thickness ASM was sampled, does not represent a parameter constantly considered for biopsy quality assessment. Stereology has been proposed as the method of choice for lung morphometric studies, as it provides accurate quantitative data (8). However, it requires that the reference space is known and sampling is unbiased (21). In endobronchial biopsies, the reference space is unknown, and sampling is biased as it is limited to carinae and by the penetrating capacity of the forceps used (31). Basal membrane length should be employed as a correction factor as it avoids the "reference trap" when the reference space is unknown (8). However, there is no method available to correct for the incomplete and variable (non-quantifiable) sampling of the ASM layer. For these reasons, we suggest to include in future studies only biopsies in which the external border of the ASM is clearly identifiable, to assure unbiased analysis. A further confounding factor when comparing different studies is the measure unit used. We have shown that data obtained using different measuring units on the same samples do not correlate, as it had been reported for human patients (15). For these reasons, direct and deliberate comparison of studies employing different units should be avoided.

Structure-function relationships

As a secondary outcome, we analyzed the relationship between structural ASM parameters and physiologic functional data. We showed that values of resistance at 5 Hz correlated with ASM% in both controls and heaves-affected horses only after bronchodilation. These findings suggest that bronchospasm is induced by the biopsy procedure at least in control animals, which can possibly alter tissue remodeling measurements. In agreements with our findings, excluding data of asthmatic children unresponsive to bronchodilator therapy ameliorated the correlation between ASM mass in endobronchial biopsies and lung function (24), supporting the fact that inhibiting bronchoconstriction normalizes ASM remodeling data. In horses with heaves, less smooth muscle (%) was present in the biopsy as lung resistance increased. This was likely caused by the thickening of the extracellular matrix layer, widening the distance between epithelium and smooth muscle, as reported to also occur in asthmatics (1). In healthy man, ASM has been shown to increase linearly with airway diameter during infancy, but when adult age is reached, the proportion of ASM within the airway wall stabilizes (7). The same seems to be true for healthy horses. On the other hand, ageing (or disease duration) progressively increases the absolute quantity of ASM (A_{ASM}) in endobronchial biopsy samples of horses with heaves, but not its ASM% value. The reasons for these findings are still unclear, but likely reflect changes in tissue viscoelasticity and the shape of the carinae with ageing. Furthermore, the significant difference observed between the A_{ASM} regression lines of horses with heaves in exacerbation and in remission indicates that such increase in A_{ASM} occurs even more rapidly during active phases of the disease, possibly driven by inflammation which increases tissue fragility.

Conclusions and perspectives

In conclusion, our study provides evidence that while not affecting biopsy morphological parameters, bronchodilation improves the relationship between ASM in the central airways and lung function. Horses with heaves produce larger biopsies during disease exacerbations, with ASM mass being positively correlated with age and negatively correlated to disease

severity as measured by lung resistance. However, endobronchial biopsy provides inappropriate samples for quantitative studies of ASM remodeling, at least in horses.

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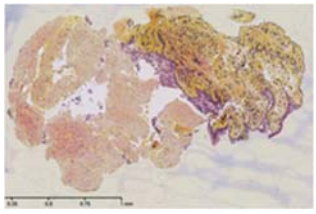
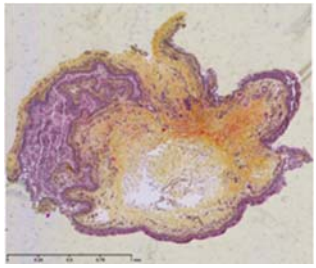
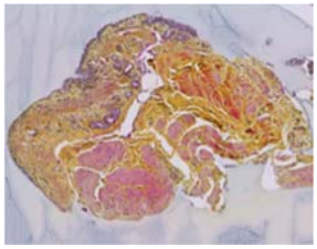
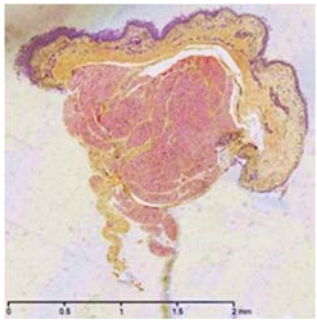
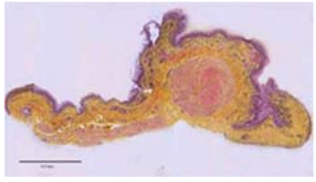
Figures and tables

Table I. Histologic quality score of biopsies.

Biopsy	Score	Description
	1	Unacceptable tissue orientation, there is no continuity between epithelium, ECM and smooth muscle
	2	Good tissue orientation, tissue architecture not completely preserved.
	3	Good tissue orientation, tissue architecture preserved at least in a consistent part of the biopsy (>50%).
	4	Optimal tissue orientation for all the biopsy, minor area where continuity between tissues is lost.
	5	Optimal tissue orientation for all the biopsy, tissue architecture is perfectly conserved. The parenchymal borders of the smooth muscle layer are clearly identifiable

Tissue orientation, architecture, and structure preservation as well as presence of ASM were evaluated in order to classify the biopsies for their quality using a score from 1 (poor quality) to 5 (optimal quality).

Table II. Correlation among different measuring methods.

	Control	**Heaves Remission**	**Heaves Exacerbation**	**All groups**	Difference between groups
			Correlation		
A_{ASM} [mm^2] - ASM ratio [%]	.186 (.62)	.383 (.44)	.424 (0.41)	.084 (.42)	-
A_{ASM} [mm^2] - A_{ASM} /MB [mm]	.082 (.76)	.778 (.15)	.355 (.46)	.118 (.38)	-
A_{ASM} /MB [mm] - ASM ratio [%]	.123 (.70)	**.007** (.93)	.606 (-.27)	**.045** (.47)	-
A_{ASM} /MB^2 [-] - ASM ratio [%]	.075 (.77)	**.041** (.83)	.598 (-.32)	.073 (.44)	-
Vv ASM [-] - ASM [mm2]	.129 (.69)	.581 (.29)	.26 (.62)	.236 (.29)	-
Vv ASM [-] - ASM ratio [%]	**.016** (.89)	**.015** (.89)	**.004** (.98)	**<.0001** (.93)	-
Vv ASM [-] - A_{ASM} /MB [mm]	**.008** (.92)	**.003** (.96)	.20 (.61)	**.0007** (.72)	-
Vv ASM [-] - A_{ASM} /MB^2 [mm]	**.031** (.85)	**.011** (.91)	.565 (.30)	**.004** (.64)	-
Vv ASM [-] - Vv ASM/Sv MB [mm]	.488 (-.36)	.093 (.74)	**.014** (.90)	**.011** (.57)	-
Vv ASM/Sv MB [mm] - A_{ASM} [mm^2]	.699 (.20)	.485 (.36)	.43 (.66)	.08 (.42)	-
Vv ASM/Sv MB [mm] - ASM ratio [%]	.80 (-.13)	**.005** (.94)	.33 (.55)	**.035** (.51)	-
Vv ASM/Sv MB [mm] - A_{ASM} /MB [mm]	**.051** (.64)	**.039** (.83)	**.028** (.86)	**.0001** (.78)	-
Vv ASM/Sv MB [mm] - A_{ASM} /MB^2 [mm]	.927 (-.05)	.098 (.73)	.339 (.58)	**.002** (.66)	-
					one-way ANOVA
A_{ASM} [mm^2]	-	-	-	-	**.04**
ASM ratio [%]	-	-	-	-	.96
A_{ASM} /MB [mm]	-	-	-	-	.32
A_{ASM} /MB^2 []	-	-	-	-	.70
Vv ASM []	-	-	-	-	.72
Vv ASM/Sv MB [mm]	-	-	-	-	.41

Data are expressed as the p (r) values resulting from Spearman (controls, heaves remission and heaves exacerbation) or Pearson (all groups) correlation tests or as p-values from one-way ANOVA test. Overall, there was poor correlation among different measuring methods, excepted for Vv ASM and ASM% and Vv ASM/SV BM and A_{ASM} /BM. Also, using different methods may lead to different results: a significant difference was indeed observed among group for A_{ASM} values ($p=0.04$) but not for the other measuring techniques ($p>0.05$).

Table III. Lung function measured by impulse oscillometry system (IOS).

	Control	Heaves Remission	Heaves exacerbation	*p*
Baseline				*Group effect*
R_3	0.0648 (±0.0177)	0.0805 (±0.0308)	0.1340 (±0.0381)[§]	**.02**
X_3	0.0074 (±0.0089)	-0.008167 (±0.0301)	-0.05683 (±0.0562)[§]	**.009**
R_5:$R_{10\ placebo}$	0.7052 (±0.19)	0.9309 (±0.2325)	1.214 (±0.1477)[§]	**.01**
R_5:$R_{10\ buscopan}$	0.8526 (±0.167)	1.219 (±0.696)	1.215 (±0.217)	**.06**
$Z_{5\ placebo}$	0.0720 (±0.0228)	0.09167 (±0.0204)	0.1050 (±0.0302)	.08
$Z_{5\ buscopan}$	0.0700 (±0.0187)	0.0767 (±0.025)	0.09833 (±0.0319)	.25
15' post sedation				
R_5:$R_{10\ placebo}$	0.7761 (±0.059)	0.952 (±0.196)	1.16 (±0.289)	.13
R_5:$R_{10\ buscopan}$	0.8854 (±0.198)	0.7447 (±0.132)[†]	0.904 (±0.172)[†]	.21
$Z_{5\ placebo}$	0.1167 (±0.055)	0.08167 (±0.0256)	0.1000 (±0.0473)	.39
$Z_{5\ buscopan}$	0.085 (±0.051)*	0.0740 (±0.0114)	0.095 (±0.0191)	.35

IOS was performed before and 15 minutes after sedation. Bronchodilator (buscopan) or placebo was administered 5 minutes after sedation. Data are expressed as mean (±SD). Resistance (R), reactance (X) and impedance (Z) values are expressed in kPa/L/s. P values reported express whether differences were observed among the 3 groups. [§]: Different from control group (one-way ANOVA and Dunns post-test). [†]: Different from R_5:R_{10} buscopan at baseline (p=0.03 for HR and p=0.02 for HE, paired t-test). *: Tends to be different from Z5 placebo at 15' post-sedation (p=0.07, paired t-test).

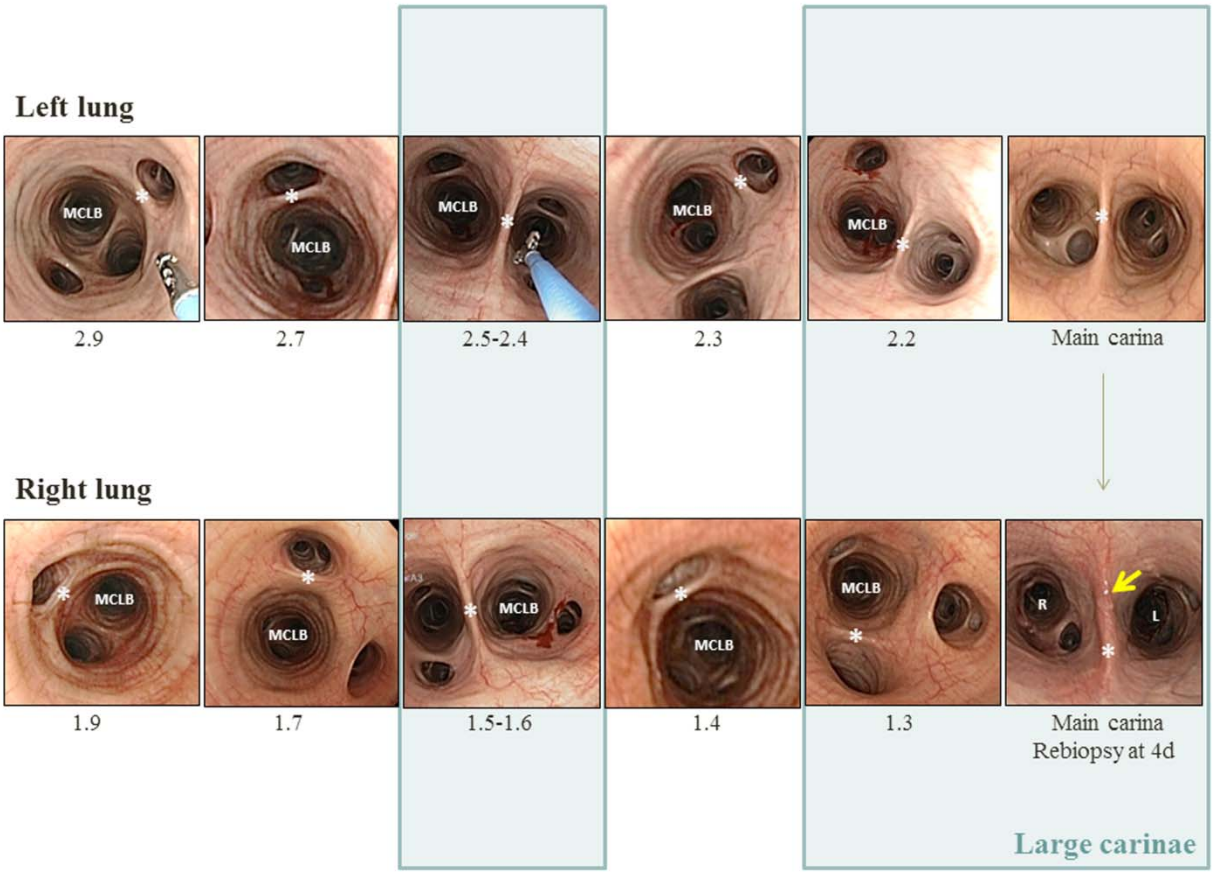


Figure 1. Biopsy collection scheme. Biopsy sites are indicated with stars. Biopsies were collected from the same sites in all horses. Biopsy sites were classified as small or large depending on carina size. Large carinae were defined as those where the accessory segmental bronchus (only for right lung), first segmental bronchus (or bronchi for the left lung) conducting air to the ventral part of the lung, and intermediate segmental bronchus stem from the main caudal lobar bronchus. Large carinae are shown within the blue boxes. The difference in carina thickness between large and small carinae can be easily appreciated. The yellow arrow indicates the site of the previous biopsy (4 days before) in the main carina; adjacent tissues are visibly inflamed and edematous. MCLB: main caudal lobar bronchus.

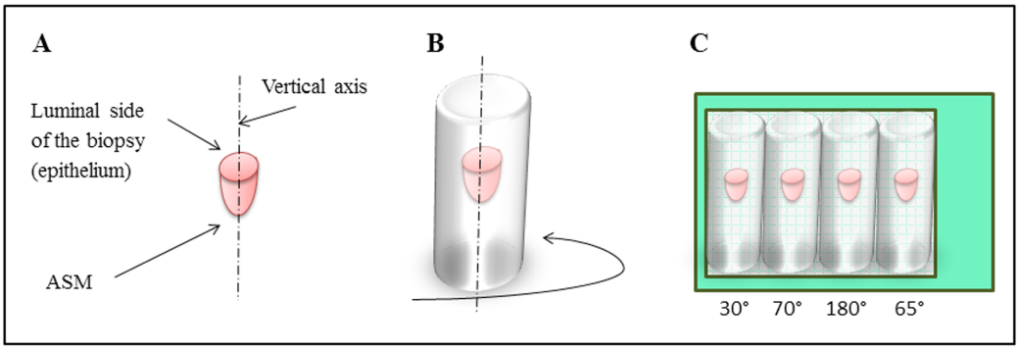


Figure 2. Biopsy processing protocol. Agar cylinders of 3 mm diameter and 20 mm in length were obtained by slowly pouring a heated (37-40°C) solution of 4% agar in manufactured molds, where biopsies were positioned with their vertical axis parallel to the vertical axis of the cylinders under stereoscopic microscope guidance (A). Cylinders were allowed to harden at room temperature (about 20°C) for 15 minutes before being randomly rotated on their vertical axis (B). A cut was made parallel to a random angle using a radial support. Agar cylinders were then positioned horizontally into the cassettes for paraffin embedding maintaining their random orientation, with their cut surfaces placed downward (C).

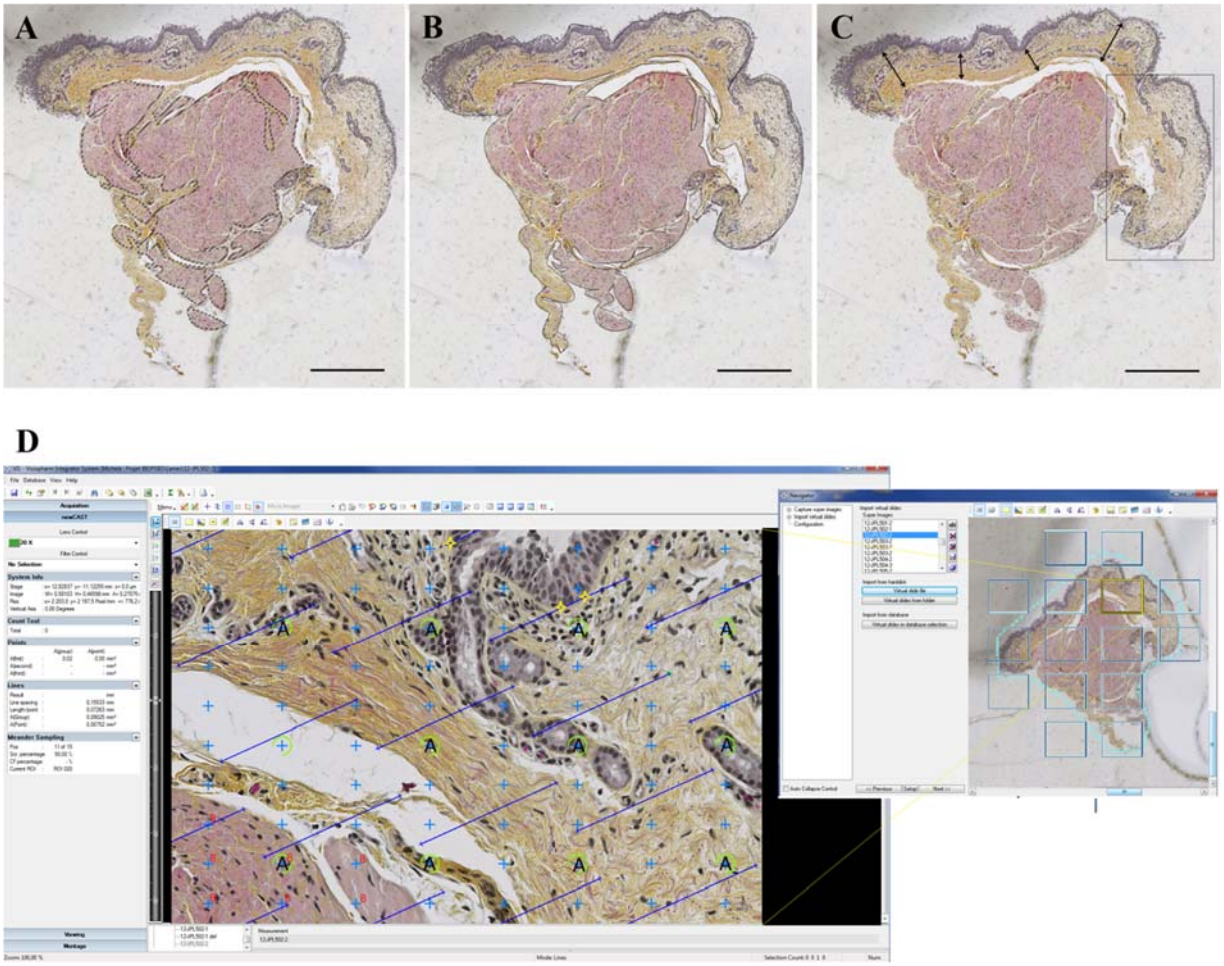


Figure 3. Histologic analysis of the biopsies. A_{ASM} and A_{tot} are clearly outlined for an illustrative biopsy in panels A and B, respectively. In C, arrows indicate the measure of the distance between the epithelium and the ASM layer. Of note, areas of the biopsies in which collagen fibers were less dense due to significant focal tissue shrinkage or other procedural artifact (as those shown within the black box) were not considered for this type of measure. In D, a frame illustrating the software used for stereological analysis of the biopsies. Left panel shows a magnification of the yellow area in the small right panel. Within the left panel, line grids were used for basal membrane surface density. Note the yellow crosses at the intersection between line probes and basal membrane, indicating I_{BM}. Also, blue crosses were used as probes for ASM mass, while blue crosses with green circles were used as probes for reference volume. Crosses marked with letters (A for P_{ref} and B for P_{ASM}) were used for counts.

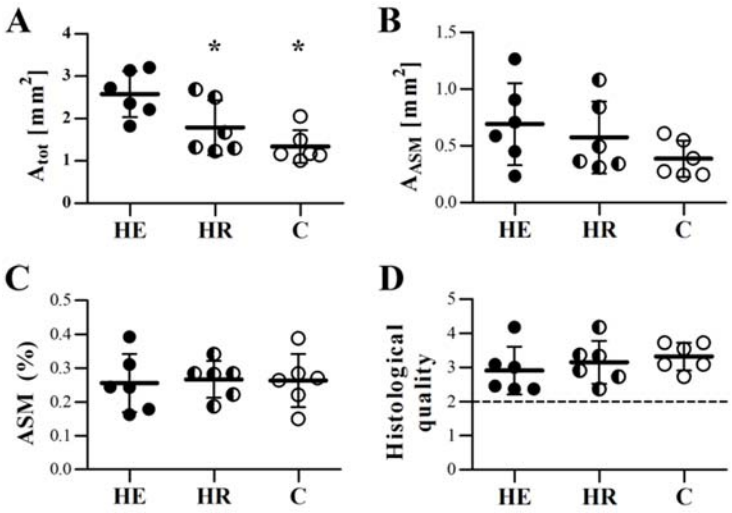


Figure 4. Effect of disease status on A_{tot} (A), A_{ASM} (B), ASM% (C) and histological quality (D) of the biopsies. Each point represents the mean value of all biopsies obtained from each animal (quality 1 to 5), excluded the re-biopsy site of the main carina. In B and C, the biopsies in which ASM was not present were included and the value of 0.00 was used for calculating the means. HE: heaves exacerbation; HR: heaves remission; C: controls.

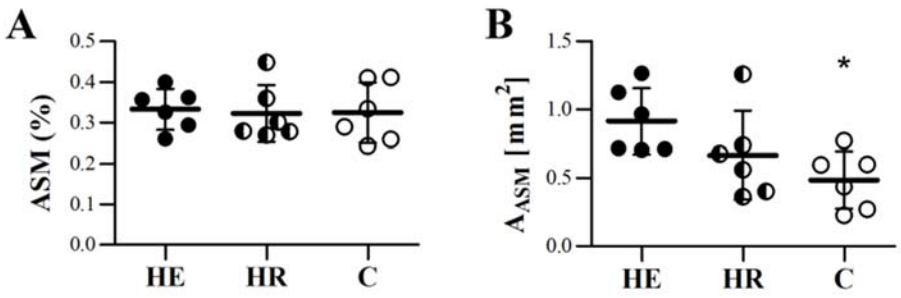


Figure 5. ASM remodeling expressed with 2 different measuring techniques in the same samples. Only good quality biopsies were used (score >2). In (A), data are expressed as ASM%, and no difference is observed among the 3 groups (p=0.96). In (B), data are expressed as A_{ASM} [mm^2], and a significant difference is detected among the 3 group (p=0.04), with A_{ASM} of controls (C) being significantly lower than those of horses with heaves in exacerbation (HE, $p<0.05$).

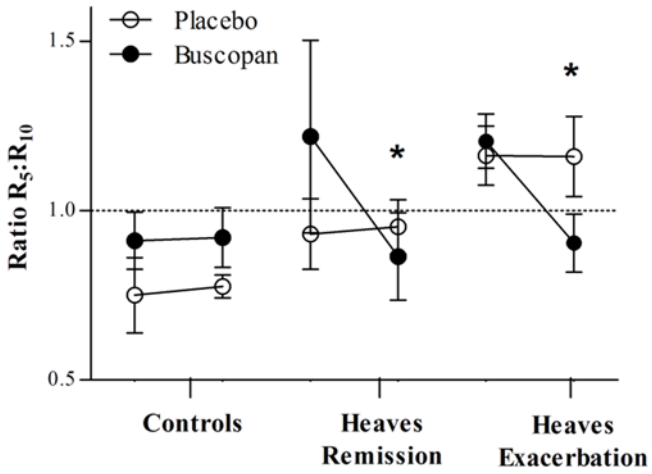


Figure 6. Bronchodilator effect on R_5:R_{10} ratio. When horses received placebo (saline, white points), no change was observed for the R_5:R_{10} ratio at baseline and 15 minutes after sedation/placebo, meaning that airflow obstruction was not significantly affected by sedation/placebo administration in the 3 groups. When horses received bronchodilator (Buscopan®, black points), the R_5:R_{10} ratio did not change in control horses but it significantly decreased in horses with heaves (both in exacerbation and in remission, $p<0.05$), indicating that airway obstruction was reduced after sedation/bronchodilator treatment in this group of animals.

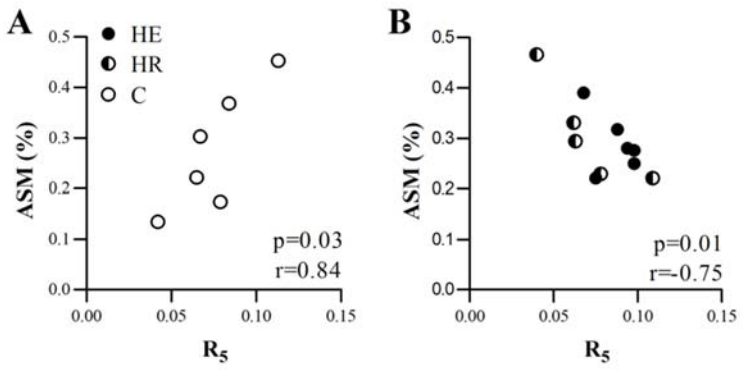


Figure 7. Correlation between ASM% and baseline peripheral lung resistance during days in which buscopan was administered prior to EBB collection in control (A) and heaves-affected horses (B). HE: heaves exacerbation; HR: heaves remission; C: controls.

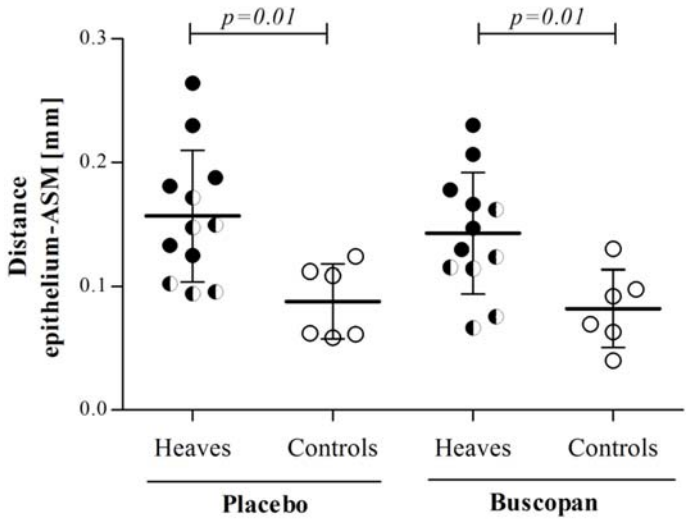


Figure 8. ECM thickness, evaluated as the distance between the bronchial epithelium and the ASM layer, is greater in horses with heaves compared to controls. Bronchodilation treatment had no significant effect on this parameter.

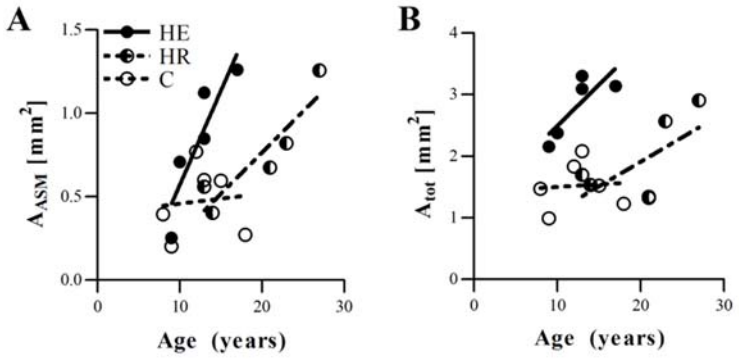


Figure 9. Correlation between age of horses and A_{ASM} (A) or total biopsy area (B). In horses with heaves, but not in controls, age is significantly correlated with A_{ASM}. Interestingly, disease exacerbation significantly shifts the regression line towards the left. A similar trend can be observed for total biopsy area (see the text for further details).

Article 2

Development of a semi-quantitative histological score for the diagnosis of heaves in endobronchial biopsies of horses

Summary

This article investigates whether structural and inflammatory changes appreciable in endobronchial biopsies of horses with heaves can be assessed using a semi-quantitative score, and whether such score correlates with functional parameters of lung health. Based on the results of this study, the score we have developed allows differentiating asthmatic horses from controls only when endobronchial biopsies are obtained during disease exacerbations. Nevertheless, the sum of all structural/morphological parameters of the score correlates with lung function in asthmatic horses both in exacerbation and in remission of the disease, but not in controls, indicating that, overall, it provides a reliable estimation of the degree of airway remodeling in equine asthma.

Contribution

I participated in study design (90%), data generation (preparation of histological sections, 50%; performing the score, 20%), communication with experienced pathologists involved in the study (70%), data analysis (100%), and preparation of the manuscript (90%).

Article submitted for publication

Submission to the *Journal of Veterinary Internal Medicine* completed on 11/24/2015.

DEVELOPMENT OF A SEMI-QUANTITATIVE HISTOLOGICAL SCORE FOR THE DIAGNOSIS OF HEAVES IN ENDOBRONCHIAL BIOPSIES OF HORSES

M. Bullone, P. Hélie, P. Joubert and J.P. Lavoie

From the Department of Clinical Sciences (Bullone, Lavoie) and the Department of Pathology and Microbiology (Hélie) of the Faculty of Veterinary Medicine, Université de Montréal, 3200 rue Sicotte, St-Hyacinthe, J2S 2M2, QC, Canada. From the Institut Universitaire de Cardiologie et de Pneumologie de Québec, Université Laval, 2725 Chemin Ste-Foy, Quebec, G1V 4G5, QC, Canada (Joubert).

Short title: Endobronchial biopsy features in heaves

Keywords: Lung, airway, remodeling, inflammation, RAO.

Abbreviations: ASM, airway smooth muscle; BALF, bronchoalveolar lavage fluid; C, control horses; EBB, endobronchial biopsy; ECM, extracellular matrix; ICC, intraclass correlation coefficient; HE, horses with heaves in exacerbation; HR, horses with heaves in clinical remission.

Corresponding author: Jean-Pierre Lavoie, Department of Clinical Sciences, Université de Montréal, 3200 rue Sicotte, St-Hyacinthe, J2S 2M2, QC, Canada.

This study work was performed at the Faculty of Veterinary Medicine of the Université de Montréal. Part of the samples analyzed in this study have been reported in a previous study investigating the physiological and technical variables affecting the quality of equine endobronchial biopsy samples[1].

Support for this study was provided by the Canadian Institutes of Health Research (#208855, JPL) and by a PBEEE-V1 Scholarship from Fonds de Recherche du Québec - Nature et Technologies (MB).

Part of this work was presented by MB at the 32nd VCRS Symposium, Kennett Square, PA, October 27-29th, 2014.

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Abstract

Background: Heaves is characterized by bronchospasm, airway remodeling, and inflammation. While bronchoalveolar lavage fluid (BALF) cytology normalizes during the asymptomatic phase of the disease, selected airway remodeling features persist in affected horses. Their identification in endobronchial biopsies would possibly facilitate the diagnosis of the disease even when horses are in clinical remission.

Hypothesis: Airway inflammation and remodeling in endobronchial biopsies permits the differentiation of horses with heaves from controls, independently of their clinical status (exacerbation or remission).

Animals: Fourteen healthy horses and 24 horses with heaves (12 in remission and 12 in exacerbation).

Methods: The score was validated by 2 pathologists using biopsies obtained from 18 horses (6 controls, 6 heaves exacerbation, and 6 heaves remission) whose lung function had been assessed with impulse oscillometry. Clinical and research application of the score was evaluated using biopsies obtained from another 20 horses (8 controls, 6 heaves exacerbation, and 6 heaves remission).

Results: The score was repeatable and could differentiate horses with heaves in exacerbation from horses with heaves in remission and controls ($p<0.0001$). The histological scores of horses with heaves in exacerbation ($r=-0.88$; $p=0.04$) and remission ($r=-0.91$; $p=0.01$) of the disease were strongly correlated with expiratory reactance values.

Conclusions and clinical significance: The proposed histological scoring system is a reliable tool for the assessment of airway inflammation and remodeling of the large airways in horses. However, it does not discriminate horses with heaves in remission from control horses. Evaluation of endobronchial biopsies might be considered in future research and clinical studies.

Introduction

Heaves is a chronic obstructive respiratory condition affecting adult horses. It is characterized clinically by periods of labored breathing at rest due to airflow obstruction[2], which results from bronchospasm, chronic pulmonary remodeling, and mucus plugging in the airways[3]. Symptoms are largely reversible with therapy. Bronchoalveolar lavage fluid (BALF) cytology reveals neutrophilic inflammation during episodes of disease exacerbation[4], but completely normalizes during periods of remission induced by antigen avoidance strategies[5,6]. Conversely, remodeling of the peripheral airways in heaves persists during disease remission[7], and correlates with residual airflow obstruction[6,8]. Whether airway wall inflammation plays a role in perpetuating the disease during asymptomatic periods of the disease has not been studied, and could provide insights into heaves pathophysiology. The identification of specific parameters of tissue inflammation or remodeling in endobronchial biopsies of affected horses could facilitate both the diagnosis of heaves during disease remission and the response to treatment.

Endobronchial biopsies (EBBs) yield bronchial samples that permit the assessment of bronchial surface epithelium, extracellular matrix (ECM), blood vessels, seromucous glands, and airway smooth muscle (ASM). Despite EBBs having been used in equine respiratory research for gene expression studies[9-12], they are rarely evaluated histologically for inflammatory or remodeling features. However, they can be easily obtained, and would allow prospective studies to be performed in both research and clinical settings. We have recently developed a detailed protocol for EBB sampling and morphometrical analysis[1]. During that study, and using histomorphometric techniques, we described histopathological features characterizing EBBs of horses with heaves, which prompted us to develop a semi-quantitative scoring system for rapid and clinically applicable bronchial tissue assessment. We hypothesized that using this score we could differentiate horses with heaves from controls, independently of the clinical state of the disease (exacerbation or remission). The aims of this study were: 1) to develop and validate a semi-quantitative scoring system for evaluating bronchial inflammation and remodeling in equine EBBs, and 2) to estimate cut-off values of the score for differentiating healthy from heaves-affected animals.

Material and Methods

Study design

Phase 1 – Previously obtained endobronchial biopsies[1] from 6 horses with heaves in exacerbation, 6 horses with heaves in remission of the disease, and 6 controls were scored by 2 board certified pathologists (PH, Diplomate ACVP, and PJ, Fellow of the Royal College of Physicians of Canada) to assess the agreement. The correlation between the score of the board-certified veterinary pathologist and the lung function was studied to assess whether the score reliably assessed the degree of remodeling and inflammation leading to airflow obstruction.

Phase 2 – Clinical application of the score. EBB tissues from 20 additional horses (8 controls, 6 horses with heaves in exacerbation and 6 in remission) were obtained through the Equine Respiratory Tissue Bank (http://www.btre.ca). They were scored by the board-certified veterinary pathologist and, together with the EBBs described in phase 1, were used for investigating the effect of the environment on the score, the ability of the score to differentiate healthy and heaves-affected horses, and for calculating cut-off values for the groups.

Phase 3 – Use of the score in research settings. To assess the potential application of this score in research setting, an equine internist (JPL) and a post-graduate student (MB) experienced at evaluating lung histological remodeling features associated with heaves were asked to score the EBBs, and their results were compared with those obtained by the board-certified veterinary pathologist.

Animals

Twenty-four horses with a diagnosis of heaves belonging to the research herd of the Respiratory Cellular and Molecular Biology Laboratory and 14 healthy horses from the teaching herd of the Faculty of veterinary medicine of the Université de Montréal were studied.

Phase 1 – Score development and validation. The characteristics of the horses studied in this phase and the experimental protocol have been previously described[1]. Briefly, horses were exposed to hay (6 horses with heaves in exacerbation and 6 controls) or kept at pasture for 2 weeks and treated with dexamethasone (0.06 mg/kg PO q24h, 6 horses with heaves in remission) for 1 week before EBBs were obtained. Impulse oscillometry testing was performed on these horses before bronchoscopy using the Equine MasterScreen IOS system[a] and FAMOS software[b], as previously described[1,13]. Bronchoscopies were performed on sedated animals (detomidine/butorphanol 0.01/0.015 mg/kg IV). Six endobronchial biopsies were obtained from previously selected sites within one randomly chosen lung of each animal. All procedures performed on the horses had been approved by the local Animal Ethics Committee (Rech-1324).

Phase 2 – Among the 20 horses included in this phase of the study, 8 were controls kept stabled (EBBs were available also after 4 months at pasture in 5 horses, and were used to investigate the effect of the environment on the score) and 12 were horses with heaves (all kept stabled for >4 weeks, with horses in remission receiving oral dexamethasone at 0.06 mg/kg q24h for 2 weeks prior to EBB collection). Lung function data obtained by impulse oscillometry were not available for these horses. Three to four endobronchial biopsies were obtained from unspecified sites of one lung for each animal.

Histology processing

Endobronchial biopsies were fixed for 24 h in 10% neutral-buffered formalin and then paraffin- embedded. Two consecutive histologic sections of 5-µm thickness were obtained from each biopsy and stained with HEPS (hematoxylin-eosin-phloxine-saffron) and modified Russell-Movat's pentachrome[14].

Scoring system

A standardized semi-quantitative grading score was developed taking into account histological features thought to be important in heaves. Figure 1 describes the inflammatory and remodeling parameters studied and their scoring. Before performing the study, the board-certified veterinary pathologist, the equine internist, and the post-graduate student were provided with a list of the parameters to be evaluated in the score and were asked to choose the biopsy considered of best quality from those available for each horse. This step allowed the operators to familiarize themselves with the variability of the parameters to be assessed in these samples. The biopsies chosen by the board-certified veterinary pathologist (one for each horse) were those used for subsequent blinded analysis. In phase 1 of the study, operators were also asked to monitor the time needed for scoring the biopsies and to make a presumptive diagnosis based on EBB findings (heaves vs control).

Statistical analysis

Phase 1 – Concordance between operators was evaluated using the Cohen's Kappa test. The Pearson test was used for correlating scores and low frequencies (0.5 Hz) expiratory lung function values.

Phase 2 and 3 – The effect of the environment on the score was assessed on the EBBs obtained from 5 control horses while stabled (>4 weeks) or at pasture (>3 months), using a paired t-test. Of these EBBs, only those obtained during stabling were included in the subsequent analysis. Also, the scores of the biopsies of 12 horses with heaves in remission (6 kept stabled and 6 kept at pasture, all treated with dexamethasone for 1 to 2 weeks) were compared to assess the effect of the environment in horses with heaves using the Mann-Whitney U test. Differences between the scores of the 3 groups of horses were evaluated with one-way ANOVA with Tukey's post-hoc test. Sensitivity and specificity of the test for diagnosing heaves were calculated for different cut-off values. The Cochran-Mantel-Haenszel test with Bonferroni correction for multiple comparisons was used to identify the parameters of the score which better differentiated horses with heaves during exacerbation or remission of the disease, and

the group of horses with heaves in remission from the controls. Interclass correlation coefficient (ICC) and Cohen's Kappa test were used to assess concordance among the operators. The Bland-Altman test was employed to calculate any significant bias between the operators.

Results

Phase 1 – Score development and validation

Agreement

The agreement between the 2 pathologists for scoring the same biopsy was good (weighted kappa = 0.52). For each biopsy, the time needed for performing the score varied from 45 seconds to 3 minutes for both pathologists (1'30''/biopsy on average).

Correlation between scores and lung function

The expiratory reactance at 0.5 Hz was significantly correlated with the biopsy score both when the horses were in remission (r=-0.91; p=0.01) and exacerbation of the disease (r=-0.88; p=0.04). Coherence values[15] of expiratory impulse oscillometry signals at 0.5 Hz were 0.81±0.14 (mean±SD) for control horses, 0.79±0.08 for horses with heaves in remission, and 0.75±0.12 for horses with heaves in exacerbation. The regression lines of both groups had similar slopes (p=0.9) but different intercepts (p=0.002). When the sums of the parameters linked to inflammation (epithelial and submucosal) were removed from the scores of horses with heaves in exacerbation, the regression line of the group in exacerbation shifted to the left without changing its slope and approached the line of the horses with heaves in remission (Figure 2). The gap remaining between these two regression lines was likely due to morphological differences still present between the 2 groups despite the treatment, most commonly represented by mucus gland hypertrophy and hyperplasia. Epithelial hyperplasia and ASM fibrosis also partially contributed to the morphological difference observed between horses in exacerbation and remission of heaves. The expiratory resistance at 0.5 Hz only tended to correlate with the biopsy score when the horses were in exacerbation of the disease (r=0.82; p=0.08).

Phase 2 – Clinical application of the score

Effect of the environment

The biopsy score was not affected by the environment in control horses (p=0.75) or in horses with heaves in remission (p=0.19). When the inflammatory parameters were analyzed separately, no difference was observed in epithelial (p=0.62) or submucosal infiltrate (p=0.37) in healthy horses kept at pasture or stabled. Horses with heaves in remission kept in both environments also had a similar degree of submucosal inflammation (p=0.24), while a tendency was observed for increased epithelial inflammation when stabled (p=0.06; fed hay, and bedded on shavings).

Histological scores

The scores were significantly different between groups (p=0.001). The score values of horses with heaves in exacerbation were significantly higher compared to those of horses with heaves in remission and controls (p=0.01 and p=0.001, respectively). No difference was observed between the scores of horses with heaves in remission and controls (p=0.8, Figure 3A). A value ≥5 of the score could differentiate a horse with heaves (in exacerbation or remission) from a control with a sensitivity of 54.2% and a specificity of 79%. A value ≥5 of the score allowed identification of horses with heaves in exacerbation compared to controls and horses with heaves in remission with 83.3% sensitivity and 77.4% specificity. The parameters of the score better differentiating horses with heaves in exacerbation from those in remission of the disease were the epithelial (p=0.02) and submucosal inflammatory infiltrate (p=0.005). None of the parameters studied could differentiate horses with heaves in remission from controls (Table 2).

Phase 3 – Use of the score in research settings

There was a moderate agreement between the board-certified pathologist and the equine internist (weighted Kappa, 0.44), and with the post-graduate student (weighted Kappa, 0.45). The interclass correlation coefficient (ICC) for the 3 observers was 69%, indicating that the level of concordance among the 3 observers was good, although a significant difference was observed among the scores of the 3 operators (analysis of variance $F(2,38)=5.17$, $p<0.01$). The observed difference was ascribed to the fact that the internist tended to slightly overestimate lower scores and underestimate higher scores compared to the pathologist (slope significantly >0, $p=0.02$, with a mean bias of 0.37), while the student tended to overestimate higher scores compared to the pathologist (slope significantly <0, $p=0.005$, with a mean bias of -1.02). Nevertheless, the scores of all operators were significantly higher for the group of horses with heaves in exacerbation compared to the groups of horses with heaves in remission or controls (Figure 3B and 3C).

Discussion

Horses with heaves are clinically asymptomatic during periods of remission of the disease and BALF cytology also normalizes. However, peripheral airway inflammation is unaffected by the clinical status of the disease[6], and could represent a means of diagnosis of heaves during remission phases. However, due to its invasiveness, sampling the peripheral airways is not practical for the monitoring of clinical cases. Using a scoring system for the assessment of inflammatory and remodeling parameters of the large airway wall, we demonstrated that bronchial inflammation differs significantly between periods of exacerbation and remission of heaves, but not between the asymptomatic phases of heaves and the healthy subjects. Our score significantly correlated with lung function in horses with heaves in remission and in exacerbation of the disease, providing a reliable measure of bronchial obstruction in this group of horses. As endobronchial biopsies are easily obtained in standing sedated horses, our findings represent a practical means for the assessment and monitoring of airway remodeling and inflammation in both clinical and research settings.

Epithelial and subepithelial inflammation were important parameters differentiating horses with heaves in exacerbation from horses with heaves in remission and controls. This finding underlines the involvement of the large airway inflammation in the pathophysiology of heaves. Interestingly, while we have shown a reduction of central airway inflammation after short-term treatment, no changes were observed in the degree of inflammation of peripheral airways after 6 or 12 months of antigen avoidance and/or inhaled corticosteroid treatment in a group of horses with heaves[6]. Whether this is due to intrinsic differences in small airway compared to large airway physiology or to the inability of inhaled treatments to reach the most distal sites remains unclear. This also suggests that large airway inflammation in heaves is intermittent, occurring only during exacerbation periods of the disease, when it could possibly drive the appearance of overt bronchospasm, increasing airway obstruction and pulmonary resistance. Conversely, small airway inflammation persists even during remission periods of heaves, perhaps acting as an active site for disease maintenance or progression. Contrary to what we hypothesized, significant differences in the bronchial inflammatory infiltrate or morphology of horses with heaves in remission and controls were not observed. As stabling

increases lung inflammation in BALF in otherwise healthy horses[5,7], we compared the scores of horses with heaves in remission and controls while at pasture vs stabled. Unexpectedly, neither the score nor the degree of inflammation in healthy horses was significantly affected by the environment. As for the horses with heaves in remission, the environment did not affect the biopsy total score but those kept stabled tended to have higher epithelial inflammation compared to those kept at pasture.

The contribution of the airway epithelium to the pathogenesis of heaves remains ill-defined. Due to its proximity to the bronchial lumen and exposure to pro-inflammatory agents, it is likely affected by, and perhaps drives the pathological process in heaves. Whether and how this translates into discernible histological changes is unclear. The almost significant effect observed in our study of the environment on epithelial inflammation in horses with heaves in remission but not in controls supports the theory of a dysregulated inflammatory response (with delayed, reduced, or lack of inhibitory mechanisms) in heaves pathogenesis. Kaup and colleagues described the histology of large airways of horses suffering from heaves in the early 90s[16]. The main differences they observed between the 2 groups involved the bronchial epithelium, in which cellular infiltration by mononuclear leucocytes[16] and mast cells[17] were described. The inflammatory cells infiltrating the epithelial layer in our study were predominantly lymphocytes and neutrophils, but these findings need to be confirmed using specific markers, at least for the mononuclear leukocytes. Nevertheless, they are in agreement with the paucity of epithelial eosinophils observed in heaves[18]. Whether mucus producing cells are increased in the airways of horses with heaves compared with controls has been investigated both in central and peripheral airways, with contrasting results[19-21]. In our study, epithelial goblet cells were increased in horses with heaves in exacerbation compared to controls. Also they were increased in horses with heaves in remission compared to controls, but the difference was not significant after correction for multiple comparisons, possibly as a result of the short duration of antigen exposure. Notably, the environment in which horses with heaves in remission were kept (pasture vs stabling) did not affect any mucus-related parameter of the score.

Both the ECM and the smooth muscle mass are increased in the peripheral airways of horses with heaves[6,8]. However, whether the submucosal structures of the large airways of horses with heaves display histologic alterations as a consequence of the disease is not well established. Endoscopic studies have observed a thickening of the tracheal septum and an increased tendency for central airway collapse in horses with heaves compared to controls[22,23]. An increased thickness of the submucosal bronchial wall, most likely due to ASM hyperplasia/hypertrophy, has also been reported in central bronchial sections[24]. These findings suggest that the whole airway wall undergoes structural modifications, thus altering its physical properties. We observed more prominent submucosal glands in horses with heaves during disease remission compared to the controls, but the difference was not significant after correction for multiple comparisons. This however integrates the results of a previous work in which the submucosal glands in large airways were reported to be larger in horses with heaves than in controls, and significantly larger in horses with heaves in exacerbation compared to those in remission[21]. Together with the tissue inflammation, likely accompanied by edema of the bronchial wall, this could account for the increased thickness of the bronchial lamina propria observed in heaves[1]. The ASM-related parameters evaluated with the score could not reliably differentiate the groups that we studied. Based on what has been reported in small airway remodeling[6], we did not expect a significant reduction of ASM mass or fibrosis to occur after only 2 weeks of treatment. However, the fact that endobronchial biopsies are inadequate tools for a reliable quantitative assessment of ASM in horses[1] could have accounted for our inability to detect morphological alterations likely present in the group of horses with heaves in remission compared to the controls.

As previously reported, the horses studied during phase 1 underwent IOS testing before and after sedation[1]. This is the first report of impulse oscillometry values obtained in horses at frequencies <1 Hz, and the expiratory coherence values indicate that measurements were of good quality (0.6 is considered the minimum coherence value for peripheral lung measurement[15,25]). The scores made by the certified pathologist were negatively correlated with low frequency expiratory reactance measured after 15 minutes of sedation for both horses with heaves in exacerbation and in remission, demonstrating the effect of both airway remodeling and inflammation on lung function. Importantly, low frequency IOS expiratory

values are not significantly affected by α_2-agonist sedation in horses[26], which justifies the use of post-sedation IOS values in our study. Also, reactance values are less affected by stabling conditions and time of the day compared to resistance values[27]. Furthermore, the head was maintained in a standard position with an angle of approximately 90° during IOS under sedation, which further standardized the measures. Sedation with α_2-agonists has the additional advantage of dilating the equine airways[28] (which explains why expiratory reactance of horses with heaves in remission and exacerbation are similar), emphasizing the remodeling component of obstruction at the expenses of bronchospasm. The observed decrease in low frequency reactance values proportional to the increase in the scores suggests that the degree of large airway remodeling significantly affects the elastic properties of the whole equine lung, and that the remodeling of the large airways is likely to parallel alterations of the peripheral airways. Even though this finding might seem counterintuitive, as we would expect airway remodeling to be proportional to airway resistance, it has been shown that airway distensibility (which decreases with increasing ECM deposition and ASM mass development) does have a measurable influence on the overall elastic properties/behavior of the lungs[29]. This concept assumes that small airways are also remodeled, which has already been demonstrated in heaves[3,6]. In further support of our data, it has been reported that increased central airway subepithelial fibrosis was negatively correlated with compliance in asthmatics[30].

Conclusions

The histological score that we have developed is a reliable tool for the assessment of airway inflammation and remodeling of the large airways in horses with heaves. The semi-quantitative assessment of the parameters we have studied was rapid and repeatable with minimum training by operators already familiar with bronchial histology. A score ≥5 allows differentiating horses with heaves from controls with a sensitivity of 50%, which rises up to 83% if the horse shows clinical signs. On the other hand, a horse with no respiratory signs and a biopsy score <5 is a healthy horses in 79% of cases. Although non-significant after correction for multiple comparisons, goblet cell hyperplasia and submucosal mucus gland hyperplasia and hypertrophy were the only parameters differentiating horses with heaves in remission from controls, with no apparent effect of the environment, warranting further investigation. The introduction of endobronchial biopsy assessment in future prospective studies and possibly in everyday practice could facilitate the diagnosis of heaves in asymptomatic horses, reveal uncovered mechanisms of respiratory diseases, as well as elucidate the role and kinetics of large airway inflammation and remodeling.

Footnotes

[a] LabManager version 4.53, Jaeger, Würzburg, Germany.

[b] IMC, Meβsysteme, Berlin, Germany.

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Figures and tables

Table 1. Differences among groups for individual parameters of the score.

Parameter	**p value**			**Direction of the effect (post-test)**
	ANOVA	Post-test HE vs HR	Post-test HR vs C	
Epithelium				
Hyperplasia	**0.009**	0.06	0.29	HE≥HR
Inflammatory infiltrate	**0.02**	**0.02**	0.92	HE>HR
Goblet cells	**0.03**	0.56	0.04*	HR≥C
Desquamation	0.19	0.59	0.19	
Extracellular matrix				
Thickened BM	0.27	0.25	0.25	
Inflammatory infiltrate	**0.01**	**0.005**	0.98	HE>HR
Mucus glands	0.28	0.42	0.47	
Airway smooth muscle				
Fibrosis	0.29	0.16	0.60	
Mucus glands	0.09	0.14	0.03*	HR≥C
ASM ending visible	0.67	0.41	0.44	

The combined scores were used for statistical analysis. HE: heaves exacerbation, HR: heaves remission, C: controls. > indicates the direction of the effect when significant differences were observed between groups (p values in bold), while ≥ indicate the direction of the effect when only tendencies to significant differences were observed. *: Non-significant after Bonferroni correction for multiple comparisons.

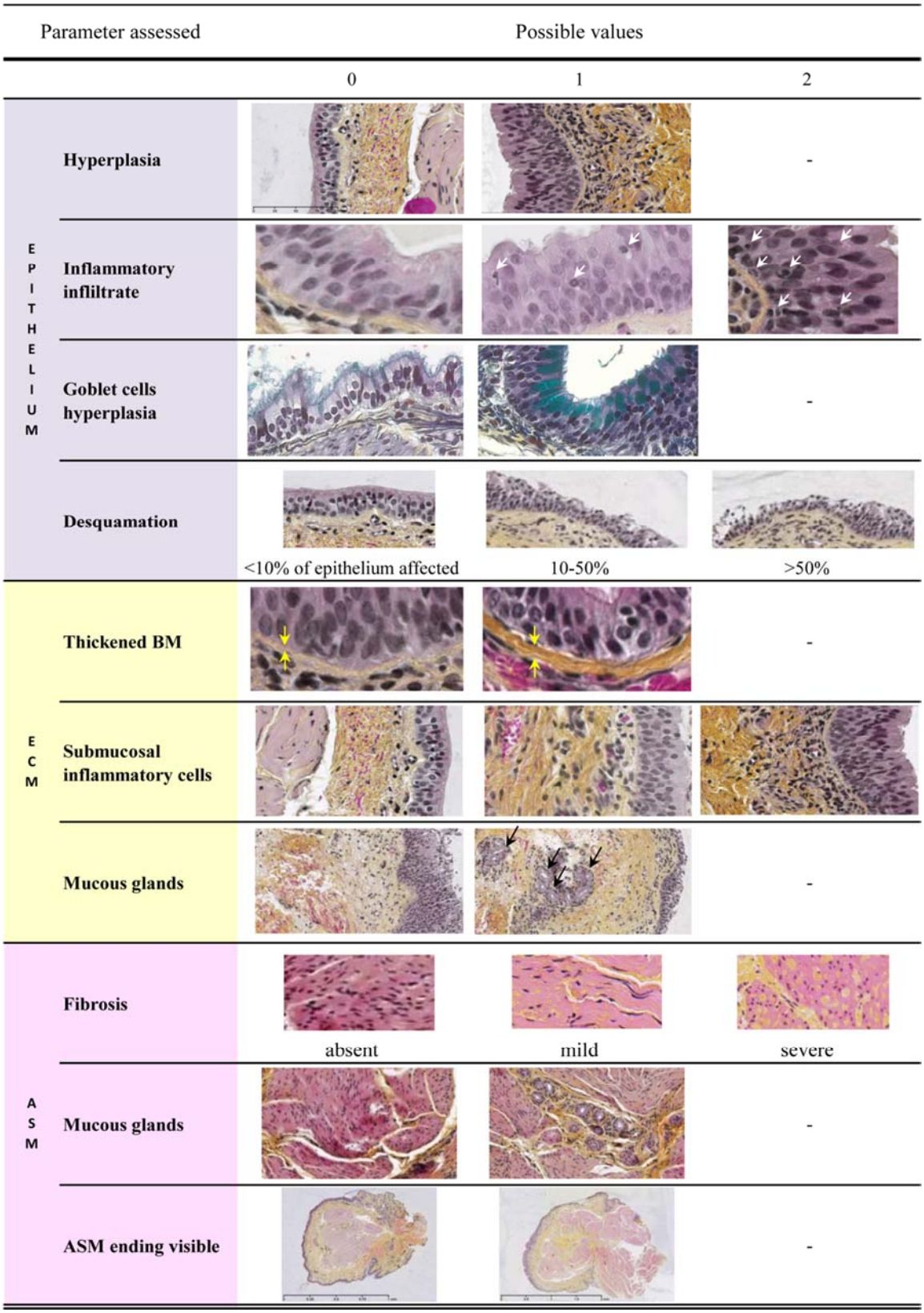

Figure 1. Histological parameters assessed with the score.

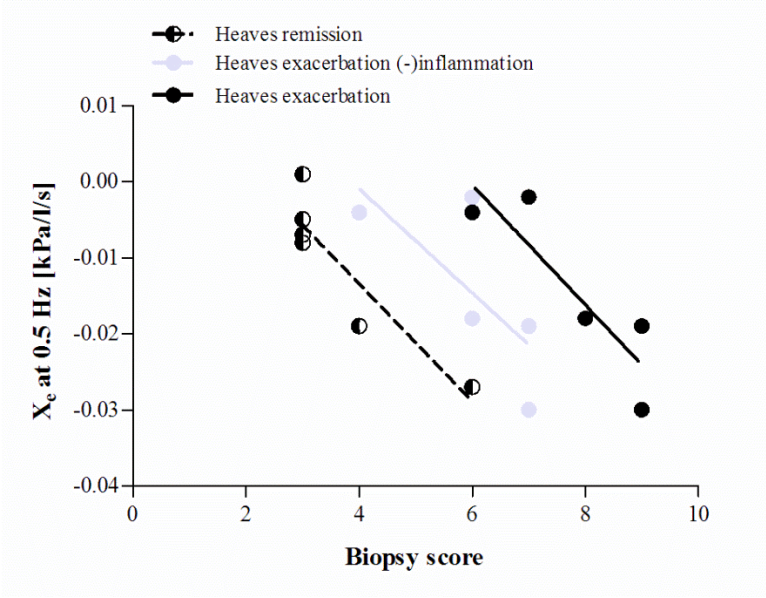

Figure 2. Effect of inflammation on the relationship between histological score and lung function in horses with heaves. Heaves exacerbation (-)inflammation: scores of HE from which the values of epithelial and subepithelial inflammation have been removed. X_e: expiratory reactance.

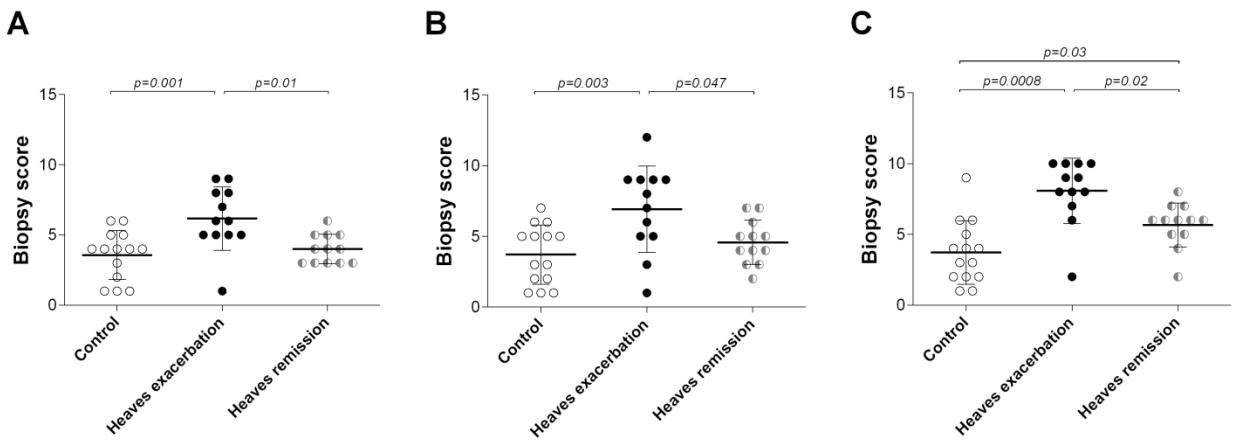

Figure 3. Effect of the group on the histological scores. Results of the board-certified pathologist (A), of the specialist in internal medicine (B). and of the post-graduate student (C). To be noted, the operators in B and C had at least a 3-year experience at evaluating pulmonary remodeling in the equine species.

Article 3

Endobronchial ultrasound reliably quantifies airway smooth muscle remodeling in an equine asthma model

Summary

Endobronchial biopsies are an unreliable tool for the quantitative assessment of the ASM mass in the central airways of asthmatic horses. This article demonstrates the reliability of endobronchial ultrasound at specifically assessing the smooth muscle layer within the central airways in the equine species. Using *ex vivo* and *in vivo* approaches, we show that the central smooth muscle mass is increased in asthmatic horses, albeit to a lesser extent compared to the peripheral one. Furthermore, based on the observed variation of remodeling occurring both inter- and intra-subject, we have developed and validated a protocol for the assessment of central airway remodeling with endobronchial ultrasound in horses.

Contribution

I participated in study design (70%), experimental procedures and data collection (management of horses and bronchoscopy, 60%; endobronchial ultrasound, 100%; lung harvesting (100%), sample processing for histology, 90%; histomorphometry and image analysis, 100%), statistical analysis (30%), and preparation of the manuscript (90%).

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ENDOBRONCHIAL ULTRASOUND RELIABLY QUANTIFIES AIRWAY SMOOTH MUSCLE REMODELING IN AN EQUINE ASTHMA MODEL

Bullone Michela[1], Beauchamp Guy[1], Godbout Mireille[1], Martin James[2], and Lavoie Jean-Pierre[1]*.

[1] Department of Clinical Sciences, Faculty of Veterinary Medicine, Université de Montréal, QC, Canada.

[2] Meakins-Christie Laboratories, Department of Medicine, McGill University, QC, Canada.

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Corresponding author: jean-pierre.lavoie@umontreal.ca (JPL)

Abstract

Endobronchial ultrasonography (EBUS) revealed differences in the thickness of the layer representing subepithelial tissues (L2) between human asthmatics and controls, but whether this measurement correlates with airway smooth muscle (ASM) remodeling in asthma is unknown. In this study, we sought to determine the ability of EBUS to predict histological ASM remodeling in normal and equine asthmatic airways. We studied 109 isolated bronchi from the lungs of 13 horses. They underwent EBUS examination using a 30 MHz radial probe before being processed for histology. ASM remodeling parameters were evaluated in EBUS images (L2 thickness, L2 area, L2 area/internal perimeter (Pi) and L2 area/Pi^2) and histological cuts (ASM area/Pi^2), and compared. EBUS was then performed *ex vivo* on the lungs of 4 horses with heaves, an asthma-like condition of horses, and 7 controls to determine whether central bronchial remodeling could be detected with this technique. An optimized approach was developed based on data variability within airways, subjects, and groups, and then validated in 7 horses (3 controls, 4 with heaves) that underwent EBUS *in vivo*. L2 area was significantly associated to ASM area in isolated lungs ($p<0.0001$), in the absence of significant bias related to the airway size. Bronchial size significantly affected EBUS ASM-related parameters, except for L2 area/Pi^2. L2 area/Pi^2 was increased in the airways of asthmatic horses compared to controls, both *ex vivo* and *in vivo* ($p<0.05$). Bronchial histology confirmed our findings (AASM/Pi2 was increased in asthmatic horses compared to controls, $p<0.05$). In both horses with heaves and controls, L2 was composed of ASM for the outer 75% of its thickness and by ECM for the remaining inner 25%. In conclusion, EBUS reliably allows assessment of asthma-associated ASM remodeling of central airways in a non-invasive way.

Keywords: Bronchoscopy, airway wall, radial miniprobe, smooth muscle layer, extracellular matrix, RAO.

Introduction

Airway smooth muscle (ASM) mass and phenotype as well as the amount and composition of extracellular matrix (ECM) mass are increased or altered in asthma [1]. Both large and small airways may suffer remodeling, the magnitude of which has been associated with disease severity [2-4]. Most human studies demonstrating remodeling of airway tissues have been performed on post-mortem specimens, especially when assessing peripheral airways or the most abaxial structures of the bronchial wall, such as ASM [5, 6]. Bronchoscopic biopsies may be valuable tools, but potentially suffer from issues of sampling [7]. There is a need for non-invasive methods to assess the airway wall structure *in vivo*, especially when assessing treatment-associated airway remodeling reversibility in asthma [4, 8].

Endobronchial ultrasonography (EBUS) is a non-invasive addition to routine bronchoscopy. Using radial mini-probes, the whole bronchial wall may be imaged and most of its structural components identified [9-11]. Two studies have shown that EBUS remodeling parameters such as the thickness of the first and second US layer (L1 and L2, representing epithelial and subepithelial tissue thickness including smooth muscle, respectively) differed between asthmatics and healthy patients [12], and also that they correlated with lung function [12, 13]. Increased thickness of the EBUS layer representing ASM and a variable portion of ECM (L2) was associated with greater values of basal membrane thickness [12]. However, whether EBUS allows for the specific assessment of ASM mass and its remodeling in asthma have not been demonstrated.

We investigated the correlation between ultrasonographic and histomorphometric measurement of ASM in a spontaneously occurring asthma-like disease of horses (heaves) [14]. We hypothesized that heaves-affected horses exhibit large airway remodeling compared to control subjects. Our objectives were to establish the comparability of measurements of airway wall structures assessed by EBUS to histology, and to develop an optimized protocol for assessing remodeling of subepithelial structures in central equine airways *in vivo*.

Material and Methods

Study design

The study was conducted in 5 consecutive phases, as summarized in Table 1. First, needle-puncture experiments were performed to describe the echographic anatomy of the laminar structures composing the equine bronchial wall. Then, isolated bronchial specimens were studied for direct comparison between EBUS and histology. *Ex vivo* examinations of equine lungs were performed as a "proof of concept" and for optimization of the technique used. *In vivo* EBUS examinations were then performed on a limited number of horses, subsequently euthanized in order to confirm our findings.

Animals

Seven horses underwent *in vivo* EBUS examination at the Veterinary Teaching Hospital (CHUV) of the Université de Montréal before being euthanized for reasons unrelated to respiratory diseases. Horses were humanely euthanized by intravenous injection of pentobarbital sodium. Available details of these animals are reported in Table 2. All procedures described on living animals were performed in accordance with the guidelines of the Canadian Council on Animal Care and approved by the Animal Ethics Committee of the Université de Montréal (Rech-1663)(Table S2 reports the ARRIVE checklist).

Isolated lungs and bronchial tissues

Isolated lungs and bronchial tissues studied in phase 1, phase 2 and phase 3 of our work were obtained at a local slaughterhouse (Viande Richelieu Meat Inc, Massueville, QC, Canada) or from autopsy samples of horses euthanized at the CHUV of the Université de Montréal for reasons unrelated to respiratory diseases. The owners of the horses gave their consent for their animals to be used for this study. The lungs studied in phase 3 were obtained from 3 of the 4 horses with heaves and from 1 of the 7 healthy horses studied in the *in vivo* experiment.

Equipment

All EBUS materials and equipment were purchased from Olympus (Richmond Hill, ON, Canada). Scans were obtained with a 30 MHz radial mini-probe (UM-S30-25R) connected to a dedicated ultrasound unit (EU-ME1). For *in vivo* EBUS, probes were inserted within a balloon-ended sheath (MH-246R) and passed through the working channel of a videoendoscope (CF-H260DL/I). EBUS experiments were recorded using iMovie on a MacBook Pro computer.

EBUS procedure

In vivo bronchoscopies were performed under sedation (detomidine 0.02 mg/kg and butorphanol 0.01 mg/kg injected IV) and topical anesthesia (lidocaine solution 0.5%). The videoendoscope was inserted through a nostril and advanced until it wedged within the main caudal lobar bronchus. Images were collected from all bronchial segments within reach, up to the main bronchi. *Ex vivo* examinations were similarly performed on lungs kept on ice until examined (<6 hours following euthanasia). One lung per animal was examined. Lungs showing macroscopic alterations were excluded.

EBUS image acquisition

Videos were analyzed at slow motion and good quality EBUS images were saved as tiff files. Quality of EBUS scans was assessed on the basis of resolution and definition of the layered structure, as well as on uniformity and completeness of the acquired scan.

Needle Puncture Experiments

Bronchial sections ~3-mm long and ~10-mm wide were randomly collected from the lungs. Needle-puncture experiments were performed as previously described [11]. Briefly, samples

were flattened and fixed with two 18G needles to a polystyrene surface (Figure 1A); a third smaller needle (27G) was then inserted longitudinally into the airway wall under stereoscopic microscope guidance. With the samples submerged in saline solution, EBUS images were captured when all needles were visible (Figure 1B). Airways were then fixed and paraffin embedded with the needles kept in place in order to reproduce histological cuts similar to the EBUS views. The layer in which the needle was identified in EBUS images was compared to the histological structure in which it was found at histology (Figure 1C).

Isolated bronchial specimen analysis (EBUS vs histology)

Eight to 10 complete bronchial sections were randomly chosen and carefully dissected from each lung. Bronchi were immersed in saline solution and EBUS images acquired before processing for histology. The following measurements were made: bronchial diameter (D, calculated as the mean of 2 perpendicular diameters measured in each airway), internal perimeter (Pi), lumen area (LA), thickness of the first and second layer (L1 and L2, respectively, calculated as the mean of 5 values measured at predetermined sites) as well as the area of the second layer (L2 area). Images were excluded from analyses if the bronchial wall was not clearly identifiable for >180° (missing angle). L2 area/Pi and L2 area/Pi^2 ratios were calculated. After formalin fixation and paraffin embedding, sections of 5-µm thickness were obtained, stained with Movat's pentachrome and digitized at 2.5-5x magnification, using a Leica camera (DCF320, Leica Microsystems, Cambridge, UK) and Photoshop CS5 (Adobe Systems Inc., San Jose, CA, USA). Histological images were scored from 1 to 4 (Table S1 in File S1), based on tissue architecture preservation and perpendicularity of the cut. Images scored 1 were excluded from analysis. Images scored 2 were considered only if the missing angle was <90°. For all airways, the mean of 2 diameters (D), internal perimeter (Pi), lumen area (LA) and ASM area (AASM) were measured (Figure 2), and AASM/Pi^2 ratio calculated. All measurements were made blindly using ImageJ (version 1.48c, NIH, Bethesda, MD, USA). When images were incomplete, the subtended angle of the analyzed part was recorded and measurements were transformed into complete measurements proportionally. Fourteen

EBUS images and 14 bronchial sections, selected randomly, were analyzed by the same observer three times at ≥ one week interval in order to assess repeatability of measures.

Composition of L2

The composition of L2 was studied using the EBUS images obtained *in vivo* and the histological sections obtained at post-mortem from the same lungs. As airways are naturally distended *in vivo* during EBUS but they contract during the fixation and embedding processes necessary for histology, we developed a method to estimate the mean thicknesses of the ECM (lamina propria) and ASM layers for maximally dilated airways (Figure 3). In brief, airways were treated as a series of concentric annuli in which the thickness of the epithelial layer was considered as negligible. In our model, Pi values measured in histological sections corresponded to the circumference of the smaller circle of the inner annulus (representing ECM), and were used to calculate its radius (r'). The radius of the larger circle (R') of the inner annulus was then calculated as a function of ECM area and Pi. The difference between the larger and the smaller radii of the inner annulus (R'-r') corresponded to the ECM thickness. The same approach was used to calculate ASM thickness (R-r), with R' being used as the smaller radius of the outer annulus (r). To validate our approach, the calculated ECM and ASM thicknesses were compared with values measured using standard histomorphometric techniques [3, 7, 16] on the same histological sections: the mean value of 5 measures performed randomly for each airway represented our measured ECM and ASM thickness.

Finally, the sum of ECM and ASM thickness calculated at histology (histologic submucosal thickness) was compared to the sum of L1 and L2 thickness (EBUS submucosal thickness). The percentage of the total airway thickness occupied by ECM and ASM at histology was calculated and related to the thickness and composition of L1 and L2.

Statistical analysis

Statistical analyses were performed with SAS v.9.3 (Cary, NC, USA). Mixed linear models with "subject" used as a random effect, and Bland-Altman tests were used to compare measurements from EBUS and histology. Mixed linear models with "group" and "bronchial size" as fixed effects were used for group comparisons including random factors to take into account the non-independent measures from the same subject for different bronchial sizes and for repetitions within bronchial size. A priori contrasts were performed, adjusting the alpha level of each comparison with the Bonferroni sequential procedure, to compare group means at each bronchial size. Two types of airways were defined depending on the size of Pi (intermediate: Pi $\leq$31 mm; large: Pi>31 mm). Mixed covariance linear models were used for studying how variables varied with Pi; the "group" was used as a fixed effect, "Pi" was used as a co-factor and "subject" as a random effect.

Variance component analyses were performed for the repeatability of EBUS and bronchial section measurements. The same analysis was also carried out within individuals for repeated measurements of L1, L2, L2 area/Pi and L2 area/Pi2. The effect of bronchial size (3 classes, based on Pi: 0-16 mm, 16-31 mm and >31 mm) was studied with a mixed linear model with "group" and "Pi class" as fixed effects. A priori contrasts were performed, again with the sequential Bonferroni procedure, to compare group means within each Pi class. The coefficient of variation (CV) was calculated for airway values obtained from 2, 3, and 4 images, to determine how many images should be analyzed to provide a reliable estimate. The level of statistical significance was set at 0.05 throughout.

Results

Phase 1 - Needle Puncture Experiments

Experiments were performed on 26 bronchial samples from 6 horses. The first hyperechoic layer (L1) corresponds to the epithelium and to a variable portion of the underlying ECM. The second hypoechoic layer (L2) corresponds to the remaining fraction of ECM and smooth muscle (Figure 4). When cartilage is present, L3, L4 and L5 correspond to the cartilage internal surface, cartilage thickness, and external cartilage surface and adventitia, respectively. Occasionally, a second, more abaxial cartilage could be identified. In the absence of cartilage, L3 corresponds to the adventitia.

Phase 2 - EBUS-histology comparison

A total of 109 isolated bronchi obtained from 13 horses were scanned and subsequently processed for histology. Repeatability of histologic measurements was excellent with an intra-class correlation coefficient of 99.0% for Pi, 98.5% for AASM, and 95.5% for A_{ASM}/Pi^2 values. Repeatability of EBUS measurements was also generally good with an intraclass correlation coefficient (ICC) of 99.6% for Pi, 81.6% for L1, 87.3% for L2, 94.2% for L2 area/Pi, and 92.3% for L2 area/Pi^2 values. Overall, a good agreement was observed between the two methods for all parameters measured, except for L2 area/Pi^2 and A_{ASM}/Pi^2 (see data in Table 3 and Figure S1). Bland-Altman tests revealed that EBUS tended to underestimate Pi when compared to histology, and differences tended to get larger as values of Pi increased (Figure S2). Also, EBUS overestimated D values for small bronchi and underestimated them for large bronchi. However, these differences in D were considered clinically negligible, as the mean difference between the 2 measurements was 0.14 mm.

Phase 3 - Ex vivo test and optimization

Four lungs obtained from horses with heaves and 7 from controls were studied after removal from the thoracic cage. None of the lungs showed significant macroscopic alterations. A mean of 8 to 10 airways was analyzed in each lung. Our first aim was to investigate whether a difference in submucosal remodeling could be observed *ex vivo* between horses with heaves and controls, in the absence of any noise generated by respiratory movements. Airways of similar sizes were analyzed in both groups (Pi [mean±SD] 20.6±4.9mm in heaves; 21.2±3.5mm in controls, p=0.65). When bronchi of both groups of horses were analyzed together, values of L1, (p=0.0003), L2 (p=0.001), and L2 area/Pi (p=0.004) were significantly larger in large rather than intermediate airways. Conversely, L2 area/Pi2 was larger in intermediate rather than in large airways (p=0.02), which we ascribe to the effect of the disease (remodeling). In both groups of horses, L1, L2 and L2 area/Pi increased with Pi, while L2 area/Pi^2 decreased (statistical details are provided in Figure 5). When analyzing only intermediate airways, the slope of the curve of the control group for L2 area/Pi^2 was not significantly different from zero (slope=-0.00022, p=0.13). The slope of the relationship between L2 area/Pi^2 and Pi was significantly higher in the heaves group than in the control group (p=0.01). L2 (p=0.009), L2 area/Pi (p=0.004) and L2 area/Pi^2 (p=0.02), but not L1 (p=0.4) values, were significantly larger in horses with heaves than in the controls. L2 area/Pi (p=0.006) and L2 area/Pi^2 (p=0.003) were also greater in intermediate airways of horses with heaves than in controls. L2 tended to be thicker in intermediate bronchi of horses with heaves than controls, although it did not reach statistical significance (p=0.06). In large bronchi, only L2 area/Pi values were greater in horses with heaves than controls (p=0.006) (Figure 5).

Secondly, we aimed at optimizing our imaging technique using an approach as similar as possible to those we use in vivo. The greatest variation observed in all EBUS parameters measured ex vivo (L1, L2, L2 area/Pi and L2 area/Pi^2) was attributable to the size of the airways in individual horses. However, analyzing 8-10 airways per horse of similar mean size allowed yielding significantly higher inter-group variation compared to inter-subject variation within the same group (Table 4). CV analysis showed that a minimum of 3 images of good

quality for each airway provided a reliable estimate of the real values of all the parameters analyzed.

Phases 4 and 5 - In vivo EBUS and histological validation

EBUS was performed *in vivo* in 7 animals (4 horses with heaves and 3 controls), all of which were then euthanized and EBUS immediately repeated on 4 of them (3 heaves and 1 control, as the lungs of the other animals were severely contaminated with blood). EBUS was performed on the same lung imaged *in vivo* using the same approach, in order to compare the results. A similar number of airways was analyzed on the two occasions (10.7±4/horse *in vivo* and 9.5±4/horse *ex vivo*, p=0.6). Three to 4 images were analyzed for each airway. Lung collapse occurring with lung removal from the thoracic cage did not significantly change the values of L2, L2 area/Pi, and L2 area/Pi^2 (p=0.46, p=0.79 and p=0.17, respectively). L1 tended to be thicker when measured *ex vivo* (p=0.09), however. This supports and validates the *ex vivo* findings we have described above.

In vivo, in both groups of horses, L1, L2 and L2 area/Pi increased with Pi, while L2 area/Pi^2 decreased (statistical details are provided in the legend of Figure 6). As previously shown *ex vivo*, the slope of the relationship between L2 area/Pi^2 and Pi was not statistically different from zero in intermediate airways of control horses (slope=-0.00026, p=0.08). The slopes of the relationships between L2 and Pi, and between L2 area/Pi and Pi were significantly higher in horses with heaves than controls (p=0.02 and p=0.04, respectively). L2 (p=0.03), L2 area/Pi (p=0.005), and L2 area/Pi^2 (p=0.04), but not L1 (p=0.27) values, were significantly larger in horses with heaves than in controls. L2 area/Pi was significantly larger in intermediate airways of horses with heaves than in controls (p=0.003). L2 and L2 area/Pi^2 values tended to be greater in intermediate airways of horses with heaves than in controls (p=0.03 and p=0.02, respectively, but not statistically significant after Bonferroni correction). In large bronchi, only L2 area/Pi values were higher in horses with heaves than controls (p=0.04) (Figure 6C). Power analysis indicated that n=6 horses per group would be needed to show a statistically significant effect 80% of the times if there was a 30% difference of L2 thickness or a 60% difference of

L2 area/Pi2 between the two groups when n≈10 bronchi of similar size are analyzed in each subject (Table 5).

Post-mortem histological analyses were performed. Morphometrical analysis of 8 to 10 bronchial sections per lung confirmed that A_{ASM}/Pi^2 was significantly larger in horses with heaves than in the controls (p=0.04) pooling all bronchi. Overall, A_{ASM}/Pi^2 values were larger in intermediate than in large bronchi (p=0.02) and a significant difference between horses with heaves and controls was observed in intermediate (p=0.03) but not in large bronchi (p=0.29). A_{ECM}/Pi^2 values were similar between the two groups across all bronchi (p=0.26) and also when measured in intermediate and large bronchi separately (p>0.1 for both, Figure 7).

Composition of L2

The mean thickness of ASM in the airways of both heaves-affected and control horses varied with airway size (p=0.0009). For this reason, we analyzed only airways with Pi<26mm, whose number and mean Pi was similar in both groups (Table 6). ASM and ECM calculated thickness were increased in the airways of asthmatic horses compared to controls (p=0.057 and p=0.02, respectively). Interestingly, both ASM and ECM thickness were overestimated when measured manually compared to when it was calculated using our derived measurements (mean±SD reduction of 33.3±12.1% for ASM and of 28.5±12.5% for ECM calculated values, Figure 8). Histologic submucosal thickness (the sum of ECM and ASM calculated thicknesses) was increased in horses with heaves compared to controls (p=0.027). The submucosal composition of the equine airways is affected by both disease and airway size (Figure 9). Indeed, the slopes of ECM% and ASM% were significantly different in the 2 groups (p=0.02) and a significant association between Pi and ECM% and between Pi and ASM% was found in horses with heaves (p=0.04) but not in controls (p=0.2). No significant differences were identified between the mean proportional content of ECM or ASM relative to the total submucosal layer between controls and heaves-affected horses (p=0.3 for both). L1 represented entirely ECM in horses of both groups. L2 represented ECM and ASM in similar proportion in both groups of horses. Indeed, on average, 22% of L2 represented ECM and

78% represented the ASM in control horses, while 28% of L2 represented ECM and 72% represented ASM in horses with heaves (p=0.6 for both) (Figure 10).

Discussion

Airway remodeling is a central feature of asthma. ASM mass is increased in central airways, more prominently in severe and fatal asthma [2]. However, its quantification *in vivo* is difficult, as imaging techniques such as computerized tomography or magnetic resonance imaging do not allow visualization of ASM, and endobronchial biopsies often provide incomplete sampling [8]. While EBUS has revealed an increase in L2 thickness in asthmatics compared to healthy subjects, which has been linked to ASM remodeling indirectly by means of bronchoprovocation tests [13], this is the first report directly comparing L2 remodeling with EBUS and ASM remodeling in histology. Using EBUS, we found that in the central airways of horses L2 is composed mainly by ASM (about 75% of its thickness) with the remaining 25% representing ECM, independently from the presence or absence of heaves-associated remodeling. Indeed, EBUS reliably quantified the ASM mass in both healthy and asthmatic animals, providing accurate estimates compared to histology, and this despite the high variation in remodeling likely to occur even within the same airway as reported in man [17]. Our *in vivo* results support EBUS usefulness for monitoring bronchial remodeling over time or during pharmacological treatments in this model. Importantly, its relative non-invasiveness will permit evaluating the same sites at different points in time.

Echographic anatomy of the equine bronchial wall is similar to that of man [11, 13] and sheep [18], in which this technique has previously been reported. The thickness of the first EBUS layer (L1), representing the bronchial epithelium and the most axial part of the underlying ECM, was not affected by disease status in our study. Contrarily, the thickness of the second EBUS layer (L2), representing the more abaxial part of the ECM and the ASM, was significantly increased in horses with heaves compared to controls. Curiously, L2 (and not L1) thickness has been shown to correlate with basal membrane thickness in human asthma although a significant difference between the thickness of L1 was observed in the same study between asthmatics and controls [12]. As L2 represents ASM and a variable part of the ECM [11] which does not include the basal membrane, its significant relationship with basal membrane thickness in asthmatics was more likely a spurious correlation rather than a histologic confirmation of EBUS findings. Indeed, it has been shown that the reticular

basement membrane thickness of central airways is correlated with submucosal airway remodeling in cartilaginous airways of asthmatics [8]. Increased basal membrane thickness has not been reported in heaves to our knowledge. However, preliminary observations from our laboratory suggest that basal membrane thickening is only an occasional finding in heaves, validating the fact that no difference was observed in L1 thickness between the 2 groups that we studied. Our results highlighted that ASM was mostly responsible for the increased L2 thickness in EBUS images of asthmatic horses compared to controls, and demonstrated that not only small [19, 20] but also large airways can exhibit remodeling in heaves. The significant association observed between values of A_{ASM} and L2 area, the absence of systematic bias for ASM quantification between histology and EBUS, and the similarity of ECM mass in the 2 groups found in this study already suggested that L2 changes in asthmatic horses mainly reflected ASM mass increases. This was confirmed by the finding that ASM represents on average 75% of L2 in both control and asthmatic horses. Horses experiencing exacerbations of the disease showed increased ECM mass in endobronchial biopsies (measured as the distance between the epithelium and the ASM layer) compared to controls [7]. In our study, we also found that ECM calculated thickness was increased in intermediate bronchi of asthmatic horses compared to controls. However, values of A_{ECM}/Pi^2 were similar in both groups. As both ASM and ECM thickness increase at increasing Pi, it is possible that despite Pi of the airways analyzed in both groups being similar, the small gap between the mean values of airway size of the 2 groups could have accounted for the statistical significance. Alternatively, as ECM represents constantly 25% of L2 and L2 thickness is increased in heaves, it is likely that ECM sustains a certain degree of remodeling in heaves, but it could be mild and thus lost with normalization (correction by Pi^2). Finally, analyses for ECM/Pi^2 were made on intermediate airways with Pi<31mm while analyses for ECM thickness were made on airways with Pi<26 mm, in order to have the same number of airways of similar Pi in both groups. It is possible that values of airways with 26mm<Pi<31mm could have accounted for the difference observed.

We have attempted for the first time to describe the composition of L2 in the asthmatic airways by studying naturally occurring equine heaves. Our results indicate that ASM accounts for approximately 75% of L2 in both healthy and diseased animals, with ECM

representing the remaining 25%. Our finding of a constant portion of L2 being occupied by ASM is in agreement with the results of a previous study in which a significant correlation between L2 thickness and airway hyperresponsiveness was found in asthmatic patients [13]. Indeed, two different studies have shown that the increase in ASM mass is potentially the most important structural determinant explaining hyperresponsiveness in asthmatics [21, 22]. Data obtained on lungs of healthy and asthmatic human lungs suggest that the proportion of the submucosa occupied by the ASM in man might be lower compared to those we found in horses in airways of similar size [5, 23]. Nevertheless, the thickness of the submucosal tissues measured at EBUS appears to be similar between the two species, considering airways of similar size [12].

We have also explored for the first time the relationship between bronchial size and ASM remodeling as estimated by EBUS (L2 thickness or L2 area). L2 thickness increased linearly with bronchial size in both asthmatic and healthy equine airways, which is in agreement with the ASM distribution along the bronchial tree (expressed as ASM thickness) in man [24]. Remarkably, such increase was steeper in asthmatic airways compared to controls. This finding, supported by our histological findings, indicates that heaves can display a phenotype similar to the type II form of asthma as defined by Ebina [23], characterized by remodeling of small [25] and large airways, at least in some cases. We also explored L2 area/Pi2 ratio, as its histological equivalent A_{ASM}/Pi^2 is commonly used for ASM mass normalization [25, 26]. Pi2 accounts for the relatively greater amount of both ASM and ECM components in small than in large bronchi [27, 28]. As previously demonstrated for A_{ASM}/Pi^2, the slope of the curve of L2 area/Pi^2 ratio plotted against airway size (expressed as Pi) was non-significant in intermediate airways of control horses, confirming appropriateness of normalization. The lack of correlation between L2 area/Pi^2 ratio and its histological equivalent A_{ASM}/Pi^2 was possibly due to amplification of the bias of Pi (underestimated by EBUS, especially in larger airways) when raised to the power of 2. However, since bias of Pi measures was constant in both groups, we judged it correct to compare their L2 area/Pi^2 ratio values in our analysis.

Variability among different airways constituted the main source of variation in our *ex vivo* study for the remodeling parameters assessed. This finding was expected due to our study

design, in which airways of different size were studied in each subject. However, the second greatest source of variation was represented by the group (heaves vs control) for all parameters studied, supporting the role of EBUS in the assessment of central remodeling in heaves. Similar findings were observed *in vivo*. A greater variation among different images of the same airway was observed *in vivo* compared to *ex vivo*, possibly due to the respiratory movements. L2 area/Pi^2 variation among different airways was greater *ex vivo* than *in vivo*. We ascribed this finding to the fact that different degrees of pulmonary collapse could have occurred in the lungs of different animals or at different levels of the bronchial tree within the same lungs. The finding that L1, L2, and L2 area/Pi are affected by airway size highlights the importance of sampling airways of similar sizes in different subjects in order to avoid sampling bias when these parameters are used.

From a clinical perspective, our results support the implementation of EBUS as a tool for assessing large airway remodeling in asthmatic patients. Despite its relatively low invasiveness (compared for instance to the endobronchial biopsy procedure) and relative ease of analysis of measurements, this technique is underemployed in asthma possibly because of its slow learning curve [29, 30], and paucity of data supporting its use to assess airway remodeling in asthma. Our findings that EBUS reliably assesses ASM mass in proximal airways in heaves, combined with the previous report correlating EBUS L2 thickness and lung function in asthma [13], support the use of this technique for evaluating the efficacy of treatments at reducing ASM mass in asthmatics. EBUS may also prove useful for defining specific asthma phenotypes based on different ASM remodeling patterns along the bronchial tree [31], possibly predicting their response to specific treatments. In this perspective, the assessment of a single airway as described in previous studies investigating airway remodeling with EBUS [12, 13] does not take into account the heterogeneity existing among different airways or even among different sections of the same airway [17], and could represent an important source of bias even when the same airway is evaluated in all subjects. Our study showed that the assessment of 8 to 10 bronchi reliably estimated the ASM mass in both heathy and asthmatic horses, and it was always performed in less than 15 minutes, which do not exceed times reported in man for a standard EBUS procedure [32].

Our study has some limitations. First, we could not obtain physiological data of the horses studied *in vivo*. Indeed, most horses undergoing euthanasia were client-owned, and the short period of time elapsing between euthanasia decision and death did not allow lung function testing. The number of horses employed in the *in vivo* phase is limited, but as *in vivo* data reflected what previously observed *ex vivo* we believe that it was sufficient to support our conclusions. Finally, the morphometric analyses performed on ECM were limited to the lamina propria, as we did not consider the ECM elements outside the ASM layer. This was mainly due to technical difficulties, as collagen fiber shredding often preventing a reliable assessment of ECM mass outside the ASM layer. Previous studies evaluating ECM remodeling in horses with heaves and asthmatic patients have used the same approach both in small and in large airways [3, 7, 16, 20].

In conclusion, our results indicate that EBUS provides reliable estimates of ASM remodeling in both asthmatic and healthy airways, and allows differentiation of such conditions based on remodeling features. Contrarily to endobronchial biopsies, it offers the possibility of studying non-carinae-derived bronchial tissue and to do it prospectively in the same airways as physical removal of bronchial tissue is not required. Finally, EBUS represent a promising technique for assessment of drug efficiency in reversing asthma-associated remodeling over time in a safe and non-invasive way.

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Figures and tables

Table 1. Study design.

Phase	Aim(s)	Methods	Samples/Animals
1	Describing equine echographic bronchial anatomy	NPE	26 bronchial samples from lungs of 6 slaughtered horses*
2	Comparing measures of interest performed with EBUS and histology	EBUS and histology	109 bronchial samples from lungs of 13 slaughtered horses*
3	*Ex vivo* technique optimization and "proof of concept"	EBUS on isolated lungs	lungs from 4 horses with heaves and 7 controls
4	*In vivo* EBUS	EBUS on horses	4 horses with heaves and 3 controls
5	Validation a. assessing the effect of lung removal from the thorax (collapse) on EBUS; b. histological validation .	EBUS and histology on post-mortem specimens of horses studied in phase 4	same horses studied in phase 4

* Lungs with obvious macroscopic alterations were excluded from the study. NPE: needle puncture experiments; EBUS: endobronchial ultrasound.

Table 2. Details of the animals studied *in vivo*.

Horse	Group	Age [years]	Breed	Disease status	Clinical score	Weight [kg]	Mucus score	Septum thickness	Bronchiectasis	DeltaP$_L$ [cmH$_2$O]	R$_L$ [cmH$_2$O/L/sec]
1	Control	12	QH	-	2/8	480	0/5	2/4	no		
2	Control	25	QH	-	3/8	450	0/5	0/4	regional		
3	Control	8	Pony	-	2/8	253	0/5	1/4	no		
4	Heaves	27	QH	Remission	4/8	438	1/5	2/4	yes		
5	Heaves	14	STB	Symptomatic	6/8	430	1/5	3/4	yes	25	1.8
6	Heaves	15	QH	Symptomatic	5/8	450	2/5	4/4	yes		
7	Heaves	27	Polo Pony	Symptomatic	6/8	450	3/5	2/4	regional		

QH: Quarter Horse; STB: Standardbred. Clinical scores were assessed as defined by Robinson [33]. Septum thickness was assessed as defined by Koblinger [15]. *Ante-mortem* BAL data were not available. Pulmonary mechanics were available only for one horse, as it belonged to our research herd.

Table 3. Mixed linear models and Bland-Altman tests for comparison between EBUS and histologic measurements.

		Mixed linear model		Bland-Altman test (EBUS-histology)				
Parameter	**N**	**p-value**	**R^2**	**Difference** mean (SD)	**Mean** mean (SD)	**Slope**[1]	**Intercept**[2]	**Systematic bias**
Perimeter [mm]	101	<0.0001	0.20	-5.04 (6.99)	22.67 (8.52)	-0.42, p<0.0001	4.46, p=0.02	yes
Diameter [mm]	101	<0.0001	0.30	0.14 (1.51)	4.7 (2.15)	-0.15, p=0.04	0.87, p=0.03	yes
Lumen area [mm^2]	21	<0.0001	0.80	-4.59 (15.98)	34.07 (42.67)	-0.15, p=0.08	0.44, p<0.98	no
A_{ASM}*L2 area [mm^2]	98	<0.0001	0.31	-0.0534 (1.843)	3.3263 (2.99)	-0.01, p=0.083	0.005, p<0.99	no
A_{ASM}/Pi*L2 area/Pi [mm]	98	0.001	0.16	0.0312 (0.0634)	0.1323 (0.06)	0.27, p=0.02	-0.004, p<0.78	yes
A_{ASM}/Pi^2*L2 area/Pi^2 [-]	98	0.45	0.030	0.0029 (0.0038)	0.0062 (0.002)	0.93, p<0.0001	-0.003, p=0.006	yes

[1] p values refer to the null hypothesis that the slope=0.

[2] p values refer to the null hypothesis that the intercept=0.

Table 4. Variance component analysis.

Ex vivo analysis				
Parameter	Variation (%)			
	Group	Subject	Airway	Images
L1	0	8.5	**72.2**	19.3
L2	27.1	3.3	**55.7**	13.9
L2 area/Pi	38.4	5.1	**41.5**	15
L2 area/Pi^2	32.5	0	**42.5**	25
In vivo analysis				
Parameter	Variation (%)			
	Group	Subject	Airway	Images
L1	7.2	12.6	**53.6**	26.6
L2	33.6	0	**43.1**	23.3
L2 area/Pi	**46.3**	0	30.2	23.5
L2 area/Pi^2	29.7	7.4	10.3	**52.6**

Table 5. Power analysis for *in vivo* EBUS.

Parameter	Expected magnitude of the effect	N
L2	15%	22
	30%	**6**
	60%	2
L2 area/Pi	15%	80
	30%	21
	60%	**7**
L2 area/Pi^2	15%	76
	30%	21
	60%	**6**

N represents the number of subjects needed to obtain a statistically significant effect of the group 80% of the times for different effect sizes (expressed as %) using the methodology described earlier.

Table 6. Data used for determining L2 composition.

	Controls	Heaves	*p value*
Histology			
N [airways/horse]	4.67±0.57	5±2.16	0.58
Pi [mm]	18.94±1.91	20.09±3.93	1.00
ECM thickness [mm]	0.0833±0.014	0.1356±0.025	**0.02**
ASM thickness [mm]	0.0762±0.008	0.1254±0.04	**0.057**
Submucosal thickness [mm]	0.160±0.011	0.261±0.05	**0.027**
Submucosal ECM % at histology	52±3.2	53.5±2.9	0.3
Submucosal ASM % at histology	48±3.2	46.5±2.9	0.3
EBUS			
N [airways/horse]	7.67±5303	8.5±3.70	0.85
Pi [mm]	16.19±1.71	19.86±1.61	0.11
L1 thickness [mm]	0.11±0.003	0.127±0.010	0.06
L2 thickness [mm]	0.177±0.022	0.269±0.021	**0.004**
Submucosal thickness [mm]	0.287±0.024	0.397±0.015	**0.024**
Submucosal L1% at EBUS	38.5±2.5	32.1±3.2	**0.037**
Submucosal L2% at EBUS	61.5±2.5	67.9±3.2	**0.037**
Composition of L1			
ECM%	100	100	1
ASM%	0	0	1
Composition of L2			
ECM%	22.1±8.9	28.1±5.7	0.63
ASM%	77.9±8.9	71.9±5.7	0.63
Others			
Shrinkage (%)	44.28±4.88	47.41±7.92	0.7

Values are presented as mean±SD of the values obtained from the airways of 4 horses with heaves and 3 controls. Bold characters indicate significance as results of t-tests. Mean values per horse were used as statistical unit.

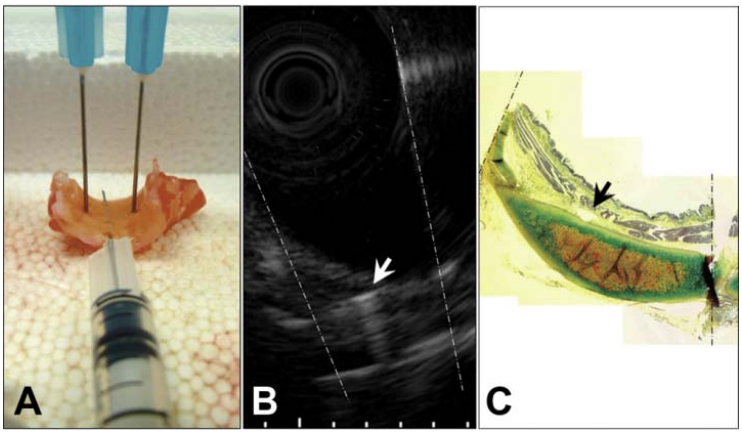


Figure 1. Illustrative images of a needle puncture experiment (NPE). Note the echo produced by the needle inserted longitudinally to the long axis of the bronchus in B (white arrow), and its corresponding histological localization as identified in C (black arrow). Dotted lines represent needles positioned transversally to the longitudinal axis of the bronchus.

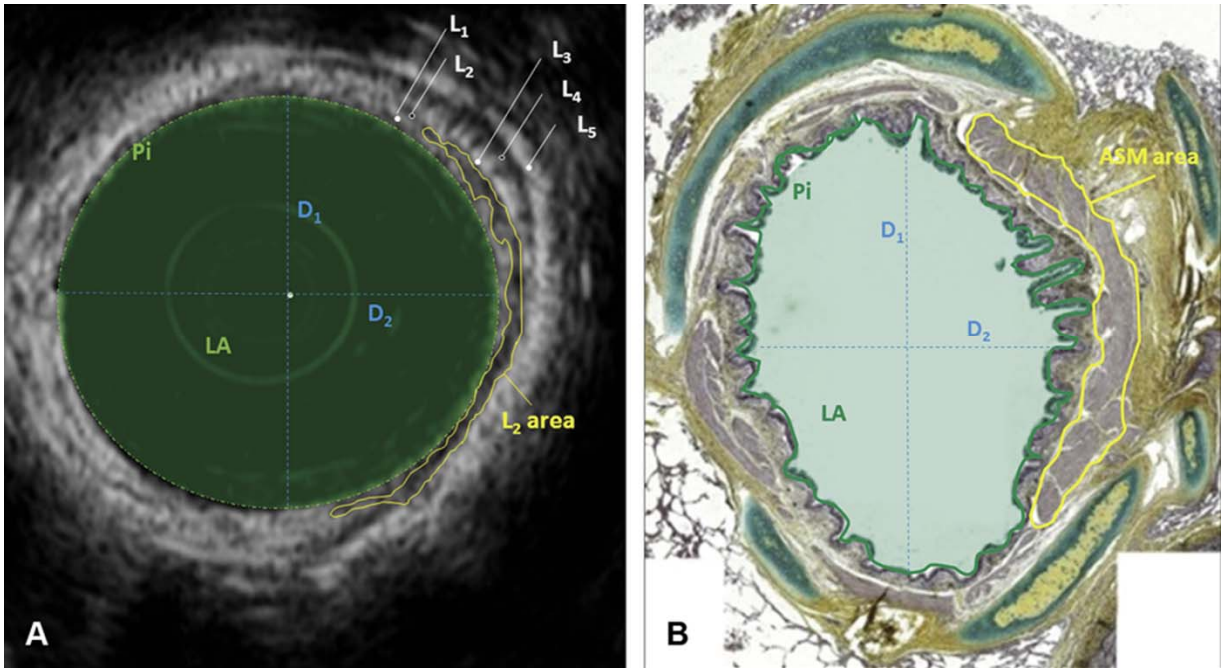


Figure 2. Measurements made on EBUS (A) and histological (B) images. Only a part of L2 area and ASM area have been encircled in yellow, to allow the reader appreciate the rest of the image. L: US layer; D_1 and D_2: perpendicular diameters (blue dotted lines); LA: lumen area (filled light green area); Pi: airway perimeter (continuous green line).

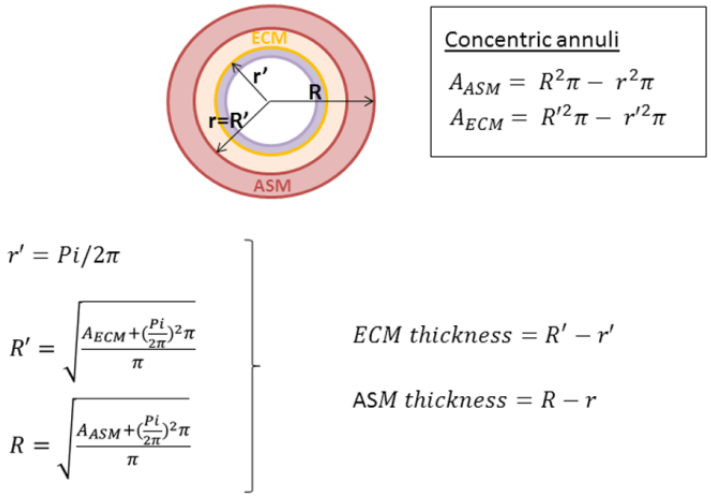


Figure 3. Scheme of a distended bronchial wall. In our mathematical model, the bronchial wall was treated as a structure composed of multiple concentric annuli. Excluding the inner annulus, representing the epithelial layer (in purple, negligible in thickness), we considered the submucosa as composed by two annuli representing the ECM (yellow) and ASM (pink). Pi corresponded to the yellow circumference in our model.

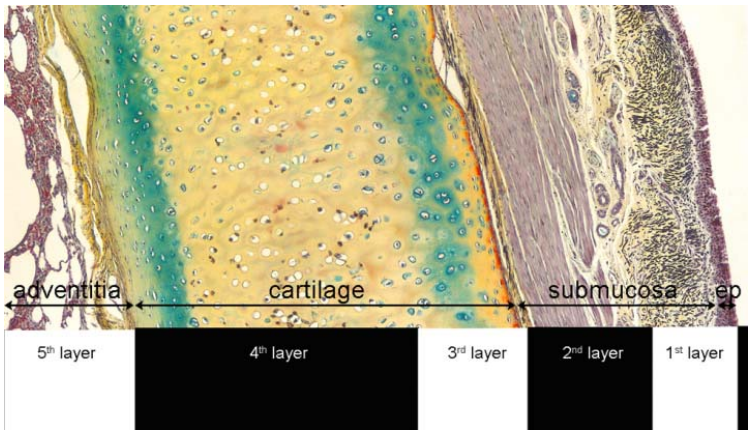


Figure 4. Comparison of EBUS and histological composition of equine airways. In the absence of cartilages, L3 was represented by the adventitia.

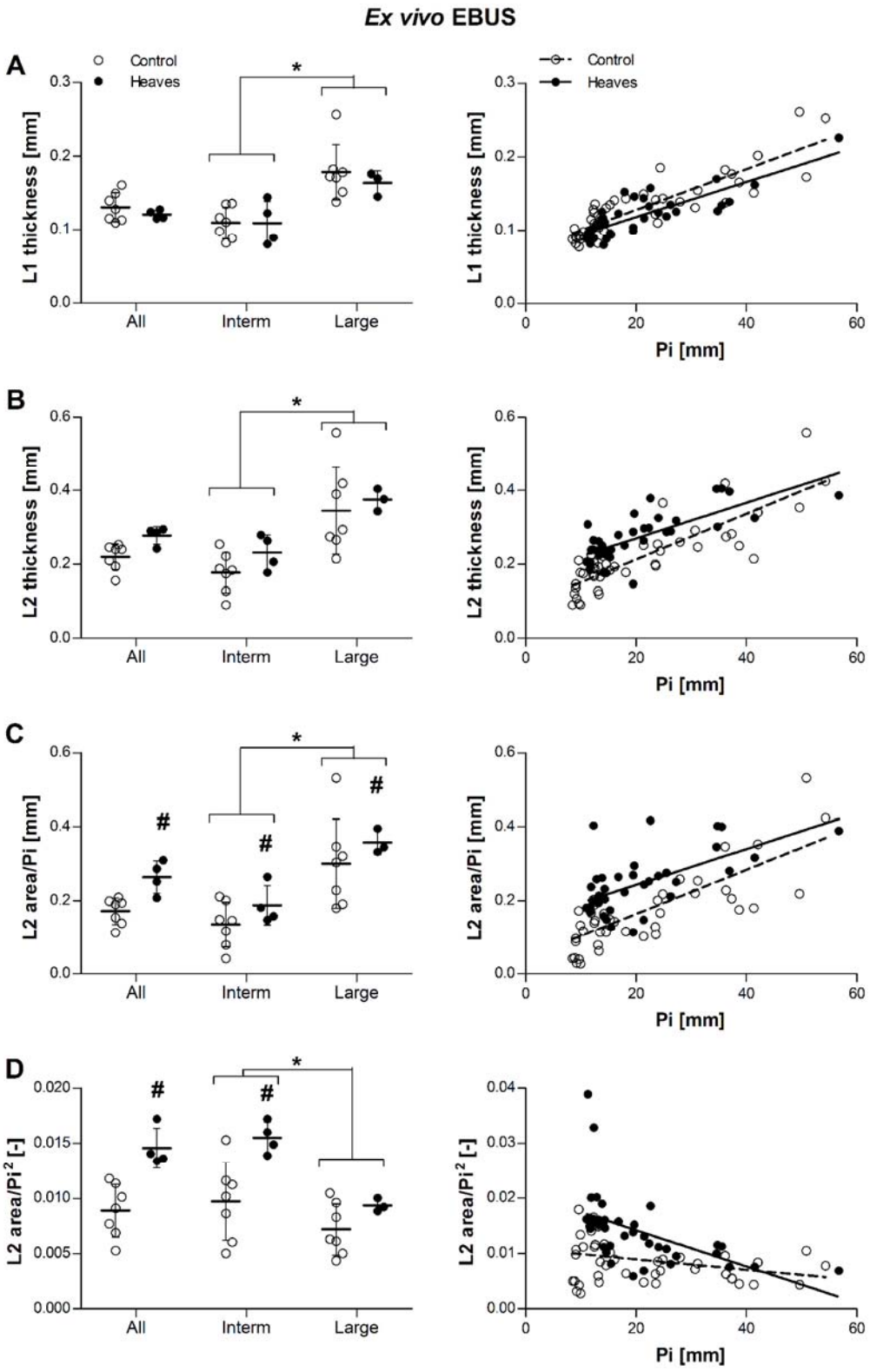


Figure 5. Effect of group and airway size on L1 thickness (A), L2 thickness (B), ratio L2

area/Pi (C), and ratio L2 area/Pi^2 (D) in *ex vivo* EBUS. Raw data (before statistical corrections) are presented as mean per animal in the graphs on the left, and as mean per airway in the graphs on the right. L1 and L2 thickness was similar in horses with heaves and controls across all bronchi (p=0.4 and p=0.1, respectively). Contrarily, L2 area/Pi and L2 area/Pi^2 ratios were greater in horses with heaves than controls across all bronchi (p=0.004 and p=0.02, respectively). L1 values were similar in intermediate (Pi<31mm, p=0.82) and large bronchi (Pi>31mm, p=0.32) analyzed separately. L2 tended to be thicker in intermediate bronchi of horses with heaves than in controls (p=0.06) but not in large bronchi (p=0.5). Both L2 area/Pi and L2 area/Pi^2 ratios were greater in intermediate bronchi of horses with heaves then controls (p=0.006 and p=0.003, respectively), but only L2 area/Pi ratios were greater also in large bronchi (p=0.04; p=0.3 for L2 area/Pi^2 ratios). Overall, L1, L2 and L2 area/Pi values were greater in large than intermediate bronchi (p=0.0003, p=0.001, and p=0.002, respectively), while L2 area/Pi^2 values were greater in intermediate than in large bronchi (p=0.02). L1, L2, and L2 area/Pi increased significantly with Pi in both groups (p<0.0001 for all), while L2 area/Pi^2 decreased significantly with increasing Pi (p<0.0001). The slope of the relationship did not differ between the two groups for L1, L2 and L2 area/Pi (p=1, p=0.14, p=0.66, respectively), but it was significantly greater in the heaves group than in controls for L2 area/Pi^2 (p=0.01). #: significantly different from controls. *: significant difference between values of intermediate and large bronchi (pooling the two groups).

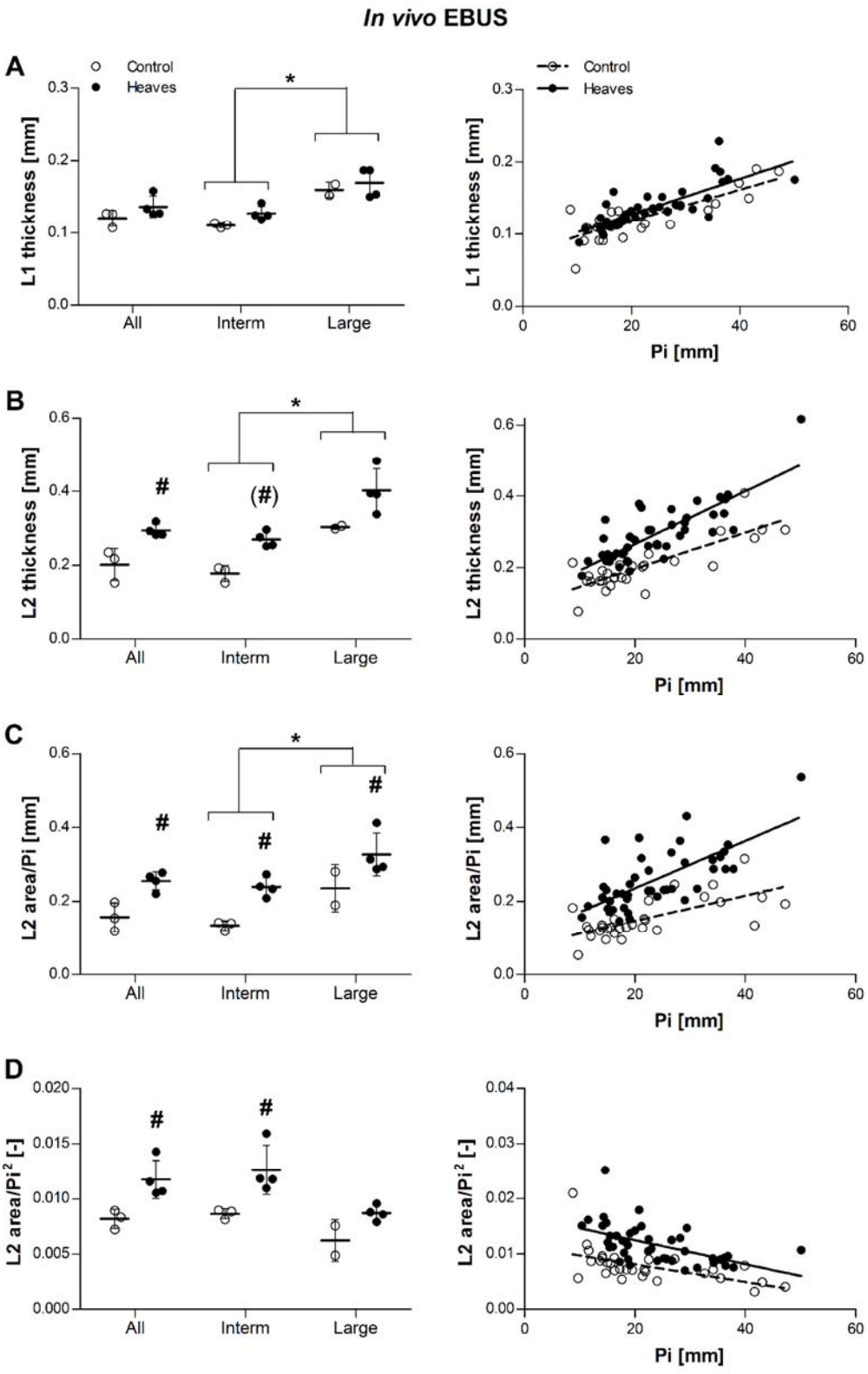


Figure 6. Effect of group and airway size on L1 thickness (A), L2 thickness (B), ratio L2

area/Pi (C), and ratio L2 area/Pi2 (D) in *in vivo* EBUS. Raw data (before statistical corrections) are presented as mean per animal in the graphs on the left, and as mean per airway in the graphs on the right. L1 was similar in horses with heaves and controls across all bronchi (p=0.27). Contrarily, L2 thickness, L2 area/Pi and L2 area/Pi^2 ratios were greater in horses with heaves than controls across all bronchi (p=0.03, p=0.005 and p=0.04, respectively). L1 values were similar in intermediate (Pi<31mm, p=0.13) and large bronchi (Pi>31mm, p=0.63) analyzed separately. L2 tended to be thicker in intermediate bronchi of horses with heaves than in controls (p=0.03, non-significant after correction) but not in large bronchi (p=0.13). Both L2 area/Pi and L2 area/Pi^2 ratios were greater in intermediate bronchi of horses with heaves then controls (p=0.003 and p=0.02, respectively), but only L2 area/Pi ratios were greater also in large bronchi (p=0.04; p=0.22 for L2 area/Pi^2 ratios). Overall, L1, L2 and L2 area/Pi values were greater in large than intermediate bronchi (p=0.002, p=0.005, and p=0.01, respectively), while L2 area/Pi^2 only tended to be greater in intermediate than in large bronchi (p=0.05). L1, L2, and L2 area/Pi increased significantly with Pi in both groups (p<0.001 for all), while L2 area/Pi^2 decreased significantly with increasing Pi (p<0.001). The slope of the relationship did not differ between the two groups for L1 and L2 area/Pi^2 (p=0.86 and p=0.49, respectively), and it was significantly greater in horses with heaves for L2 and L2 area/Pi (p=0.02 and p=0.04, respectively). Filled dots represent heaves and open dots represent controls. #: significantly different from controls. *: significant difference between values of intermediate and large bronchi (pooling the two groups).

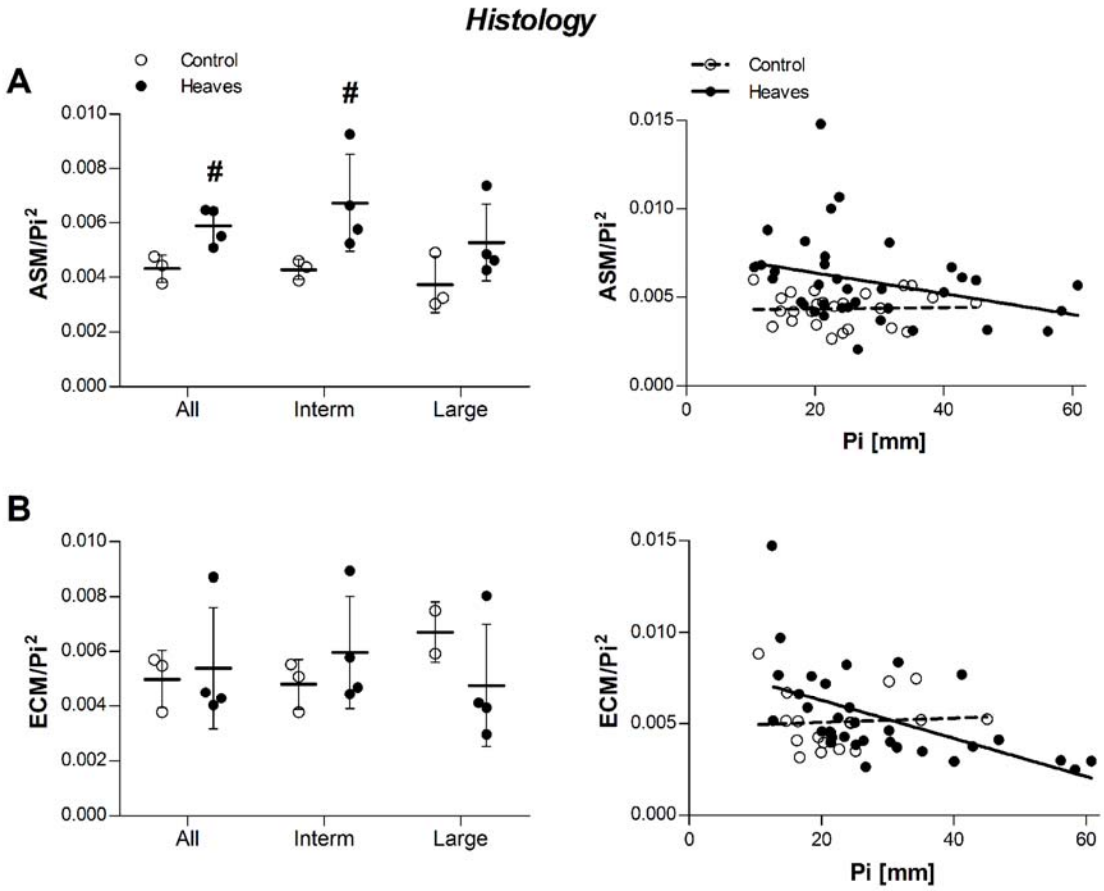


Figure 7. Effect of group and airway size on A_{ASM}/Pi^2 (A) and A_{ECM}/Pi^2 (B) measured on histological sections. Raw data (before statistical corrections) are presented as mean per animal in the graphs on the left, and as mean per airway in the graphs on the right. Histological analysis confirmed *in vivo* and *ex vivo* EBUS findings concerning ASM remodeling. The slope of the A_{ASM}/Pi^2 relationship was similar between the 2 groups (p=0.26), but heaves-affected horses had significantly higher values compared to controls (p=0.04). The slope of the A_{ECM}/Pi^2 relationship tended to be different between the 2 groups (p=0.07), but heaves-affected horses had similar values compared to controls (p=0.26). #: significantly different from controls. *: significant difference between values of intermediate and large bronchi (pooling the two groups).

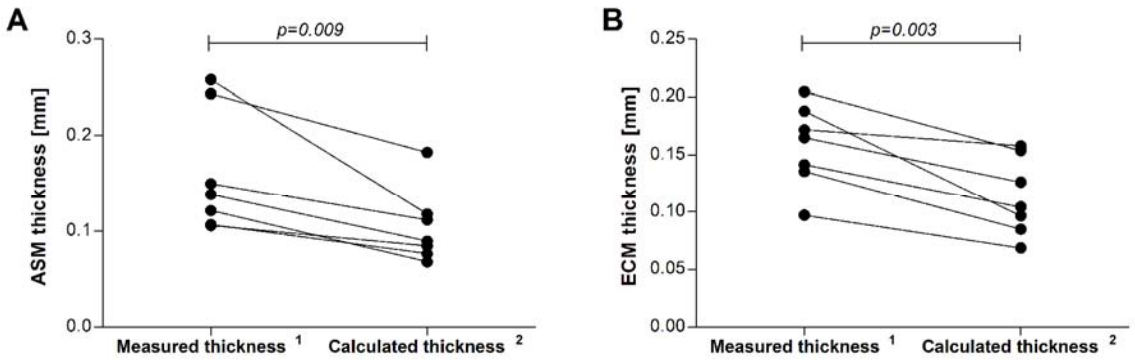


Figure 8. Effect of the method used for measuring ASM (A) and ECM (B) thickness in histologic bronchial samples. Results are shown as mean values per horse. [1]Mean value of 5 measures of ASM or ECM thickness performed manually and randomly around the bronchial circumference (ECM was measured from the basal membrane to the ASM inner border, avoiding regions where obvious collagen fiber shredding occurred). [2]Thickness of the ASM or ECM calculated as function of the ASM or ECM area and airway Pi, as if it was a continuous and homogeneous layer for an airway completely distended (Pi corresponds to the inner circumference of an annulus). Statistical differences were calculated with paired t-tests.

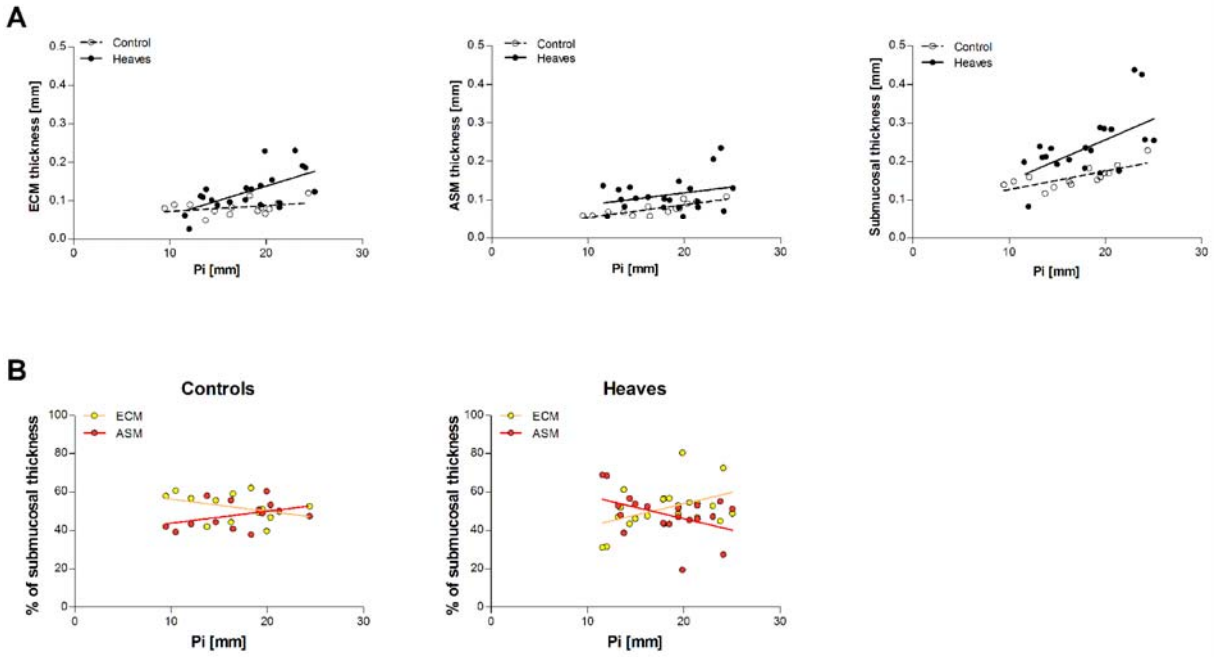


Figure 9. Composition of the submucosal tissues at histology. Values are presented as percentage of the submucosal tissue occupied by ECM and ASM for each airway. N=14 for controls and 20 for heaves. On average, a greater percentage of submucosal thickness was occupied by ASM in smaller airways of horses with heaves compared to controls. This tendency reverses as the airway size increases. However, as the submucosal thickness is increased in horses with heaves compared to controls, the absolute thickness of the ASM layer still remains greater in horses with heaves than in controls for airways with Pi<26mm.

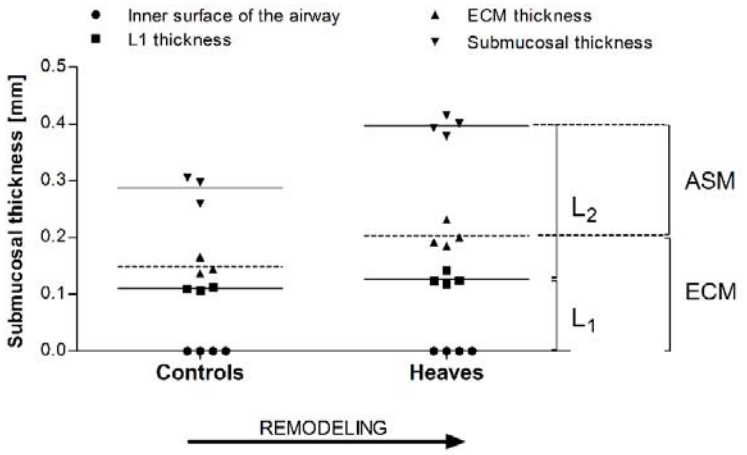
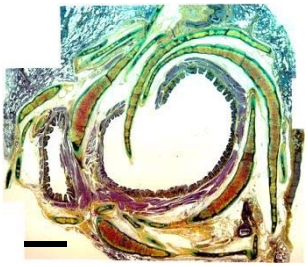
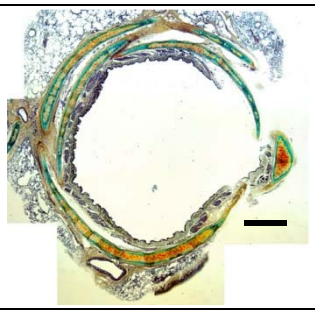
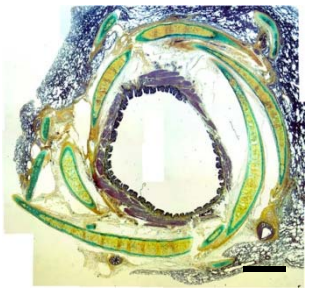
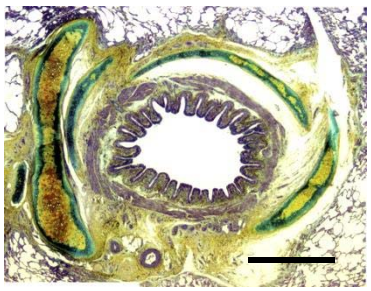


Figure 10. Composition of L2 is similar in asthmatic and control horses. The inner 25% is occupied by ECM while the remaining outer 75% is occupied by ASM. Notice that most of the ECM thickness lies behind the echo of L1.

Supporting information

Table S1. Histological quality score.

Airway section	Score	Description
	1	Airway wall missing for >90° or cut oblique to the perpendicular axis of the airway or significantly altered tissue architecture
	2	Cut perpendicular or slightly oblique to the airway longitudinal axis or airway wall missing for >45°, well preserved tissue architecture
	3	Cut perpendicular to the airway longitudinal axis, airway wall components (epithelium/ASM) missing for 15-45°, well preserved tissue architecture
	4	Cut perpendicular to the airway longitudinal axis, complete airway wall, very well preserved tissue architecture

Images with score 1, 2 and 3 were taken at 2.5x magnification, while image scored 4 was taken at 5x magnification (scale bars = 1 mm in all figures).

Table S2. The ARRIVE Guidelines Checklist.

The ARRIVE Guidelines Checklist

Animal Research: Reporting In Vivo Experiments

Carol Kilkenny[1], William J Browne[2], Innes C Cuthill[3], Michael Emerson[4] and Douglas G Altman[5]

[1]The National Centre for the Replacement, Refinement and Reduction of Animals in Research, London, UK, [2]School of Veterinary Science, University of Bristol, Bristol, UK, [3]School of Biological Sciences, University of Bristol, Bristol, UK, [4]National Heart and Lung Institute, Imperial College London, UK, [5]Centre for Statistics in Medicine, University of Oxford, Oxford, UK.

	ITEM	RECOMMENDATION	Section/ Paragraph
Title	1	Provide as accurate and concise a description of the content of the article as possible.	
Abstract	2	Provide an accurate summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings and conclusions of the study.	
INTRODUCTION			
Background	3	a. Include sufficient scientific background (including relevant references to previous work) to understand the motivation and context for the study, and explain the experimental approach and rationale. b. Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study's relevance to human biology.	
Objectives	4	Clearly describe the primary and any secondary objectives of the study, or specific hypotheses being tested.	
METHODS			
Ethical statement	5	Indicate the nature of the ethical review permissions, relevant licences (e.g. Animal [Scientific Procedures] Act 1986), and national or institutional guidelines for the care and use of animals, that cover the research.	
Study design	6	For each experiment, give brief details of the study design including: a. The number of experimental and control groups. b. Any steps taken to minimise the effects of subjective bias when allocating animals to treatment (e.g. randomisation procedure) and when assessing results (e.g. if done, describe who was blinded and when). c. The experimental unit (e.g. a single animal, group or cage of animals). A time-line diagram or flow chart can be useful to illustrate how complex study designs were carried out.	
Experimental procedures	7	For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example: a. How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s). b. When (e.g. time of day). c. Where (e.g. home cage, laboratory, water maze). d. Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used).	
Experimental animals	8	a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range). b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naïve, previous procedures, etc.	

Housing and husbandry	9	Provide details of: a. Housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish). b. Husbandry conditions (e.g. breeding programme, light/dark cycle, temperature, quality of water etc for fish, type of food, access to food and water, environmental enrichment). c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.	
Sample size	10	a. Specify the total number of animals used in each experiment, and the number of animals in each experimental group. b. Explain how the number of animals was arrived at. Provide details of any sample size calculation used. c. Indicate the number of independent replications of each experiment, if relevant.	
Allocating animals to experimental groups	11	a. Give full details of how animals were allocated to experimental groups, including randomisation or matching if done. b. Describe the order in which the animals in the different experimental groups were treated and assessed.	
Experimental outcomes	12	Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioural changes).	
Statistical methods	13	a. Provide details of the statistical methods used for each analysis. b. Specify the unit of analysis for each dataset (e.g. single animal, group of animals, single neuron). c. Describe any methods used to assess whether the data met the assumptions of the statistical approach.	
RESULTS			
Baseline data	14	For each experimental group, report relevant characteristics and health status of animals (e.g. weight, microbiological status, and drug or test naïve) prior to treatment or testing. (This information can often be tabulated).	
Numbers analysed	15	a. Report the number of animals in each group included in each analysis. Report absolute numbers (e.g. 10/20, not 50%[2]). b. If any animals or data were not included in the analysis, explain why.	
Outcomes and estimation	16	Report the results for each analysis carried out, with a measure of precision (e.g. standard error or confidence interval).	
Adverse events	17	a. Give details of all important adverse events in each experimental group. b. Describe any modifications to the experimental protocols made to reduce adverse events.	
DISCUSSION			
Interpretation/ scientific implications	18	a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature. b. Comment on the study limitations including any potential sources of bias, any limitations of the animal model, and the imprecision associated with the results[2]. c. Describe any implications of your experimental methods or findings for the replacement, refinement or reduction (the 3Rs) of the use of animals in research.	
Generalisability/ translation	19	Comment on whether, and how, the findings of this study are likely to translate to other species or systems, including any relevance to human biology.	
Funding	20	List all funding sources (including grant number) and the role of the funder(s) in the study.	

References:
1. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLoS Biol* 8(6): e1000412. doi:10.1371/journal.pbio.1000412
2. Schulz KF, Altman DG, Moher D, the CONSORT Group (2010) CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials. *BMJ* 340:c332.

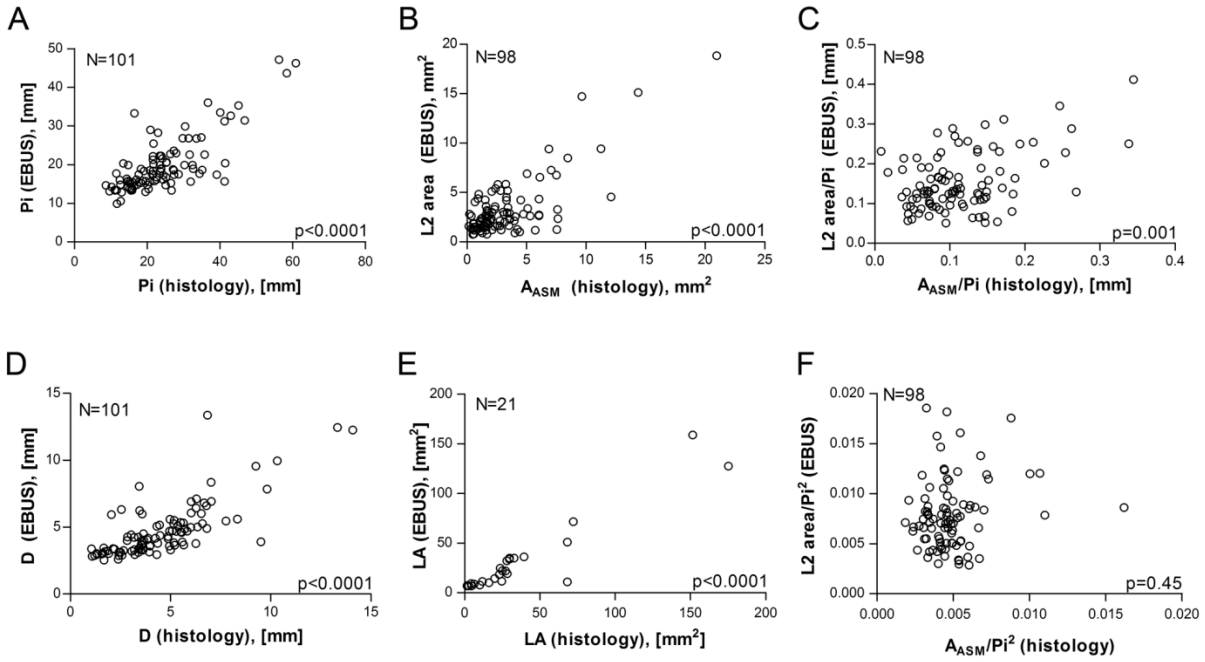


Figure S1. Reliability of EBUS compared to histology (mixed lineal model). Results of mixed linear models for the association between the measures of perimeter (A), diameter (D), lumen area (E), ASM area vs L2 area (B), ASM area/Pi versus L2/Pi (C) and ASM/Pi^2 versus L2 area/Pi^2 (F) obtained with EBUS and histology. Each dot represents the mean of the measures made for a single airway.

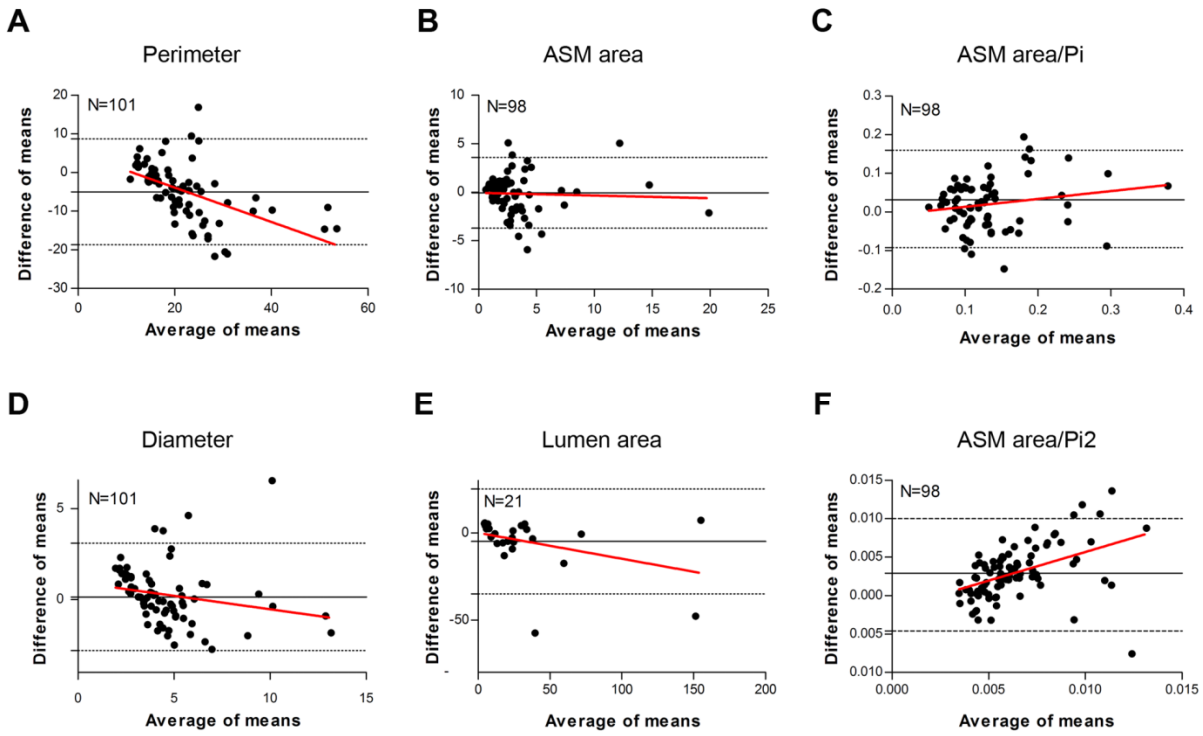


Figure S2. Reliability of EBUS compared to histology (Bland-Altman tests). Comparison of measures of perimeter (A), ASM area (B), ASM area/Pi (C), diameter (D), lumen area (E) and ASM area/Pi^2 (F) obtained with EBUS or histologic images studied with Bland-Altman tests. Each dot represents the mean of the measures made for a single airway. Continuous lines represent the mean difference between the two measuring methods (EBUS-histology). Dotted lines define the area where 95% of the differences should occur assuming a normal distribution of the differences. A negative difference means that EBUS values are smaller than the corresponding histology measurements.

Article 4

Fluticasone/salmeterol synergistic effects on asthma-associated airway remodeling and inflammation

Summary

Combined inhalation of corticosteroids and long acting β2-agonists (ICS/LABA) is more effective than ICS monotherapy at controlling asthma exacerbations. However, whether ICS/LABA can achieve a better control of inflammation or remodeling is not well established, especially at the peripheral airway level. In equine asthma (heaves), the enhanced therapeutic effect of ICS/LABA over ICS monotherapy was associated with a reduction of ECM deposition, mainly observed within the large airways, and also with a decreased neutrophilia in the bronchoalveolar lavage. However, ICS/LABA was not advantageous over ICS monotherapy for reversing ASM remodeling and submucosal inflammation.

Contribution

I contributed to the study design (40%), treatment administration (80%), data collection and analysis (lung function, 90%; bronchoscopies, 70%; thoracoscopies, 25%; post-operative follow-up, 90%; histology processing and staining of samples, 90%, immunohistochemistry, 90%; histomorphometry, 100%; statistical analysis, 70%), and preparation of the manuscript (90%).

Article in preparation for submission

Manuscript formatted for the *American Journal of Respiratory Medicine and Critical Care.*

FLUTICASONE/SALMETEROL SYNERGISTIC EFFECTS ON ASTHMA-ASSOCIATED AIRWAY REMODELING AND INFLAMMATION

Michela Bullone[1], Amandine Vargas[1], Yvonne Elce[1]*, James G. Martin[2], Jean-Pierre Lavoie[1].

[1] Université de Montréal, Faculty of Veterinary Medicine, Department of Clinical Sciences, 3200 rue Sicotte, St-Hyacinthe, J2S 2M2, Quebec, Canada.

[2] McGill University, Meakins Christie Laboratories, McGill University Health Centre Research Institute, 1001 Decarie Blvd, Montreal, H4A 3J1, Quebec, Canada.

* Current address: University of Edinburgh, Royal (Dick) School of Veterinary Studies, Easter Bush Campus, EH25 9RG, United Kingdom.

Corresponding author: Jean-Pierre Lavoie, Department of Clinical Sciences, Université de Montréal, 3200 rue Sicotte, St-Hyacinthe, J2S 2M2, Quebec, Canada. Phone: [redacted]. Fax: [redacted]. Email address: jean-pierre.lavoie@umontreal.ca.

Authors' contributions:

Conception and design: JPL, MB, AV, JM. Data acquisition: MB, AV, YE. Analysis and interpretation: MB. Drafting the manuscript: MB. Revising the manuscript for important intellectual content: JPL, JM. Final approval of the manuscript: MB, AV, YE, JPL, JM.

Running title: ICS/LABA synergy in remodeling reversal in asthma

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At a Glance Commentary

Scientific knowledge on the subject:

Asthma is incurable, perhaps because peripheral airway remodeling is only partially reversible by the administration of anti-inflammatory therapies such as inhaled corticosteroids (ICS). Recent findings indicate that bronchospasm may contribute to remodeling of the airways independently of inflammation. This suggests that the combination of ICS and bronchodilators such as long acting β2-agonists (ICS/LABA) could achieve a better control of asthmatic airway remodeling compared to ICS monotherapy.

What this study adds to the field:

We studied equine heaves, a model of neutrophilic asthma, to assess the contribution of ICS, LABA, and their combination to reversing remodeling and inflammation in central and peripheral airways. Our results suggest that the enhanced therapeutic effect of ICS/LABA over ICS monotherapy in asthmatic patients may be associated with a reduction of extracellular matrix deposition, mainly observed within the large airways, and possibly also with a decreased neutrophilia in the bronchoalveolar lavage fluid. However, ICS/LABA does not provide any additional benefit to ICS monotherapy in terms of peripheral airway smooth muscle remodeling and submucosal inflammation as both induce a 30% decrease of the airway smooth muscle mass in 3 months.

This article has an online data supplement, which is accessible from this issue's table of content online at http://atsjournals.org.

Abstract

Rationale: Asthmatic airways are inflamed and undergo remodeling. Inhaled corticosteroids and long-acting β2-agonist combinations are more effective than inhaled corticosteroid monotherapy in controlling disease exacerbations, but their efficacy compared to inhaled corticosteroids on bronchial remodeling and inflammation is unknown.

Objectives: To evaluate the contribution of inhaled fluticasone and salmeterol, alone or combined, to the reversal of inflammation and remodeling in central and peripheral airways.

Methods: Asthmatic horses with ongoing airway remodeling and inflammation were treated with fluticasone, salmeterol, fluticasone/salmeterol, or with antigen avoidance (6 horses per group) for 12 weeks. Lung function, central and peripheral airway remodeling and inflammation were assessed at multiple time points.

Measurements and Main Results: Fluticasone/salmeterol and fluticasone monotherapy decreased peripheral airway smooth muscle remodeling after 12 weeks ($p=0.007$ and $p=0.02$, respectively). On average, a 30% decrease was observed with both treatments. Fluticasone/salmeterol achieved this effect mainly by reversing myocyte hypertrophy, while no precise pattern was observed in horses treated with fluticasone monotherapy. In central airways, fluticasone/salmeterol synergistically reversed extracellular matrix remodeling after 12 weeks, both within the lamina propria (decreased thickness, $p=0.005$) and within the smooth muscle layer ($p=0.004$). Both fluticasone/salmeterol and fluticasone monotherapy decreased submucosal inflammation within central airways ($p<0.05$), while only fluticasone/salmeterol decreased bronchoalveolar neutrophilia ($p=0.03$) to the same extent as antigen avoidance already after 8 weeks.

Conclusions: Fluticasone/salmeterol combination synergistically decreased extracellular matrix remodeling in central airways and bronchoalveolar lavage neutrophilia. Fluticasone/salmeterol and fluticasone monotherapy equally reversed peripheral airway smooth muscle remodeling and central airway inflammation.

Keywords: extracellular matrix, smooth muscle, endobronchial ultrasound, lung biopsy, animal models.

Introduction

Bronchial wall remodeling is a hallmark of asthma (1). Airway smooth muscle (ASM), whose increase is proportional to disease severity (2-4) and caused by cellular hyperplasia and hypertrophy (5), is the major contributor to airway obstruction (6-8). Multiple studies have focused on central ASM remodeling using samples obtained by endobronchial biopsy (EBB) (2, 4). However, EBBs provide partial-thickness samples of the central airways. Furthermore, only carinae can be studied (9), while post mortem studies on entire lungs have shown that, in nonfatal asthmatics, the most marked alterations occur in the peripheral airways (3, 10).

Compelling evidence suggests that both inflammation and bronchospasm-induced mechanical stress may contribute to airway remodeling (11, 12). Combining anti-inflammatory and bronchodilating treatments has a synergistic effect on disease control, possibly ascribable to their enhanced anti-inflammatory effects (13). Nevertheless, few studies have addressed the advantages of their combination on airway remodeling reversal in asthma. Inhaled corticosteroids (ICS) and long-acting β2-agonists (LABA) can reverse subepithelial fibrosis and angiogenesis (14, 15), whereas their effect on ASM is unclear. One study investigating how ICS monotherapy affects ASM remodeling suggested that asthma-associated established structural changes might be reversible only in peripheral airways (16). However, a reduction of parenchymal myofibroblasts rather than ASM mass could have accounted for the difference observed. Ultimately, whether ICS or the ICS/LABA combination can reverse established airway remodeling and whether they are equally effective centrally and peripherally remains undetermined.

Peripheral airway dysfunction is associated with poor asthma control (17) and the effectiveness of currently employed asthma treatments at this level is questionable (18). Indirect small airway assessment in asthma is limited by the paucity of diagnostic tools available (19). Direct sampling is challenging and requires invasive procedures such as transbronchial biopsies or partial lung resection (20), whose implementation in human studies is limited by ethical considerations. In this respect, animal models represent the best available option for investigating small airway pathophysiology and response to treatment in asthma. Adult horses exposed to barn antigens can develop equine asthma (heaves), a disease

characterized by central and peripheral remodeling and inflammation (21, 22). Equine lung samples can be easily harvested, which makes asthmatic horses ideal models for the translational study of the reversibility of remodeling in asthma (21). A 6-month ICS treatment can partially reverse peripheral ASM remodeling in equine asthma, while submucosal ECM content decreases only after 1 year (23). We hypothesized that combination therapy with ICS/LABA enhances peripheral remodeling reversal compared to ICS alone, by synergistic anti-inflammatory and anti-remodeling effects. This study was designed for evaluating the specific contribution of anti-inflammatory and bronchodilator treatments, alone or combined, to lung function, inflammation and remodeling reversal in the equine asthma model.

Methods

Animals and experimental design

After 5 weeks of antigen exposure consisting of stabling and hay-feeding, asthmatic horses were treated for 12 weeks with one of four treatments. In study I, horses were either moved to a low-antigenic environment (pasture, considered the gold standard approach for controlling airway inflammation in heaves (23)) or administered inhaled fluticasone/salmeterol (Advair® 250 HFA MDI, GlaxoSmithKline, 2500/250 μg q12 h). During study II, horses were administered either inhaled fluticasone (Flovent® HFA MDI, GlaxoSmithKline, 2500 μg q12 h) or salmeterol (Sigma-Aldrich, 250 μg q8 h (24)). Horses receiving pharmacological treatments were kept in the offending environment (stable and hay diet). Pulmonary function, bronchoalveolar lavage (BAL), EBBs, peripheral lung biopsies, and endobronchial ultrasound (EBUS) were performed at regular intervals as described in the online supplement (**Figure E1**). The protocol was approved by the Ethics Committee of the Université de Montréal (Rech-1324).

Histomorphometry

Morphometry was performed on Russell-Movat-stained tissues using ImageJ (NIH, Bethesda, USA) and newCAST (Visiopharm, Hoersholm, Deanmark). In peripheral lung biopsies, the area occupied by ASM, extracellular matrix (ECM), and elastic fibers (EF) as well as myocyte nuclei were measured/counted manually (ASM, ECM, nuclei) or by point counting (EF), and corrected by the square of the basal membrane length (Pi^2) to account for variation in airway size (25). ASM bundle composition (fraction occupied by myocytes, collagen, and elastin) was assessed by point counting. ASM cell size was indirectly calculated (ASM area x myocyte fraction of ASM bundle/myocyte nuclei). In EBBs, ASM area, ASM composition, ASM cell size, and lamina propria thickness (epithelium-ASM distance) were assessed (26). Proliferating myocytes ($PCNA^+$/α-smooth muscle $actin^+$ cells) were identified using immunohistochemistry (27) and counted manually. Myocyte proliferation density was

expressed as proliferating cells per ASM area. Airway inflammation was assessed semi-quantitatively (score range: 0-2).

Statistical analysis

Statistical analysis was performed with SAS v.9.3 (Cary, NC, USA). Studies I and II were analyzed separately. Data not normally distributed were log10 transformed. A repeated-measures linear model was employed with "time" as within-subject factor, "treatment" as a between-subjects factor, and "disease severity" as a cofactor. *A priori* contrasts with Benjamini-Hochberg correction were used for testing differences among groups and times (all time-points vs. baseline). Inflammation scores were analyzed with the Cochrane-Mantel-Haenszel test for ordinal data. Paired t-test was used for analysis of residual bronchoconstriction after bronchodilator administration and peripheral ASM mass. A linear mixed model with "subject" as random-effect was used for studying associations between different parameters. Depending on data distribution, Spearman or Pearson test was used for correlations.

Results

Animals

Table 1 reports physiological details of the horses studied.

Lung function

At baseline, all horses had severe airway obstruction as demonstrated by their increased pulmonary resistance and elastance compared to normal values (**Figure 1A,C**). The combination of fluticasone/salmeterol normalized pulmonary resistance and elastance from week 1 to 12. Antigen avoidance induced a similar rapid decrease in elastance, while resistance decreased only after 4 weeks (**Figure 1A**), and remained above normal values in 4/7 horses at 12 weeks. When fluticasone and salmeterol were administered separately, they both decreased resistance and elastance after 1 week of treatment, which was maintained until week 12 only by fluticasone. The effect of salmeterol on pulmonary function was partly lost in 3 out of 6 horses after 8 weeks of treatment and was only temporarily restored by a single dose of oral dexamethasone (0.06 mg/kg) administered at the end of week 8 (28) (**Figure 1C**). At week 12, the bronchoreversibility test showed the presence of residual bronchoconstriction in antigen avoidance and salmeterol-treated horses (**Figure 1B,D**).

Airway luminal inflammation

Antigen exposure induced BAL fluid (BALF) neutrophilia (>20%) in all groups at baseline. Antigen avoidance reduced neutrophilia after 1 week, but only 3/7 horses had normal values (<5%) by week 12. Despite ongoing antigen exposure, fluticasone/salmeterol reduced BALF neutrophilia starting from week 8, with 3/6 horses reaching normal values at week 12 (**Figure 2A**). Neither fluticasone nor salmeterol monotherapy affected BALF neutrophilia (**Figure 2B**). Total cell counts were higher in animals treated with fluticasone/salmeterol and fluticasone compared to antigen avoidance and salmeterol treated animals, respectively ($p<0.001$).

Peripheral remodeling reversal

Peripheral airway remodeling was assessed in 10±4 [mean±SD] airways/horse/time during study I and 10±3 during study II (range: 3-25). Average airway Pi was 811±221 μm in study I and 883±456 μm in study II (range: 160-1996 μm); it was similar between groups and time points studied.

At baseline, the ASM mass was similar in all treatment groups (**Figure 3A-B**). It was comparable to previously reported values of ASM remodeling in asthmatic horses (23, 27, 29). In the peripheral ASM layer, ECM was mainly comprised of elastin with rare collagen deposition (**Table 2**). Overall, peripheral ASM mass was greater in severe compared to moderate asthmatic horses, independently of treatment or time (p=0.004). On average, fluticasone/salmeterol reduced ASM mass by 27% (range: 3-45%) at 12 weeks when compared to baseline (p=0.007, **Figure 3A**). Conversely, antigen avoidance had no effect on ASM mass. Inhaled fluticasone alone, but not salmeterol, also reversed ASM remodeling at 12 weeks (p=0.02, average reduction 33.6%, range: 4-62%, **Figure 3B**), to the same extent observed with fluticasone/salmeterol.

Values of ASM mass at baseline and at 12 weeks were correlated in fluticasone/salmeterol (r=0.84, p=0.03), antigen avoidance (r=0.85, p=0.03), salmeterol (r=0.81, p=0.048), but not in fluticasone-treated horses (r=-0.07, p=0.9). ASM cell size (**Figure 3C**) and ECM fraction of ASM (**Figure 4E**) were decreased with fluticasone/salmeterol, contributing respectively to 80% and 20% of the observed reduction of peripheral ASM mass at 12 weeks. Conversely, fluticasone alone did not affect myocyte hypertrophy or ECM fraction of the ASM layer (**Figure 3D, 4F**). Further details on ECM fraction remodeling reversal are provided online (**Figure E2**). Antigen avoidance resulted in a transient decrease of myocyte area at 4 weeks (**Figure 3C**). The number of ASM nuclei/Pi^2 remained unvaried along the study period despite the fact that the quantity of proliferating myocytes per ASM area decreased with time in all groups (**Figure 4B-C**, **3E-F**). In both studies, ASM mass correlated with ASM nuclei/Pi^2 at baseline (r=0.92, p<0.0001 in study I; r=0.87, p=0.0003 in study II) and after 12 weeks (r=0.87, p=0.0002 in study I; r=0.8, p=0.001 in study II), whereas after 4 weeks of treatment the correlation was significant only for fluticasone-treated horses (r=0.85, p=0.03).

The normalized quantity of collagen and elastin in the lamina propria of peripheral airways was unaffected by 12 weeks of either treatment studied (**Figure 5**).

Central airway inflammation

Horses of all groups had severe inflammation of the epithelial and subepithelial compartments of their central airways at baseline. The effect of the treatments on central airway inflammation is reported in **Figure 7**. Epithelial inflammation was reduced by fluticasone/salmeterol after 1 week of treatment and continued to decrease until 12 weeks. The anti-inflammatory effect of fluticasone in bronchial epithelium was evident starting at week 4. Antigen avoidance and salmeterol did not exert any beneficial effect on epithelial inflammation. Subepithelial inflammation was reduced after 8 weeks of fluticasone/salmeterol treatment, while the effect was observed after 4 weeks with fluticasone alone. Only a trend ($p=0.05$) could be observed at 12 weeks for antigen avoidance treatment, while salmeterol did not affect subepithelial inflammation at all.

Central remodeling reversal

On average, 5 endobronchial biopsies/horse/time were analyzed in both studies (range: 3-6), of which 3.5±1.8 considered of good/optimal quality in study I and 3.8±1.4 in study II, with no differences between groups and time points studied.

At baseline, EBUS yielded values similar to those previously published using this technique in asthmatic horses, indicating increased ASM (25). While antigen avoidance was ineffective, 12 weeks of fluticasone/salmeterol reduced submucosal remodeling as indicated by EBUS (**Figure 6B**). A concomitant decrease in ASM area within endobronchial biopsies was observed (**Figure 8A**), and coincided with a reduction of the ECM fraction of central ASM (**Figure 8D**) and with a decreased density of proliferating myocytes (**Figure 8B**). Fluticasone and salmeterol administered separately did not affect ASM area (**Figure 9A**). Only fluticasone decreased myocyte proliferation at 4 and 8 weeks (**Figure 9B**). Conversely, we observed a

tendency towards reduction of the ECM fraction within the ASM bundles in salmeterol-treated horses after 12 weeks (**Figure 9D**). Further details on reversal of ECM fraction remodeling are available online (**Figure E3**). Overall, the biopsy ASM area correlated with the collagen fraction of ASM in study I ($p=0.03$) whereas only a trend was detectable in study II ($p=0.1$), independently of treatment or time. Despite the fact that myocytes of all groups reached a similar size starting at 4 weeks of treatment, and maintained this size until week 12, the reduction was not significant in fluticasone/salmeterol and in salmeterol treated horses, due to the smaller size of their myocytes at baseline (**Figure 8C**, **9C**). The thickness of the lamina propria decreased to values similar to those previously reported in healthy horses (26) after 12 weeks of fluticasone/salmeterol (**Figure 8E**), whereas it was partially decreased by salmeterol alone after 4 and 8 weeks. The effect was lost at 12 weeks (**Figure 9E**). Neither antigen avoidance nor fluticasone therapy affected lamina propria thickness.

Comparison between central and peripheral ASM

ASM bundle composition differed in peripheral and central airways, independently of treatment or time. Overall, ECM fraction was higher centrally than peripherally ($p=0.0002$). Within the ECM fraction, collagen predominated centrally while elastin prevailed peripherally ($p<0.0001$, **Table 2**). A linear relationship was observed in study I between central and peripheral myocyte size ($p=0.007$), independently of treatment or time.

Relationship between EBUS and histological findings in central and peripheral airways

Central ASM remodeling assessed with EBUS (L2 area/Pi^2: area of the subepithelial layer corrected by the perimeter squared) negatively correlated with endobronchial biopsy ASM area at baseline in all horses ($r=-0.62$, $p=0.04$) and at 12 weeks only in fluticasone/salmeterol-treated horses ($r=-0.85$, $p=0.06$). A negative correlation was expected as an increased thickness of the lamina propria prevents a deep sampling within the smooth muscle layer. Similarly, EBUS-assessed central ASM remodeling correlated with peripheral ASM mass

(ASM/Pi^2) at baseline in all horses (**Figure 6C**) and at 12 weeks only in fluticasone/salmeterol-treated horses (r=0.88, p=0.03).

Discussion

In the present study we examined the dynamics and kinetics of airway remodeling reversal following antigen avoidance or administration of ICS, LABA, or their combination in an equine asthma model. We also investigated whether the administration of fluticasone/salmeterol was advantageous over fluticasone monotherapy for pulmonary remodeling reversal and inflammation control. Notably, both peripheral and central airways were studied, together with the specific contribution of salmeterol monotherapy, which is precluded in human asthma due to safety concerns (30). The results indicate that fluticasone/salmeterol combination has a synergistic anti-inflammatory effect on luminal neutrophilia but it is equally effective as fluticasone monotherapy at reversing peripheral ASM remodeling in 3 months. Also, fluticasone/salmeterol synergistically facilitates ECM remodeling reversal in the central airways.

ASM is increased in asthma, which greatly contributes to airway obstruction (1). The efficacy of asthma treatments in reversing established ASM remodeling, particularly peripherally, is not established. With this perspective, the importance of the present study is three-fold. Firstly, it confirms that peripheral ASM remodeling reversal is possible in vivo (23). Secondly, it provides insights into the kinetics and dynamics of such reversal. Lastly, it corroborates the hypothesis that peripheral remodeling is partly irreversible by currently-employed asthma treatments. The observation of persistently increased ASM and uncontrolled small airway dysfunction in asthmatic patients considered adequately treated (5, 10, 31) further supports this theory. However, it raises the question as to whether current treatments are adequate. Asthma treatment efficacy is assessed using spirometry and patient-reported symptom perception tests, two unreliable indicators of peripheral airway disease (32). Persistent small airway inflammation in a patient otherwise responding to therapy is thus not surprising in asthma, and may foster remodeling. Equine asthma can be effectively controlled with high-dose ICS or oral corticosteroids as reliever therapy and low-dose ICS as maintenance therapy (23). Nevertheless, in the present study we have administered high-dose fluticasone or combined fluticasone/salmeterol as both relief and maintenance therapy, in order to assess whether supplemental treatment may improve small airway dysfunction. In agreement with our hypothesis, fluticasone/salmeterol enhanced the control of BALF

inflammation (typically neutrophilic in asthmatic horses) compared to ICS monotherapy. However, and unexpectedly, reversal of ASM remodeling did not improve beyond that achieved with fluticasone alone. Several reasons may explain these results. The most obvious would be that a portion of ASM remodeling is irreversible. Including the current study, three different studies performed in asthmatic horses have achieved, on average, a maximal 30% decrease of peripheral ASM, independently of dose, duration and type of treatment (23). «Premodeling» (congenital remodeling preceding symptoms) has been hypothesized in asthma (33) and would be consistent with our findings, but has not been confirmed. Alternatively, a disproportion between the detrimental effects of inflammation during the establishment of remodeling and the beneficial effects of inflammation control during reversal of remodeling could also explain our observations. Based on a mathematical model of ASM growth in disease, years of complete inflammation control may be required to counterbalance the ASM mass increase caused by a short period of severe airway inflammation (34). Conversely, persistent mild-degree inflammation, as observed in asthma despite treatment (35, 36), would freeze ASM mass in its remodeled state, preventing reversal. As the reduction of BALF inflammation was observed at 12 weeks in our study, the treatment effect may have been too short (<4 weeks) to fully appreciate any consequence for ASM remodeling. Also, the inappropriateness of BAL cytology for the assessment of small airway inflammation (36) and the ability of salmeterol to enhance peripheral mucociliary clearance (37-39) could have introduced a bias to our results. However, at least for mucociliary clearance, an effect on total cell number rather than neutrophil percentage would have been expected. The reported ability of β2-agonists to potentiate corticosteroid-induced neutrophil survival (40) also argues against the possibility that BALF neutrophilia amelioration in our study is artefactual. The molecular basis of the synergistic anti-inflammatory effect of ICS/LABA combinations has been documented in vitro (13). Some studies also suggest that salmeterol may selectively inhibit IL-8 concentration in BALF and neutrophil influx into asthmatic airways in vivo (41, 42). Our findings support the potential of fluticasone/salmeterol to reduce pulmonary neutrophilia in asthma. Importantly, this effect was not observed with fluticasone or salmeterol monotherapies, which highlights the requirement for synergy. Assuming that neutrophilic inflammation control is maintained over time by fluticasone/salmeterol, further studies will be needed to understand whether it can potentiate reversal of ASM remodeling.

Increased or altered ECM deposition occurs in the submucosa of asthmatic airways, possibly as a consequence of fibroblast and myofibroblast activation, and myocyte switching from a contractile to a synthetic phenotype (43). The present study demonstrates the ability of fluticasone/salmeterol to synergistically reverse increased ECM deposition, which is in agreement with in vitro data (13). Also, it shows that the onset and magnitude of this effect differs across bronchial structures (lamina propria vs. smooth muscle) and sites (peripheral vs. central). Within the ASM layer, ECM deposition was reversed both peripherally and centrally by fluticasone/salmeterol, while they were ineffective as monotherapies. On average, this effect explains 20% of the reduction in ASM observed in peripheral airways and 50% in central airways, assuming that changes observed by EBUS proportionately reflect ASM dynamics (25). Contrarily, significant effects within the lamina propria were observed only centrally. At this level, fluticasone and salmeterol monotherapies showed either a partial or transient effect, while their combination normalized the lamina propria thickness based on data obtained in healthy horses (26). This is in agreement with the previous observation that 6 months of fluticasone treatment did not reduce peripheral collagen deposition within the lamina propria in asthmatic horses (23). The existence of intrinsic differences between central and peripheral fibroblasts may explain these results (44). Alternatively, the lower peripheral deposition of inhaled drugs (45) could also have contributed. The clinical consequences of a decreased ECM deposition in asthma are unclear, and may vary depending on the airway compartment involved (46). In asthmatic horses, collagen deposition in the lamina propria of peripheral airways correlated with pulmonary resistance when bronchospasm is absent (47). Further studies including bronchoprovocation tests before and after reversal of remodeling could help elucidate the role of ECM deposition, or its reversal, in asthma.

Myocyte hyperplasia and hypertrophy are recognized features of ASM remodeling in asthma (5). Whether currently-administered treatment can reverse ASM cell hypertrophy and/or hyperplasia in human asthma is unknown. Using the equine asthma model, characterized by ASM hypertrophy and hyperplasia (29), previous studies have shown that 1 year of fluticasone monotherapy reduced peripheral ASM hyperplasia (23). Our results confirm and expand these observations. Indeed, while all the treatments tested decreased the density of proliferating myocytes, none of them reversed peripheral hyperplasia and only the combination of

fluticasone/salmeterol was effective in reducing peripheral hypertrophy after 12 weeks. In contrast, central ASM hypertrophy was reversed in less than 4 weeks by all treatments, suggesting that is it not related to tissue inflammation. Our finding that the transient decrease of central myocyte proliferating density occurred earlier with fluticasone/salmeterol compared to fluticasone alone is supported by in vitro studies (48). Additionally, myocardin, a protein promoting contractile over proliferative phenotype switching in smooth muscle cells (49), is increased by oral corticosteroid administration in asthmatic central ASM (50), supporting an anti-proliferative effect of corticosteroids in vivo. In summary, hyperplasia was not reversed by 3 months of either treatment; centrally hypertrophy, when present, was rapidly reversed by all treatments, independently of tissue inflammation, while the reversal of hypertrophy in the periphery required a longer time.

Conclusions

This study shows that both fluticasone and fluticasone/salmeterol decrease peripheral ASM mass in less than 3 months in neutrophilic asthma. The synergistic anti-inflammatory effect exerted by fluticasone/salmeterol on BALF neutrophils was not mirrored by a simultaneous decrease of peripheral ASM mass compared to fluticasone monotherapy. Fluticasone/salmeterol also showed a synergistic effect on ECM remodeling within the lamina propria and ASM of central airways, which was uncoupled from submucosal inflammation control. ASM hypertrophy reversal occurred earlier centrally than peripherally during periods of asthma control. Peripheral hyperplasia probably requires more than 3 months of treatment to reverse. Salmeterol alone transiently decreased ECM deposition within the bronchial wall, but did not control inflammation. These results were obtained using a naturally-occurring equine asthma model. All the animals studied had intermittent clinical signs for more than 4 years on average (range: 2-11 years) and manifested established airway remodeling. Our findings could help understanding the effect of currently administered treatments on airway remodeling in human asthma.

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Figures and tables

Table 1. Horses' description.

	Study I			Study II		
	Antigen avoidance	Fluticasone/ Salmeterol	*p value*	Fluticasone	Salmeterol	*p value*
N	7*	6		6	6	
Age [years]	18.5±6.3	15.3±4.5	*0.36*	14.3±5.6	15.2±3.7	*0.66*
Weight [kg]	487±91	544±115	*0.34*	513±57	543±74	*0.82*
Sex [f/m]	4/3	5/1	*0.56*	5/1	3/3	*0.54*
Disease duration† [years]	4.6±2.6	3.3±1.4	*0.42*	4.5±3.5	4.0±1.3	*0.69*
Disease severity [moderate/severe]	3/4	5/1	*0.56*	3/3	3/3	*1*

Values are expressed as means ± S.D. * One horse in the antigen avoidance group underwent all sampling except peripheral lung biopsies. †: For horses with unknown history, disease duration was estimated as starting one year before the moment they joined the research herd, a possible underestimation.

Table 2. ASM bundle composition during equine asthma exacerbations (baseline).

	Peripheral ASM bundles			Central ASM bundles		
	Myocytes	Collagen	Elastin	Myocytes	Collagen	Elastin
Antigen avoidance	87±6	0.6±0.6	12±6	84±6	15±6	0.9±0.7
Fluticasone/Salmeterol	84±6	0.4±0.4	15±6	77±6	20±6	2±1.4
Fluticasone	88±5	1.2±1.2	11±5	84±6	15±6	0.9±0.8
Salmeterol	91±3	0.7±0.6	8±3	83±3	16±3	1±1

Values are expressed in percentage [%] and as means ± S.D. ASM: airway smooth muscle.

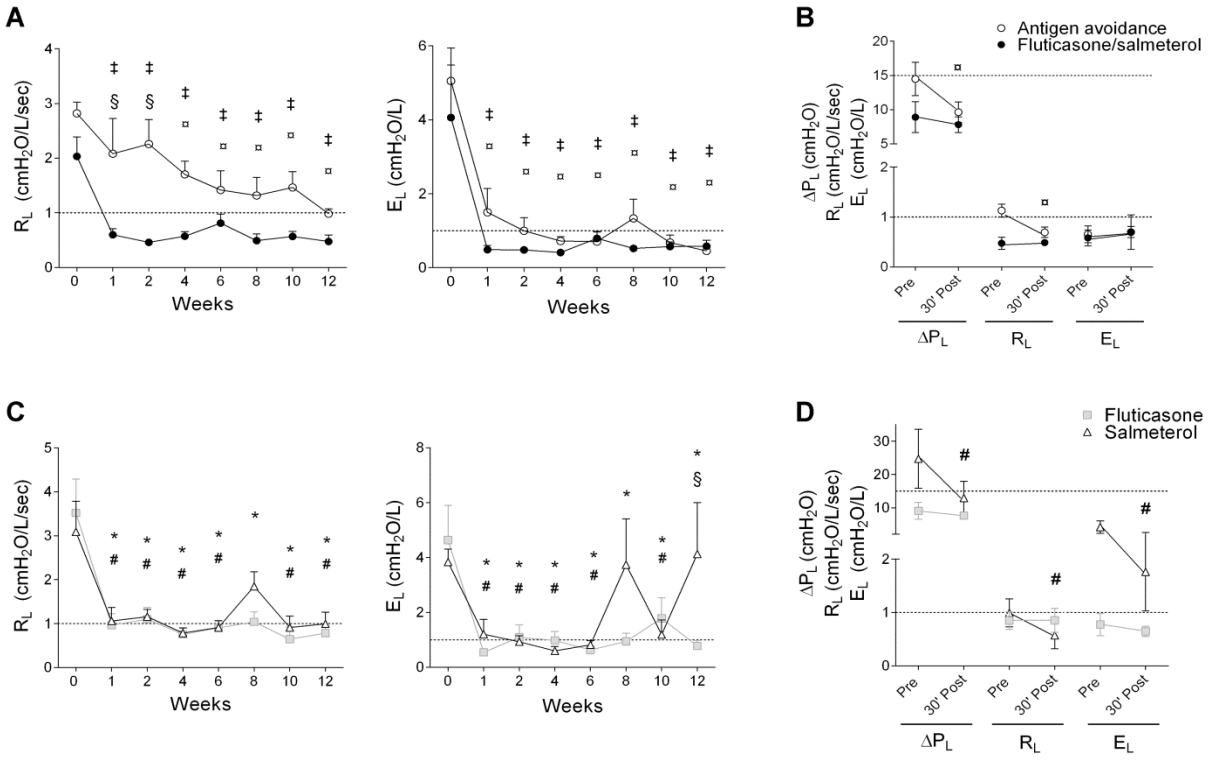

Figure 1. Results of pulmonary mechanics and bronchodilator tests performed in study I (A, B) and II (C, D). Bronchodilator response was evaluated before and 30 minutes after the administration of albuterol 500 µg by inhalation in study I (B) and N-butyl-scopolamine 0.03 mg/kg intravenously in study II. Values are expressed as means ± SEM. Dashed lines represent normal threshold in healthy horses. R_L: pulmonary resistance; E_L: pulmonary elastance; ΔP_L: transpulmonary pressure; ‡: different from baseline of the same group for fluticasone/salmeterol ($p<0.0001$), ¤: different from baseline for antigen avoidance ($p<0.05$), *: different from baseline for fluticasone ($p<0.0001$), #: different from baseline for salmeterol ($p<0.0001$), §: difference between groups at the time point indicated ($p<0.05$).

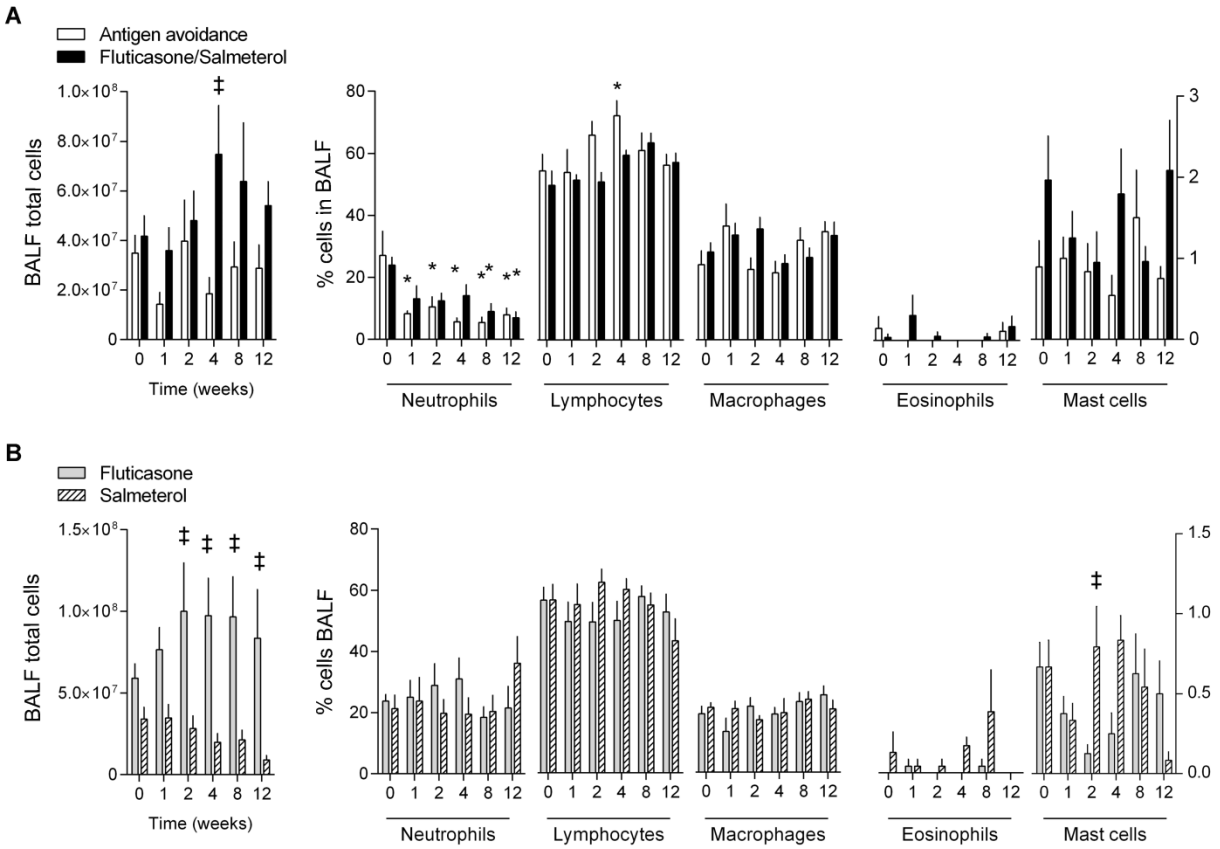

Figure 2. Bronchoalveolar lavage fluid cytology of study I (A) and II (B). Values are expressed as means ± SEM. *: different from baseline of the same group ($p<0.05$); ‡: difference between groups at the time point indicated ($p<0.05$). BALF: bronchoalveolar lavage fluid cytology.

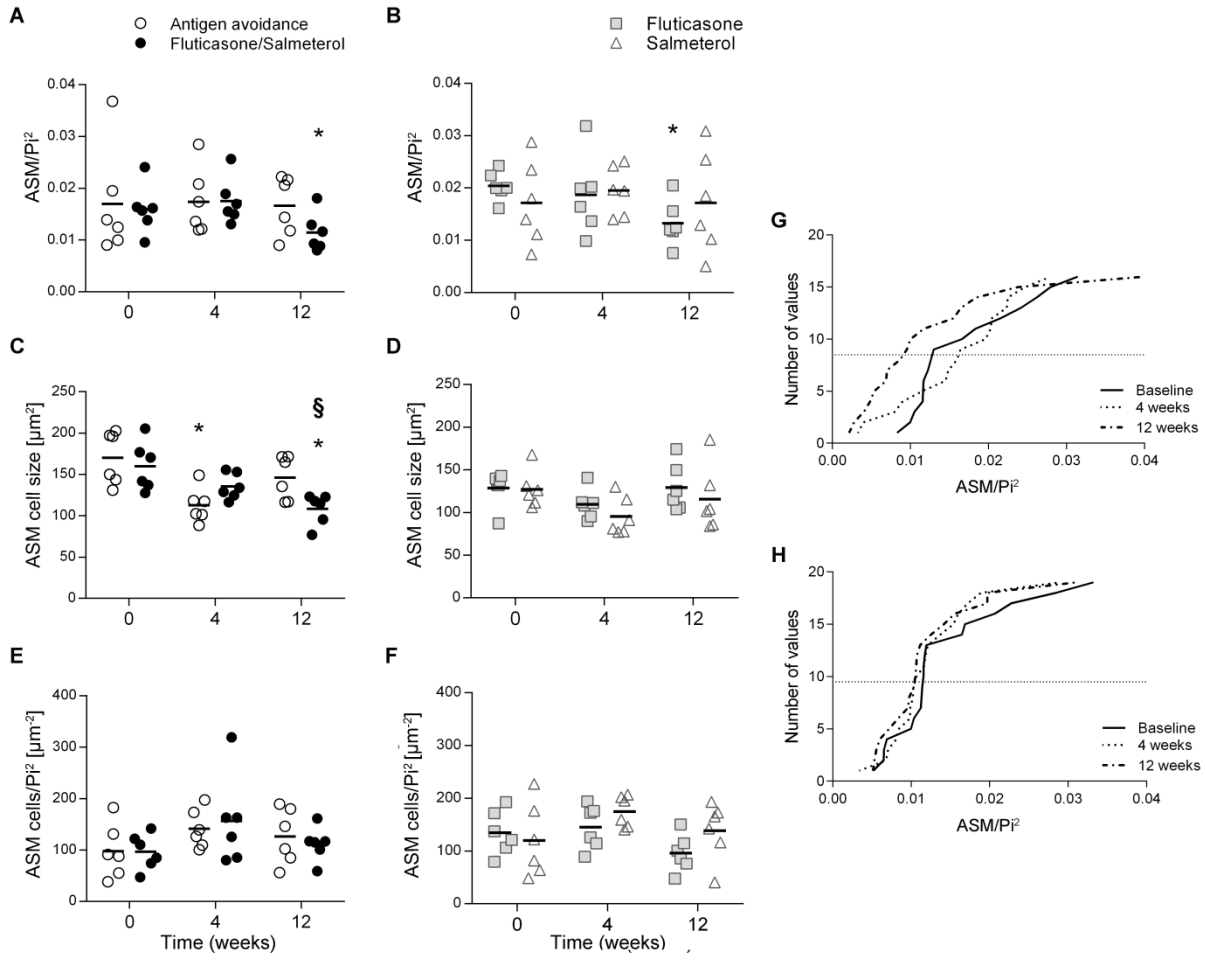

Figure 3. Peripheral airway smooth muscle remodeling. The corrected quantity of ASM, as well as ASM cell hypertrophy and hyperplasia were assessed by histology in study I (A, C, and E, respectively) and II (B, D, and F). *: Different from baseline of the same group ($p<0.05$); §: difference between groups at the time point indicated ($p<0.05$). ASM: airway smooth muscle; Pi: internal perimeter of the airway. Panels G and H show representative examples of the cumulative frequency distribution of peripheral ASM remodeling (ASM/Pi^2 data) in one horse treated with fluticasone/salmeterol (G, n=16 airways/time) and another one treated with antigen avoidance (H, n=19 airways/time). Dashed lines identify median values.

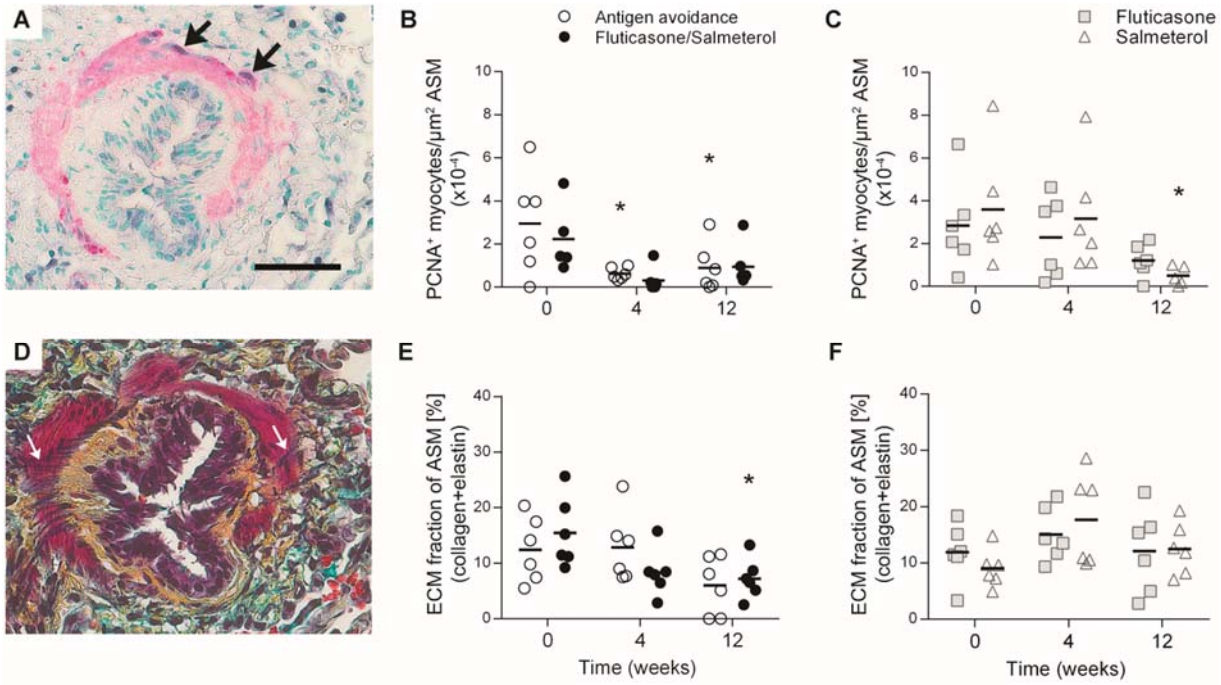

Figure 4. Proliferative and synthetic activities of peripheral ASM cells in study I (B, E) and II (C, F). Panel A shows a representative example of a peripheral airway stained by immunohistochemistry with anti-alpha-smooth muscle actin (pink) and PCNA (dark blue), counterstained with methyl-green. Black arrows indicate $PCNA^+$ myocyte nuclei. Panel D illustrates a peripheral airway stained with the Russell-Movat pentachrome technique. White arrows indicate black-stained elastic fibers lying within the ASM layer. Scale is the same for all images. Each symbol represents one horse (mean value of multiple measures performed). Scale bar: 50 µm. *: Different from baseline of the same group ($p<0.05$). ASM: airway smooth muscle; PCNA: Proliferating Cellular Nuclear Antigen. ECM: extracellular matrix.

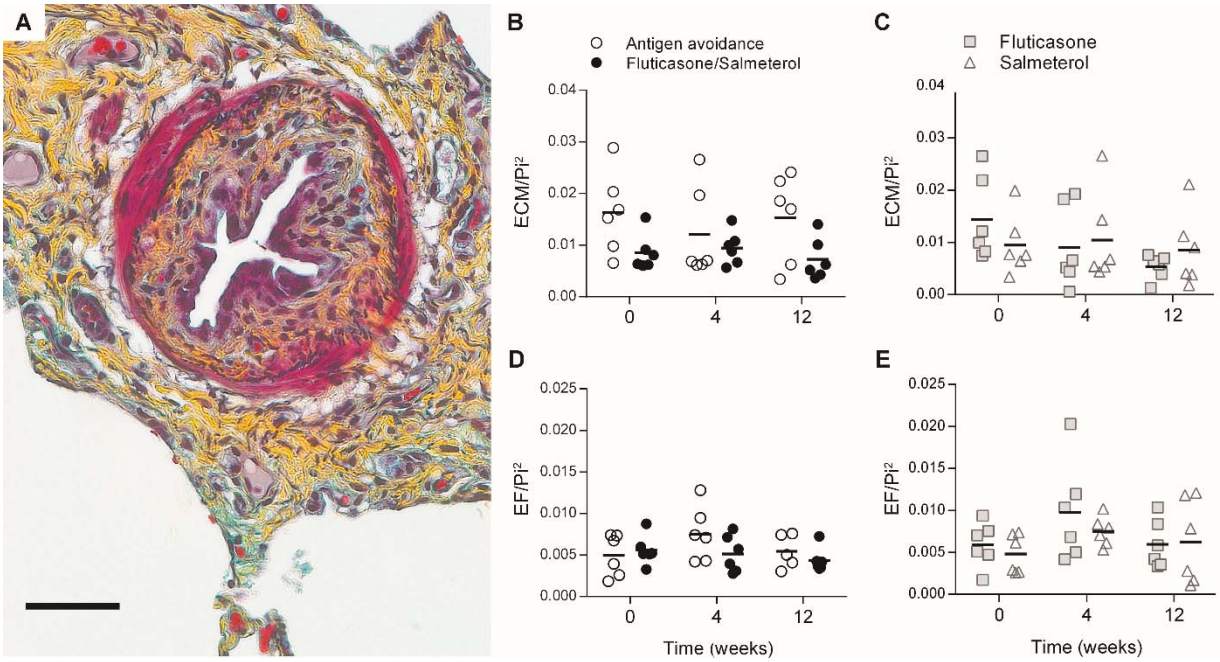

Figure 5. Peripheral remodeling of the lamina propria. A) Representative image of a small airway of an asthmatic horse in which smooth muscle stains dark pink, collagen stains yellow, and elastin stains black (Russell-Movat penthachrome staining). Scale bar: 50 µm. B-E) Corrected area of total ECM and elastin lying within the lamina propria of small airways of the horses participating in study I (B, D) and II (C, E). Each symbol represents one horse (mean value of multiple measures performed). ECM: extracellular matrix; EF: elastic fibers; Pi: internal perimeter.

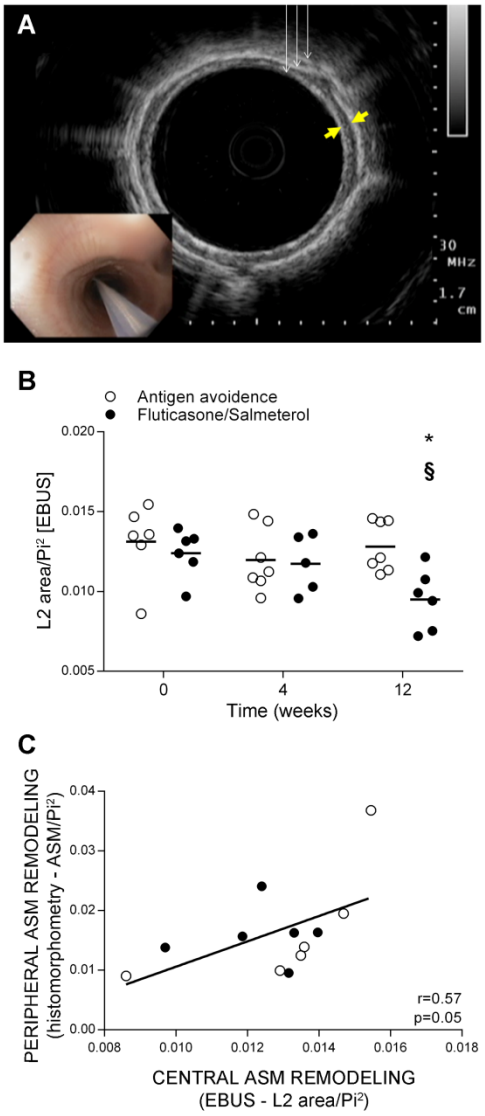

Figure 6. Assessment of central airway remodeling with EBUS. A) Representative image of a central airway obtained with EBUS in a horse with asthma. Thin white arrows indicate the 3 hyperechoic layers of the bronchial wall representing, from the airway lumen going outwards, the bronchial epithelium, the inner, and the outer borders of the bronchial cartilage. The distance between the thick yellow arrows illustrates the thickness of L2. B) EBUS-assessed central airway remodeling in study I. C) Correlation between EBUS-assessed central airway remodeling and peripheral ASM remodeling assessed at histology in study I. Each symbol represents one horse (mean value of multiple measures performed). L2: second layer; EBUS: endobronchial ultrasound; ASM: airway smooth muscle; Pi: internal perimeter. *: Different

from baseline of the same group ($p<0.05$); §: difference between groups at the time point indicated ($p<0.05$).

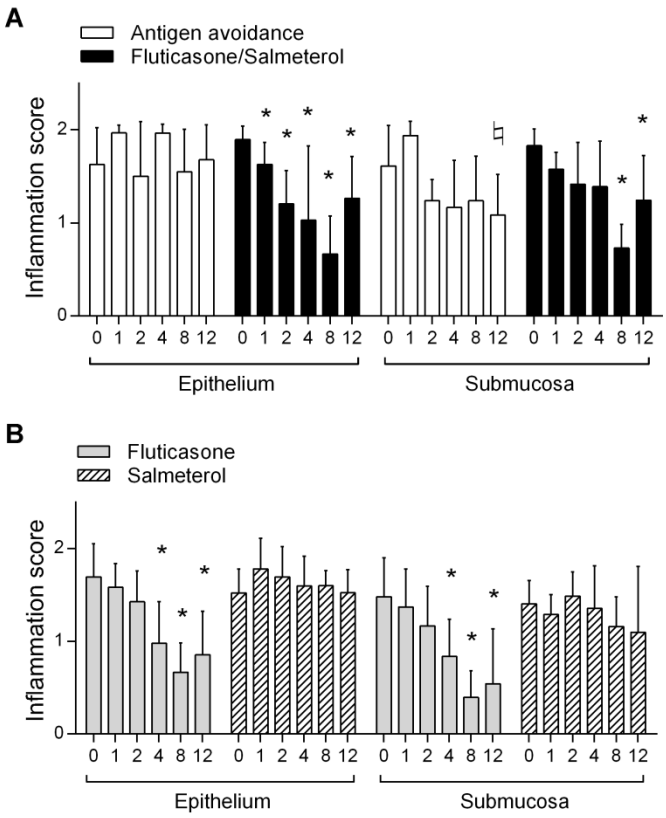

Figure 7. Central airway inflammation was assessed with a semi-quantitative score in study I (A) and II (B). The x axis indicates time (weeks). Values are presented as means ± SEM. *: Different from baseline of the same group ($p<0.05$).

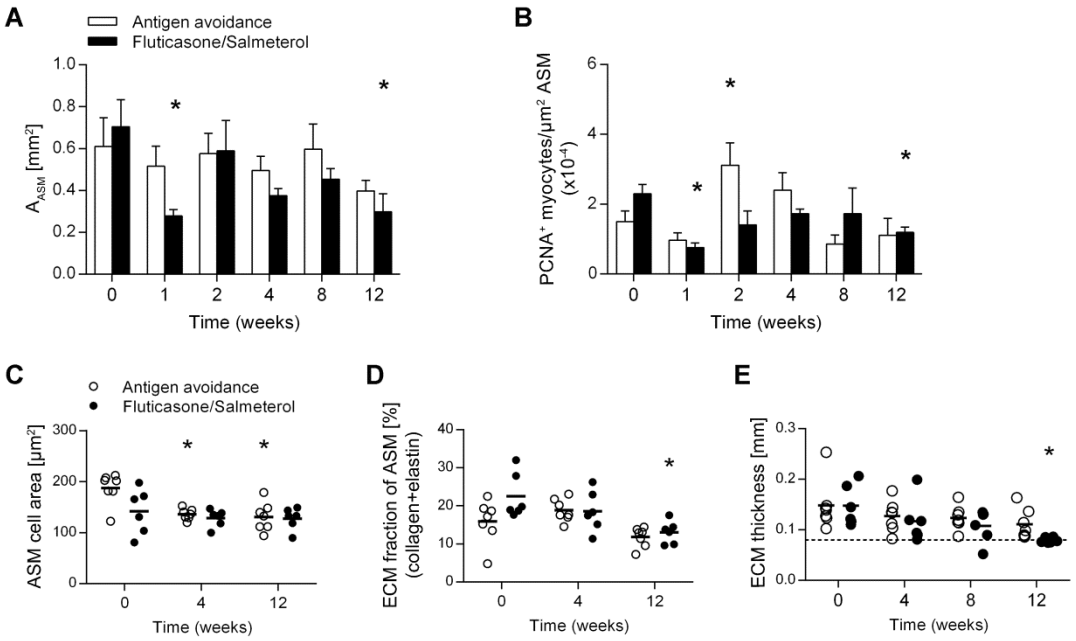

Figure 8. Endobronchial biopsy findings in study I. Uncorrected ASM area (A), myocyte proliferation density (B), myocyte size (C), ECM fraction of the ASM (D) and thickness of the ECM in the lamina propria were assessed. Values are shown as means ± SEM in panels A and B. In panels C, D, and E, each symbol represents one horse (mean value of multiple measures performed). Dashed line in panel E corresponds to the mean thickness of ECM reported in healthy horses in a previous study (37). *: Different from baseline of the same group ($p<0.05$).

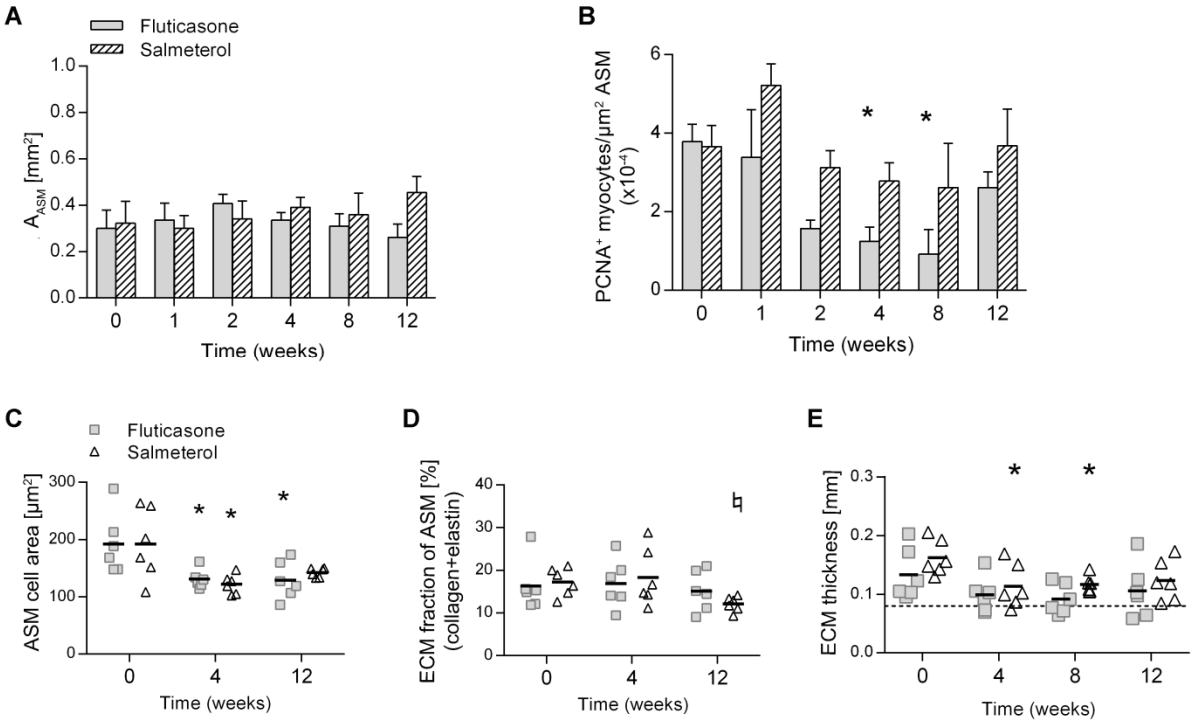

Figure 9. Endobronchial biopsy findings in study II. Uncorrected ASM area (A), myocyte proliferation density (B), myocyte size (C), ECM fraction of the ASM (D), and thickness of the ECM in the lamina propria were assessed. Values are presented as means ± SEM in panels A and B. In panels C, D, and E, each symbol represents one horse (mean value of multiple measures performed). Dashed line in panel E corresponds to the mean thickness of ECM reported in healthy horses in a previous study (37). *: Different from baseline of the same group ($p<0.05$). ♮: $p=0.05$ from baseline of the same group.

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Supporting information

ONLINE DATA SUPPLEMENT

Synergistic effect of inhaled fluticasone and salmeterol on pulmonary neutrophilia and collagen deposition within the airway smooth muscle of asthmatic horses

Michela Bullone, Amandine Vargas, Yvonne Elce, James G. Martin, Jean-Pierre Lavoie.

Methods

Animals and experimental design

Nineteen horses with a known history of asthma were studied. After a 5-week antigen exposure consisting of stabling and hay-feeding, horses were treated for 12 weeks with one of four treatments. In study I, horses were either moved to a low-antigenic environment (pasture, considered the gold standard approach for controlling airway inflammation in equine asthma (1), n=7 horses) or administered inhaled fluticasone/salmeterol (Advair® 250 HFA MDI, GlaxoSmithKline, 2500/250 μg q12 h, n=6). During study II, horses were administered either inhaled fluticasone (Flovent® HFA MDI, GlaxoSmithKline, 2500 μg q12 h, n= 6) or salmeterol (Sigma-Aldrich, 250 μg q8 h, n=6 (2)). Horses receiving pharmacological treatments were kept in the offending environment (stable and hay diet). Fluticasone/salmeterol combination and fluticasone alone were administered by means of a equine aerosol chamber (AeroHippus, Trudell Medical International). Salmeterol powder was solubilized in sterile PBS at a concentration of 250 μg/ml, using Tween20 (Sigma-Aldrich) to facilitate solubility (3). Salmeterol was administered using a face-tight mask equipped with a mobile ultrasonic nebulizer (SaHoMa, NEBU-TEC International, Germany). Pulmonary function was performed at baseline (week 0), after 1 and 2 weeks of treatment, and then every other week throughout the duration of the study. Residual bronchoconstriction was assessed at the end of the treatment period using inhaled albuterol, (500 μg, in study I (4)) or N-butyl-scopolammonium bromide (0.3 mg/kg IV, in study II). Study design is summarized in **Figure E1**. Briefly, bronchoalveolar lavage (BAL) and EBBs were collected at baseline, and after 1, 2, 4, 8, and 12 weeks of treatment. Peripheral biopsies were obtained at baseline and after 4 and 12 weeks of treatment. Endobronchial endoscopy (EBUS) was performed only during study I, at baseline and after 4 and 12 weeks of treatment. All the procedures performed were approved by the Ethics Committee of the Université de Montréal (#Reach-1234).

Lung function

Lung function was assessed as previously described (5). Briefly, transpulmonary pressure was estimated by means of an esophageal balloon and respiratory flow detected using a heated pneumotachograph connected to a face-tight mask placed on the horse's nose. Pulmonary resistance and reactance were then computed using dedicated software (Labdat/Anadat program on MS-DOS, and flexiWare software, SCIREQ, Canada). A single-compartment linear model of the lung was employed, expressed as: $P_L = (E_L \times V_T) + (R_L \times \dot{V}) + k$, where P_L: transpulmonary pressure, E_L: pulmonary elastance, V_T: tidal volume, R_L: pulmonary resistance, $\dot{V}$: respiratory flow, and k: transpulmonary end-expiratory pressure.

Bronchoscopy

Bronchoscopies were performed on standing sedated horses (detomidine/butorphanol administered IV at 0.015/0.015 mg/kg). BALF was first obtained from a lung, randomly chosen for each animal at baseline and then systemically changed at every time points, stored on ice and processed within 2 hours for cytology assessment (5). Differential cell counts were performed on a minimum of 400 cells on Wright-Giemsa stained cytospins of non-filtered BALF aliquots of 400 µm each. When cell density was too high to allow reliable counting, cytospins of 200 µm aliquots were prepared. Six to eight EBBs were obtained from the contralateral lung as previously described (6), fixed in PFA for 24 hours, and paraffin-embedded. Care was taken to avoid biopsying twice the same bronchial bifurcation. EBUS images were obtained from the lung used for the BAL procedure following a protocol previously developed and validated in our laboratory (7).

Thoracoscopy

Thoracoscopies were performed on standing sedated horses (detomidine/butorphanol 0.015/0.015 mg/kg IV), restrained in a stock. Large peripheral lung biopsies (>5 cm^3) were

obtained by means of a cautery device (Ligasure, Covidien) and endoscopic staplers (Endo GIA, Covidien) as described in previous reports (8, 9), fixed in PFA for 72 hours, and paraffin-embedded.

Histomorphometry

Four-µm-thick histological sections were used for both histological and immunohistochemical staining. Morphometry was performed on Russell-Movat-stained tissues using ImageJ (NIH, Bethesda, USA) and newCAST (Visiopharm, Hoersholm, Deanmark). EBBs of good to optimal quality (6) and peripheral bronchi with a major to minor axis ratio<1.5, ASM surrounding at least 70% of their circumference, and intact epithelium were studied at 40x magnification. In peripheral biopsies (10), the area occupied by ASM, extracellular matrix (ECM), and elastic fibers (EF) as well as myocyte nuclei were measured/counted manually (ASM, ECM, nuclei) or by point counting (EF), and corrected by the square of the basal membrane length to account for variation in airway size (7). ASM bundle composition (fraction occupied by smooth muscle, collagen, and elastin) was assessed by point counting, each point corresponding to an area of 88 μm^2. ASM cell size was indirectly calculated (ASM area x smooth muscle fraction of ASM bundle/myocyte nuclei). In EBBs, total biopsy area (A_{tot}), ASM area (A_{ASM}), ASM% (A_{ASM}/A_{tot}), ASM composition, ASM cell size, and lamina propria thickness (epithelium-ASM distance) were assessed (6). Proliferating myocytes ($PCNA^+$/α-smooth muscle $actin^+$ cells) were identified using immunohistochemistry (11) and counted manually. Myocyte proliferation density was expressed as number of proliferating cells per ASM area. Epithelial and subepithelial inflammation was assessed semi-quantitatively with a score ranging from 0 to 2.

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Figures

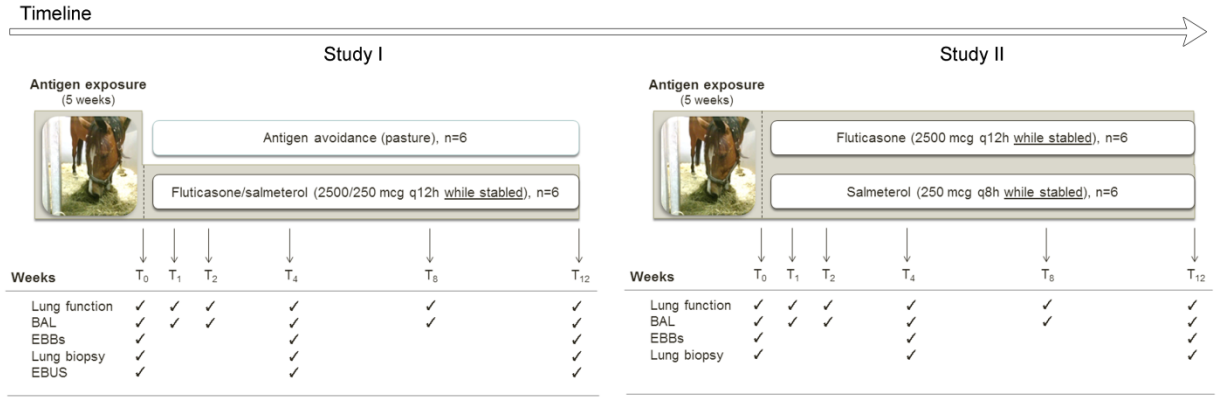

Figure E1. Study design. BAL: bronchoalvelar lavage; EBB: endobronchial biopsies; EBUS: endobronchial ultrasound.

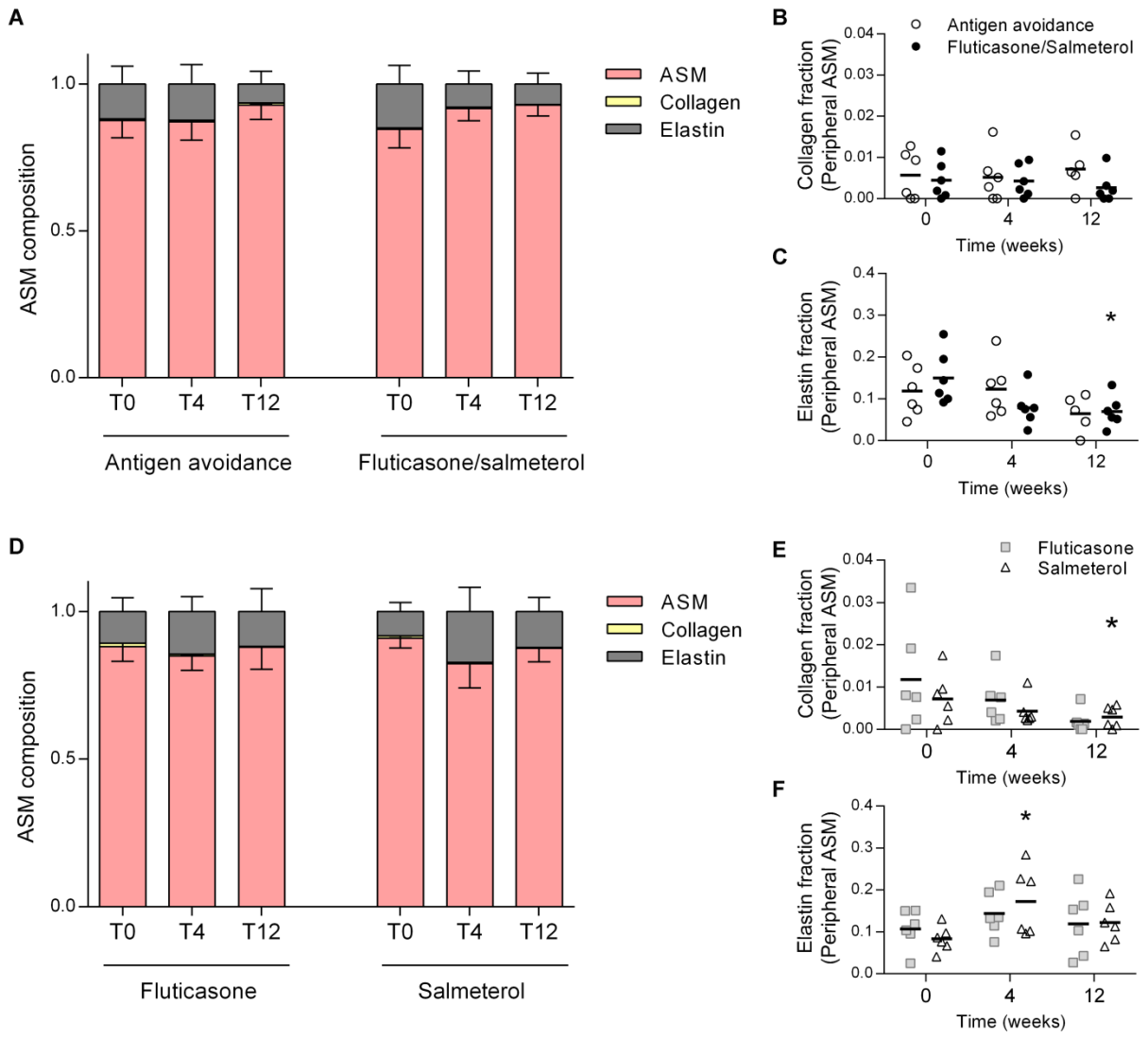

Figure E2. Composition of peripheral ASM bundles and effect of treatments. Panels A and D represent the total composition of peripheral ASM bundles in study I and II, respectively. Bars represent means ± SEM. The specific contribution of collagen and elastin is reported in panels B and C for study I, and in panels E and F for study II. Each point represents one horse (mean value of multiple measures). *: Different from baseline of the same group ($p<0.05$). ASM: airway smooth muscle.

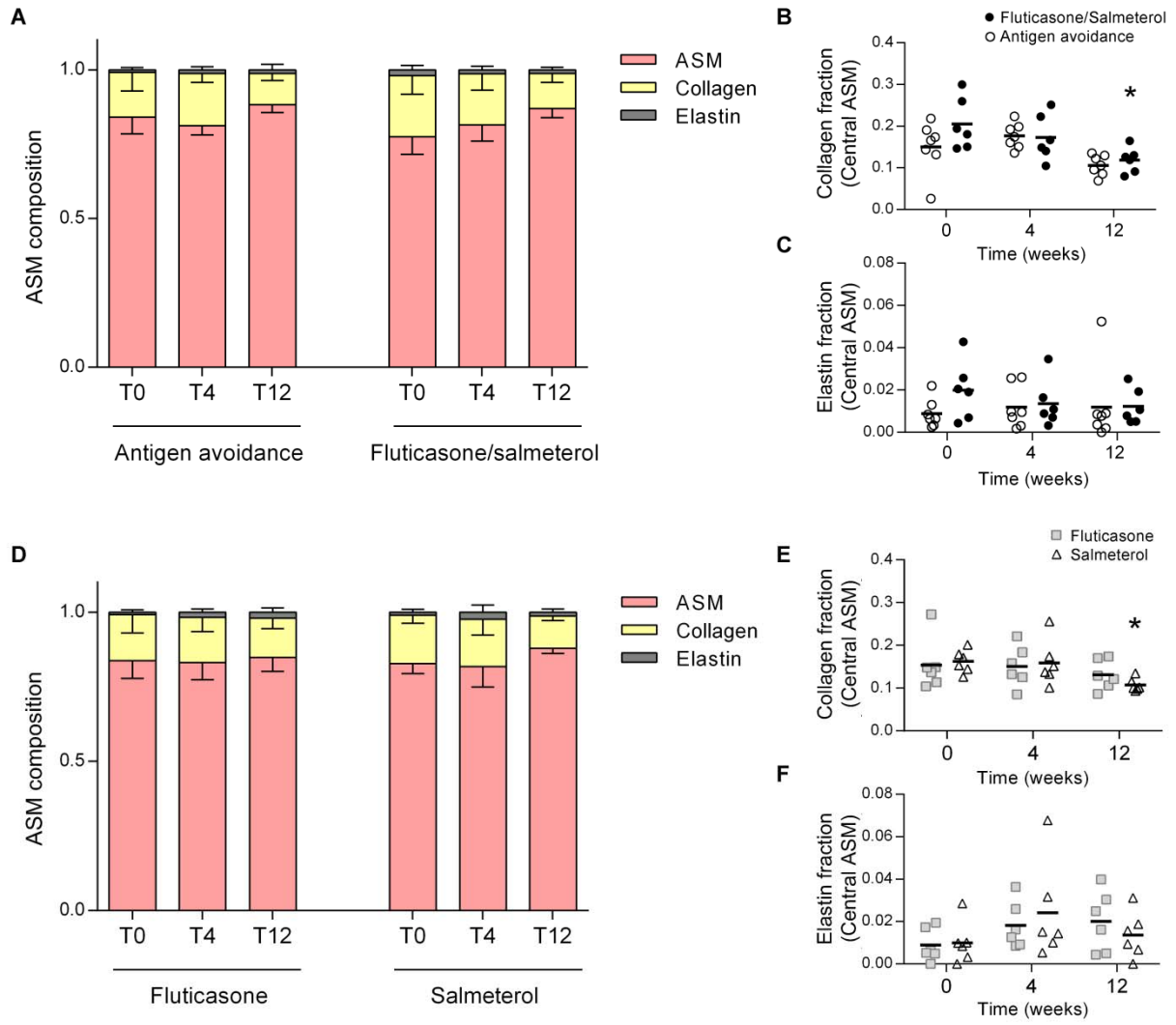

Figure E3. Composition of central ASM bundles and effect of treatments. Panels A and D represents the total composition of central ASM bundles in study I and II, respectively. Bars represent means ± SEM. The specific contribution of collagen and elastin is reported in panels B and C for study I, and in panels E and F for study II. Each point represents one horse (mean value of multiple measures). *: Different from baseline of the same group ($p<0.05$). ASM: airway smooth muscle.

Article 5

Environmental heat and airborne pollen concentration are associated with increased asthma severity in horses

Summary

During the experimental procedures described in *Article 4* of this thesis, we have observed a worsening of the clinical signs of asthmatic horses during periods of hot environmental conditions, which we initially attributed to an alteration of their breathing pattern used in order to maintain thermoregulation. This study seeks to evaluate how environmental variables, namely temperature, relative humidity, and pollen concentration in the air, affect the breathing pattern and lung function of asthmatic horses during periods of disease exacerbation.

The results obtained suggest that in asthmatic horses, airway obstruction worsens when they are exposed to rapid increases of environmental heat, whereas their breathing pattern remains unchanged.

Contribution

I participated in study design (90%), management of the horses (100%), data collection (70%) and analysis (100%), as well as preparation of the manuscript (90%).

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ENVIRONMENTAL HEAT AND AIRBORNE POLLEN CONCENTRATION ARE ASSOCIATED WITH INCREASED ASTHMA SEVERITY IN HORSES

Michela Bullone, Ruby Y. Murcia, Jean-Pierre Lavoie

Department of Clinical Sciences of the Faculty of Veterinary Medicine, Université de Montreal, 3200 rue Sicotte, J2S 2M2, St-Hyacinthe, QC, Canada.

Corresponding author: Jean-Pierre Lavoie, Department of Clinical Sciences of the Faculty of Veterinary Medicine, University of Montreal, 3200 rue Sicotte, J2S 2M2, St-Hyacinthe, QC, Canada. Email: jean-pierre.lavoie@umontreal.ca.

Short title: Environmental heat and severe equine asthma.

Keywords: Horse, RAO, heaves, temperature, relative humidity, enthalpy.

Word count: 3894

Ethical considerations: All the procedures described have been approved by the Animal Ethics Committee of the Université de Montréal (Rech-1324).

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Authorship: MB contributed to study design, data collection, analysis and interpretation, preparation and approval of the manuscript. YM contributed to study execution, preparation and approval of the manuscript. JPL contributed to study design, data interpretation, preparation and approval of the manuscript.

Competing interests: None.

Summary

Reason for performing the study – Clinical exacerbations of severe equine asthma are more frequently reported during winter, when horses are exposed to airborne dusts during stabling. However, we have also observed a worsening of clinical signs on days of heatwave.

Objectives – We sought to investigate the association between environmental temperature and humidity and clinical signs of asthmatic horses during clinical exacerbation of the disease.

Study design – Retrospective longitudinal study.

Methods – Historical data of 14 severe asthmatic horses exposed to a dusty environment and evaluated using a previously validated clinical score system were analyzed. Barn temperature and relative humidity values were obtained, and air enthalpy (h) was calculated. Correlation tests were used for studying the relationship between mean daily clinical scores of horses and environmental variables. Lung function parameters recorded at 4-day intervals during hot (25°C) and warm (18°C) barn conditions were compared using paired t-test.

Results – Significant positive correlations were observed between the mean daily clinical score and temperature ($r=0.58$, $p=0.01$) and air enthalpy ($r=0.55$, $p=0.02$). Maximal daily temperature correlated with airborne pollen concentrations ($r=0.51$, $p=0.0002$). Higher barn temperature and enthalpy, in theabsence of changes in the management of horses, were associated with increased transpulmonary pressure ($p=0.005$), pulmonary resistance ($p=0.008$), and elastance values ($p=0.005$).

Conclusions – Providing a cold environment could help to attenuate the severity of airway obstruction in uncontrolled exacerbation of severe equine asthma. Furthermore, variations in environmental heat and associated pollen concentrations should be taken into account when evaluating the response to therapy in clinical or research settings.

Introduction

Severe equine asthma (also known as Recurrent Airway Obstruction, RAO, or heaves) is a chronic obstructive respiratory condition affecting 15 to 20% of adult horses living in temperate climate [1]. The risk of disease exacerbation increases during winter months [1; 2], when horses are stabled for extended periods of time and exposed to endotoxins, molds, mites, as well as other dust particulate matter present in hay and straw [3; 4]. Although not described in asthmatic horses, cold-induced bronchoconstriction could also play a role [5].

We have occasionally observed unexpected worsening of clinical signs in severe asthmatic horses during hot summer months, driving the hypothesis that hot environmental conditions could negatively affect lung function in affected horses. A cholinergic-mediated reflex inducing bronchoconstriction in response of breathing hot humid air has been shown in asthmatic patients [6] and could be present also in the equine form of the disease, given the similarities among the two conditions [7]. Alternatively, the increased respiratory effort observed could have been secondary to thermoregulation strategies leading to altered breathing patterns with minimal changes in lung function (i.e. pulmonary resistance and elastance). Finally, pollens have been implicated as triggers for clinical manifestations of the summer form of equine asthma (SPAOPD) [8]. While evidence linking severe equine asthma exacerbations to these antigens is lacking [9], they could act as non-specific irritants for the reactive airways of affected horses. This study was therefore undertaken with the aim to investigate retrospectively the short-term effect of environmental temperature, humidity, and antigenic load (airborne pollens and spores) on the clinical status of asthmatic horses during clinical exacerbation of the disease.

Methods

All the procedures described were performed as part of another study and approved by the local Ethics Committee (Rech-1324). Environmental data were obtained and analyzed retrospectively. Fourteen severe asthmatic horses aged 15.1±4.4 years (mean±SD; range: 7-30) and weighting 519±64 kg were studied. There were 5 Quarter Horse, 4 Standardbred, 2 Canadian, 2 Paints and 1 Arab mixed breed, of which 4 were geldings and 10 females. Study design is summarized in Fig 1. All horses had been kept at pasture for at least 4 months before the beginning of the experimental phase of the study. Antigen exposure started on the 15th of April 2014 and was protracted for 6 weeks. During this period, horses were stabled and fed hay. Stabling conditions (bedding, ventilation, number of animals kept within the facility, hay type/batch and quantities administered) remained the same for the duration of the study. Horses were turned out in a paddock 2 to 6 hours/day in the afternoon. An 8-point clinical score previously validated in horses and ranging from 1 (normal) to 4 (severe effort) for both nasal and abdominal effort during breathing [10] was performed between 8:00 and 10:00 a.m. during the first 5 weeks of antigen exposure. Scoring was made by one of 3 trained operators in optimal agreement (interclass correlation coefficient>0.8), as this is part of the antigen challenge monitoring protocol of our laboratory. During the 6th week, pulmonary mechanics were performed in the stable where horses were housed between 8:00 and 10:00 a.m., on Monday (retrospectively considered a “hot” day based on the average temperature for this time of the year in our geographical area: 25°C, 60% relative humidity, RH, 55.27 kJ/kg enthalpy (h), indoor values at 8:00 a.m.; versus 19°C, 71% RH, 43.65 kJ/kg h outdoor) and on Friday (retrospectively considered a “warm” day: 18°C, 61% RH, 37.82 kJ/kg h indoor at 8:00 a.m., versus 15.5°C, 77% RH, 36.82 kJ/kg h outdoor). Briefly, transpulmonary pressure was measured with an esophageal balloon catheter connected to a pressure transducer, and breathing flow signals obtained from a heated pneumotachograph connected to a mask. Pulmonary resistance and elastance values were derived using the flexiWare 7.6 software[a].

Temperature and relative humidity outside and within the stable at 8:00 a.m. were obtained from www.meteoblue.com and from the archives of the barn in which horses were housed, respectively. Temperature and humidity in the stable are recorded twice daily (8:00 a.m. and

4:00 p.m.). The concentrations of outside airborne pollens and spores were obtained from the Aerobiology Research Laboratories[b] information service. Measurements were performed at a station located 50 km west from the stable where the horses were kept. A complete list of the airborne allergens tested is provided online (Supplementary item 1). Enthalpy (h, expressed in kJ/kg) of the ambient air was calculated using the formula: $h = T + x\ (2500 + 1.9T)$, where T is temperature and x is the specific humidity (or moisture content) of humid air. Further details on enthalpy calculation are provided online (Supplementary item 2). Enthalpy was chosen as it approximates to which extent a given combination of temperature and humidity affects heat dissipation.

Statistical analyses were performed with SAS/STAT software[c] and Prism 5[d]. A regression model was used to identify data to be included in the analysis. In order to avoid biases due to the concomitant effects of antigen exposure and season-related increase in temperature on the horses' clinical scores, a piecewise regression model was employed for differentiating the initial rising phase of the clinical score curve, where barn antigen exposure is likely to exert a predominant effect, from the following plateau, where the effect of antigen exposure has reached a stable phase. We fitted a model including two different slopes and an inflexion point. The equation for the first segment before the inflexion time is $score = a + b*time$ and the equation for the second segment is $score = a + b*time + c*(time\text{-}inflexion\ time)$. Only data obtained during the second segment of the curve (stable phase) were studied. The effect of the environmental variables on the mean daily clinical score obtained from the horses was analyzed using Pearson or Spearman correlation test, depending on data distribution. Indoor and outdoor meteorological variables were compared with Pearson correlation tests. The effect of hot vs warm environment on lung function was assessed with paired t-tests. Pearson correlation coefficient was also calculated to determine whether the pairing was effective (that is, whether the direction and magnitude of the variation induced by the warm vs the hot conditions were similar in all horses). Normal distribution of data was assessed with the Kolmogorov-Smirnov test. P-values <0.05 were considered significant.

Results

Fig 2 shows the time-trend of the mean clinical score (daily mean of all the horses studied, panel A) together with the environmental variables studied (panel B and C). The non-linear model indicates that the slope of the curve ('b') was significantly greater than 0 before the inflexion point (confidence interval not including 0), but it became not different from 0 after the inflexion point (confidence interval includes 0). The estimated inflexion point corresponded to 1st May 2014. These findings provided the rationale for including only the data observed after the first 15 days of antigen exposure into statistical analysis.

From day 15 to 35, significant correlations were observed between the daily mean of 14 individual clinical scores of the horses and the indoor temperature ($r=0.58$, $p=0.01$, Fig 3A) and enthalpy ($r=0.55$, $p=0.02$, Fig 3B). There was also some evidence of a correlation between the mean clinical score and the indoor RH, but it was not statistically significant ($r=0.44$, $p=0.08$, Fig 3C). Indoor and outdoor temperature ($r=0.94$, $p<0.0001$) and RH ($r=0.62$, $p=0.002$) recorded at 8:00 a.m. during the whole study period as well as indoor and outdoor enthalpy values ($r=0.85$, $p<0.0001$) were strongly correlated.

Overall, during the period studied, daily airborne pollen concentrations correlated strongly with outdoor maximal daily temperature ($r=0.51$, $p=0.0002$), while spore concentrations correlated with minimal daily temperature and RH ($r=0.44$, $p=0.002$, and $r=0.29$, $p=0.047$, respectively). The most abundant outdoor airborne pollens during the period studied were tree pollens (deciduous trees > coniferous trees), with only limited concentrations of grass pollens. Most of the airborne spores were produced by ascomycetes (i.e. *Oospora spp*) and fungi imperfecti (i.e. *Alternaria spp*, *Aspergillus spp*). Mean clinical scores of the horses were not correlated with the total concentrations of airborne pollens ($r=0.35$, $p=0.15$) or spores ($r=0.30$, $p=0.23$) of the same day. However, a significant correlation was observed with total pollen but not with spore concentration of the previous day ($r=0.5$, $p=0.03$; and $r=0.21$, $p=0.41$, respectively). Significant correlations were observed between mean clinical score and specific airborne concentrations of pollens (mainly from *Pinaceae* (pine, fir, spruce), *Betula* (birch),

and *Morus* (mulberry)) and spores from *Oospora spp* (powdery mildew). Further details are provided online (Supplementary item 1).

Lung function values fell on warm days compared to hot days, as demonstrated by the reduction of transpulmonary pressure (p=0.005), pulmonary resistance (p=0.008) and elastance values (p=0.005, Fig 4). On average, a 32%, 27%, and 36% decrease was detected for transpulmonary pressure, pulmonary resistance, and pulmonary elastance, respectively. The statistical pairing was effective for all 3 parameters (r=0.56, p=0.03 for transpulmonary pressure; r=0.69, p=0.007 for resistance; and r=0.75, p=0.002 for pulmonary elastance), indicating that a similar improvement in lung function occurred proportionally in all subjects when environmental heat was reduced. Respiratory rate (p=0.48) and tidal volume (p=0.12) were not significantly affected by temperature and RH variations. The pairing was effective for tidal volume (r=0.6, p=0.02) but not for respiratory rate (r=0.3, p=0.18). As environmental conditions on the days preceding the lung function test could have exerted a carryover effect, their description is provided in Table 1.

Discussion

Winter is considered a risk factor for exacerbations of severe equine asthma [1; 2], as horses spend more time in stables during this season, inhaling increased concentrations of molds and dusts. However, worsening of clinical signs of affected subjects has been reported also during summer months [11], even when horses were kept outdoor for most of the time [12]. During 2 consecutive years, on periods of high environmental temperatures for our geographical area, we observed a worsening of the clinical signs of asthmatic horses kept at pasture (8 weeks post-exacerbation) or stabled and contemporarily treated with inhaled corticosteroids or bronchodilators. Results from this study indicate that an increase of environmental temperature and humidity (determinants of humid air enthalpy and strongly associated with the pollen and spore air content) negatively affects the lung function of asthmatic horses during disease exacerbations, further worsening airway obstruction. Transpulmonary pressure, resistance, and elastance values significantly improved over few days as a consequence of a reduction in environmental heat, in spite of unchanged breathing strategy or hay and bedding dust exposure.

Increased environmental temperature and humidity, especially if sudden, hinders heat dissipation in animals, which in turn induces changes in their breathing strategy as a physiological response to avoid hyperthermia. Heat dissipation in horses occurs by evaporative cooling mainly from the skin and in part from the upper respiratory tract [13]. We initially postulated that the apparent deterioration of clinical conditions observed in asthmatic horses during hot environmental conditions would be the result of heat-induced thermoregulatory mechanisms altering their breathing pattern. A significant increase in respiratory frequency is indeed observed in horses in response to heat stress, and prevents hyperthermia during resting conditions [14; 15]. Asthmatic horses in exacerbation already have an increased respiratory rate compared to healthy animals, and mucus often covers an important portion of the tracheal mucosa, possibly hampering adequate thermoregulation in these animals. Furthermore, severe asthmatic horses are usually aged [2], which could further reduce their thermoregulatory ability [16] and increase the risk of hyperthermia even during resting conditions compared to healthy animals. However, contrarily to our initial hypothesis,

the worsening of the horses' clinical conditions observed with increased temperatures was not associated with an altered respiratory strategy to improve thermoregulation, as breathing frequency or tidal volume were similar during warm and hot days.

Breathing hot humid air increases bronchial temperature and causes bronchospasm in many species, especially in the presence of airway inflammation [6; 17; 18], as occurring in equine asthma. Interestingly, breathing hot humid air at increased respiratory frequencies induces a cholinergic-mediated bronchoconstriction also in human asthmatic patients [6], a condition that shares many pathophysiological similarities with equine asthma [7]. In our study, the significant correlation observed between environmental enthalpy and clinical scores, and significant increase in pulmonary resistance and elastance observed on the hotter day suggest that airway obstruction worsens when heat dissipation is prevented by increased temperature and/or RH, supporting the involvement of heat-induced bronchospasm in heaves pathobiology. The rapid development of severe airway obstruction after stabling a cohort of horses previously kept outdoors during winter in Quebec [19] and the identification of spending <15h/day outdoors during winter months as a risk factor for equine asthma exacerbation [1] provide further evidence for the occurrence of heat-induced bronchospasm in diseased horses. It also stresses the importance of even moderate temperature increases as bronchoconstriction triggers rather than absolute cutoffs. However, further studies are needed to confirm this theory and the mechanisms implicated.

Within the range of environmental conditions studied, heat dissipation is prevented to a greater extent by increases in temperature than in RH (i.e. RH should increase of 7-8% in order to produce the same effect on enthalpy as a 1°C-increase in temperature), which could explain why a more severe airway obstruction was detected on the hot day compared to the warm in the presence of similar RH but different temperature values. Furthermore, the correlation between RH and clinical scores did not reach significance at the 5% level but there was some weak evidence of a relationship, and this in spite of a significant correlation of the scores with temperature and enthalpy, which further highlight the great effect of temperature on heat dissipation. The study power was, however, only 0.54 for RH, and doubling the time points studied would have been necessary in order to raise the power to 0.8 with the same alpha level

(0.05). However, as enthalpy is determined by the integration of temperature and RH, both of them can be considered as causal factors associated to environmental heat.

Increased temperature during spring and summer months is associated with increased airborne pollens and molds [8]. Pollens are considered triggering factors for exacerbations of SPAOPD [8], but evidence directly linking severe equine asthma exacerbations to these antigens is lacking. Nevertheless, they could act as non-specific irritants for the reactive airways of affected horses, and it has been estimated that up to approximately 30% of the variance in equine asthma prevalence in veterinary hospitals could be explained by the sum of climatic factors and their effect on aeroallergen concentrations in ambient air [11]. As the horses studied spent a few hours per day at pasture, we investigated whether airborne concentrations of pollens and spores could have affected disease severity. Our findings confirm and even strengthen the evidence for a correlation existing between daily outdoor temperature and RH values and airborne pollen and spore levels. Airborne pollen but not spore concentrations were correlated with the horses' clinical scores, suggesting that they could play a role in disease severity. It is interesting to notice that the correlation was significant between the clinical scores and the pollen concentration of the previous day, as horses spent their afternoon outside and the scores were performed early in the morning. Also, outdoor concentration of pollens were increased on average 3-fold on the hot compared to the warm day during which pulmonary function tests were performed. In particular, increases in birch (*Betulla*, 5.4-fold increase on the hot day), ash (*Fraxinus*, 12.6-fold), mulberry (*Morus*, 5.5-fold), and oak (*Quercus*, 12-fold) pollens were most marked. The same trend was observed on the 3 days preceding the hot and the warm days. *Alternaria* and *Aspergillus/Penicillum* spore concentrations were also higher (4-fold and 6-fold, respectively) on the 3 days preceding the hot compared to the 3 days preceding the warm day. An association between monthly prevalence referrals for equine asthma exacerbations in veterinary hospitals and pollen counts measured 3 months before was observed for *Quercus*, *Fraxinus*, and *Morus spp* in a previous study, as well as with *Alternaria* spore counts measured during the same month [11]. Although these data would support an association between the increase in airborne pollens and equine asthma pathobiology, it is not possible to separate the specific role of environmental temperature/humidity and inhalable allergens based on our observations. However, the same is

true in clinical practice. With this study we have shown that a correlation exists between environmental heat and the severity of clinical signs in severe equine asthma. Albeit both heat-induced bronchoconstriction and airway irritation caused by airborne particulates are likely to act synergically, environmental heat can be more easily predicted, assessed, and, at least partially, contained by means of preventive measures (i.e. improved ventilation).

In conclusion, our study indicates that high environmental temperature and humidity can worsen the clinical signs of horses with severe equine asthma during disease exacerbation due to impaired lung function. Whether and in which proportion the negative effect of high environmental temperature and RH on lung function is worsened by inhalable pollens and molds, or by other undefined factors, remains to be ascertained. Nevertheless, these findings highlight the necessity of providing a temperate environment to severe asthmatic horses, especially during disease exacerbation or when exposure to stable antigens cannot be avoided. Also, changes in environmental temperature should be taken into account when evaluating the response to therapy in clinical or research settings.

Footnotes

[a] SCIREQ Scientific Respiratory Equipment Inc., Montreal, QC, Canada.

[b] Aerobiology Research Laboratories, Nepean, ON, Canada.

[c] SAS Institute Inc., Cary, NC, USA.

[d] GraphPad Software Inc., La Jolla, CA, USA.

Supporting information

Supplementary item 1: List of the airborne pollens and spores studied, and results of their correlation with clinical scores of the horses (Bonferroni correction for multiple comparisons was applied).

Supplementary item 2: Details for enthalpy calculation.

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Figures and tables

Table 1. Environmental characteristics at the moment of lung function tests.

	Hot day	**Warm day**	***p*** ***(paired t-test)***
Breathing frequency* [Hz]	0.344 (±0.084)	0.304 (±0.123)	0.48
Tidal volume* [L]	5.6 (±0.9)	6.2 (±1.5)	0.12
Indoor temperature (barn) 8h a.m.	25°C	18°C	-
Indoor RH (barn) 8h a.m.	60%	61%	-
Outdoor temperature 8h a.m.	19°C	15.5°C	-
Outdoor RH 8h a.m.	71%	77%	-
Indoor temperature (barn) 8h a.m. (mean previous 3 days)	18.5°C	15.9°C	-
Indoor RH (barn) 8h a.m. (mean previous 3 days)	62.3%	61%	-
Outdoor temperature 8h a.m. (mean previous 3 days)	14°C	11.3°C	-
Outdoor RH 8h a.m. (mean previous 3 days)	88%	83%	-
Pollens [P/m^3]	249.2	85.4	-
Spores [P/m^3]	1737.1	2137.2	-
Pollens [P/m^3] (mean previous 3 days)	102.1	34.8	-
Spores [P/m^3] (mean previous 3 days)	2121.2	4040.5	-

RH: relative humidity; P/m^3: particles per cubic meter of air. *: daily mean ± SD of individual values observed in horses.

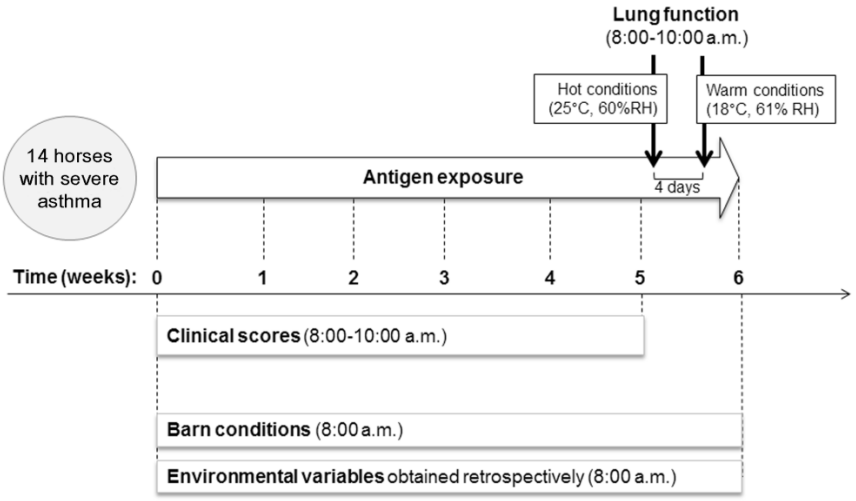

Figure 1. Experimental design. RH: relative humidity.

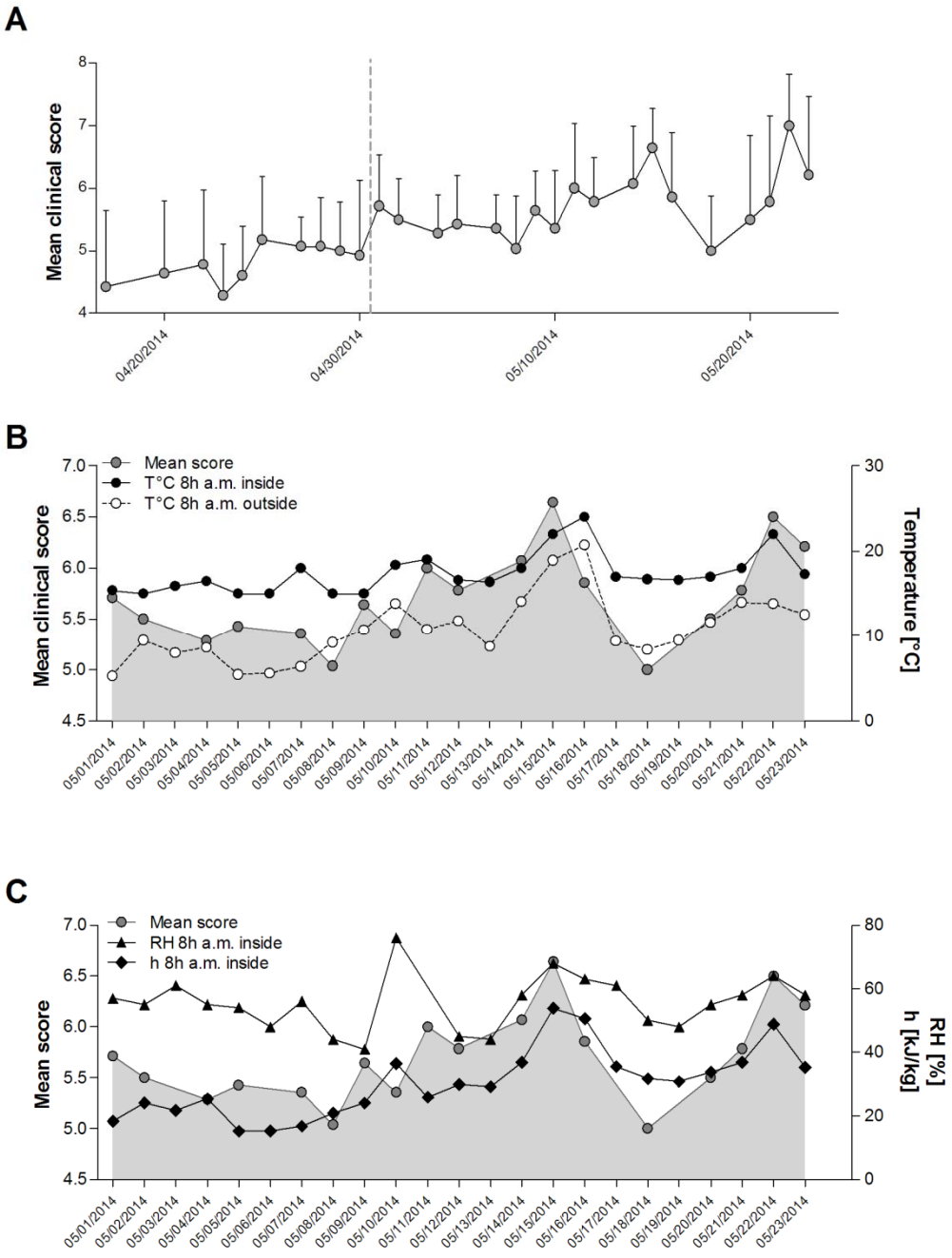

Figure 2. (A) Time trend of daily mean clinical score of the group of horses studied (n=14, error bars correspond to S.D.) for the whole period of antigen exposure. Data on the left of the dashed line were not considered for statistical analysis. (B, C) Time trend of daily mean clinical score, indoor and outdoor temperature, indoor relative humidity (RH) and enthalpy measured at 8:00 a.m. during the period studied.

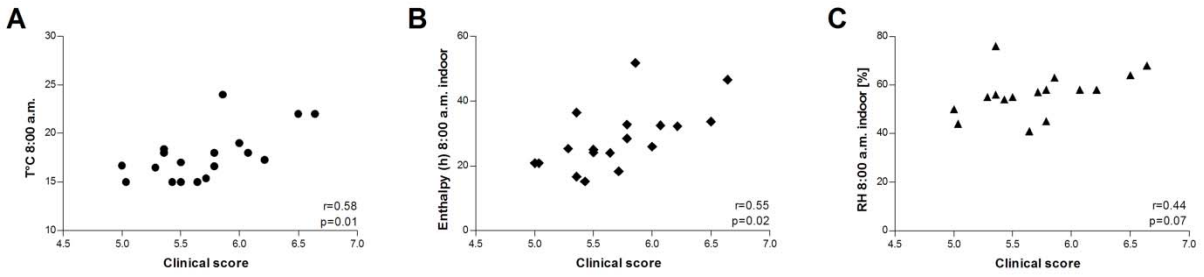

Figure 3. Correlations of the mean clinical score (daily mean of the clinical scores of the horses studied, n=14) and (A) temperature, (B) enthalpy, and (C) RH measured at 8:00 a.m. in the stable where horses were housed.

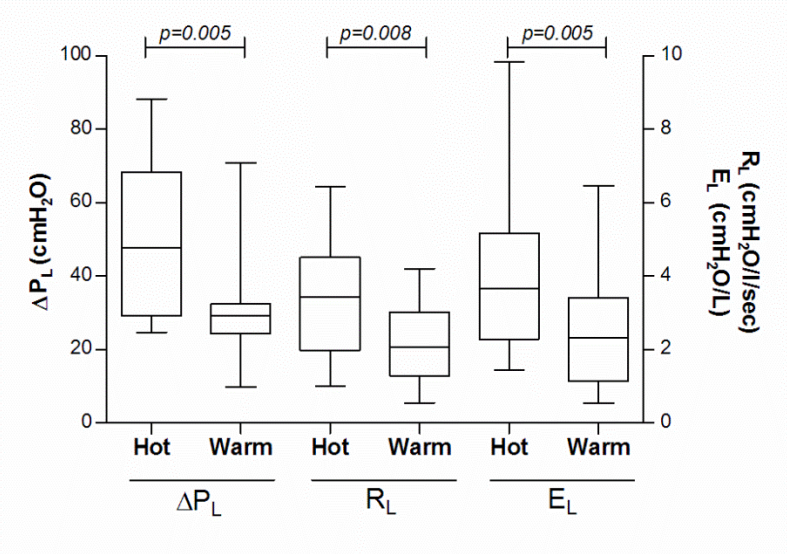

Figure 4. Effect of temperature variation on pulmonary mechanics in asthmatic horses during disease exacerbation. Data are presented as median, 25th to 75th percentiles (boxes), and min-max values (whiskers). ΔP_L: transpulmonary pressure; R_L: pulmonary resistance; E_L: pulmonary elastance.

Supporting information

ONLINE SUPPLEMENTARY ITEM 1

Results

Table S1. Aeroallergens studied and correlation with mean clinical score of the horses.

	Correlation between mean clinical score and allergen concentration on the same day		**Correlation between mean clinical score and allergen concentration on the previous day**		**Correlation between mean clinical score and mean allergen concentration on the previous 3 days**	
	r	*p*	*r*	*p*	*r*	*p*
POLLENS						
TOTAL GRASSES	0.4320	0.0734	0.4978	0.0355	-0.4927	0.0378
CYPERACEAE (Sedge family)	0.08835	0.7274	0.2193	0.3820	-0.3711	0.1294
GRAMINEAE (True Grasses)	0.5649	0.0146	0.5073	0.0317	-0.6222	0.0058
TOTAL TREES	0.3535	0.1501	0.5034	0.0332	0.06615	0.7943
TOTAL CONIFEROUS TREES	-0.1465	0.5619	0.05179	0.8383	0.6944	0.0014
CUPRESSACEAE (Cedar,Cypress,Juniper)	-0.3874	0.1122	-0.1697	0.5008	0.6512	0.0034
LARIX (Larch, Tamarak, Pseudotsuga)						
PINACEAE (Pine, Fir, Spruce)	**0.8081**	**<0.0001**	0.6617	0.0028	-0.4143	0.0874
TSUGA CANADENSIS/TSUGA HETEROPHYLLA (Hemlock)	0.3512	0.1530	0.3043	0.2195	-0.2341	0.3498
TOTAL DECIDUOUS TREES	0.3928	0.1069	0.4982	0.0354	-0.1654	0.5120
ACER (Boxelder, Maple)	-0.3435	0.1628	-0.04966	0.8448	0.4956	0.0365
AESCULUS (Buckeye, Horse Chestnut)						
ALNUS (Alder)	-0.2623	0.2930	-0.1423	0.5734	0.1577	0.5321
BETULA (Birch)	0.6084	0.0074	**0.7782**	**0.0001**	-0.2891	0.2446
Birch look-a-likes (Hornbeam, Hop-Hornbeam)	0.5452	0.0193	0.5855	0.0107	-0.3383	0.1697

CARYA (Hickory)						
CASTANEA (Chestnut)						
CELTIS (Hackberry)						
CORYLUS (Hazelnut)						
FAGUS (Beech)	0.3325	0.1776	0.5107	0.0303	-0.6146	0.0066
FRAXINUS (Ash)	0.5386	0.0211	0.6173	0.0064	-0.1536	0.5427
JUGLANS (Walnut)	0.007570	0.9762	0.3340	0.1755	-0.3576	0.1451
MORUS (Mulberry)	0.5085	0.0312	**0.7916**	**<0.0001**	0.01882	0.9409
OLEACEAE (Ligustrum, Syringa)						
PLATANUS (Sycamore)						
POPULUS (Aspen, Poplar)	-0.2434	0.3305	-0.2339	0.3502	0.4050	0.0955
PRUNUS, MALUS, CRATAEGUS (Apple, Plum, Pear, Hawthorn)	0.7110	0.0009	0.6840	0.0017	-0.4160	0.0860
QUERCUS (Oak)	0.6948	0.0014	0.6056	0.0077	-0.3992	0.1008
SALIX (Willow)	0.2063	0.4114	0.3464	0.1591	-0.1284	0.6117
SAMBUCUS (Elderberry)						
TILIA (Basswood, Linden)						
ULMUS (Elm)	-0.2914	0.2407	-0.3500	0.1545	0.6136	0.0068
Misc. Trees and Shrubs	0.2093	0.4046	0.3512	0.1529	-0.2797	0.2610
TOTAL WEEDS						
AMBROSIA (Ragweed)						
ARTEMISIA						
CRUCIFERAE (Mustards)						
LYTHRUM (Purple Loostrife)						
Misc. Compositeae						
Misc. Weeds						
PLANTAGO (Plantain)						
RUMEX (Dock, Rhubarb, Sorrel)						
S. PESTIFER (Russian Thistle)						
SOLIDAGO (Goldenrod)						
TYPHA (Cattail)						
UMBELLIFERAE (Wild Carrot)						
URTICACEAE (Nettles & Pellitory)						

Unidentified pollen						
FILICINAE (Ferns)						
SPORES						
TOTAL ASCOMYCETES	0.3080	0.2137	0.1860	0.4598	0.1757	0.4855
ASCOBOLUS						
CALOPLACA, XANTHORIA						
CHAETOMIUM	0.3512	0.1530	0.3043	0.2195	0.3043	0.2195
DIATRYPACEAE	0.07938	0.7542	0.04626	0.8554	-0.3862	0.1134
LEPTOSPHAERIA						
Leptosphaeria and Look-a-likes	0.2094	0.4043	-0.002069	0.9935	-0.2501	0.3168
Leptosphaeria look-a-likes						
MASSARIA						
MASSARINA	-0.5206	0.0268	-0.4216	0.0814	-0.6774	0.0020
Misc. Ascospores	0.4682	0.0500	0.2580	0.3013	0.2408	0.3357
OOSPORA (Powdery mildew)	**0.7854**	**0.0001**	0.4447	0.0645	0.4108	0.0904
PERONOSPORA(Downey mildew)	0.1657	0.5112	0.3735	0.1268	0.4539	0.0585
PLEOSPORA	0.09940	0.6948	-0.2439	0.3293	-0.4434	0.0654
SORDARIA						
VENTURIA	0.3614	0.1406	0.3280	0.1839	0.3075	0.2145
TOTAL BASIDIOMYCETES	0.08682	0.7319	0.1546	0.5402	-0.02377	0.9254
BOLETUS						
BOLETUS, MELANOGASTER						
CALVATIA						
COPRINUS, COPRINELLUS	0.2418	0.3337	0.3368	0.1717	0.3559	0.1472
GANODERMA	0.09364	0.7117	-0.06435	0.7997	0.01991	0.9375
Misc. Basidiospores	0.2112	0.4002	-0.007768	0.9756	-0.3680	0.1330
PANAEOLUS						
UREDINALES (Rusts)	0.04597	0.8563	0.01837	0.9423	0.1159	0.6471
USTILAGINALES (Smuts)	0.3404	0.1669	0.4049	0.0956	0.5628	0.0150
TOTAL FUNGI IMPERFECTI	0.1261	0.6181	0.3039	0.2202	-0.03618	0.8867
ALTERNARIA	0.3321	0.1781	0.3497	0.1549	0.2310	0.3565
ARTHRINIUM						

ASPERGILLUS, PENICILLIUM	0.1458	0.5638	0.09235	0.7155	0.2563	0.3047
BOTRYTIS	0.06359	0.8021	0.4003	0.0998	0.1453	0.5651
CERCOSPORA						
CLADOSPORIUM	0.01240	0.9610	0.2760	0.2677	-0.08992	0.7227
CURVULARIA						
DRECHSLERA	0.5148	0.0288	0.1639	0.5159	-0.1706	0.4984
EPICOCCUM	-0.1801	0.4746	-0.1321	0.6013	-0.05498	0.8285
FUSARIELLA						
FUSARIUM						
FUSICLADIUM	0.3043	0.2195	-0.01136	0.9643	0.2308	0.3569
HELICOMYCES	0.2503	0.3165	0.2104	0.4020	0.3868	0.1128
LACCARIA						
Misc. Fungi Imperfecti	0.07032	0.7816	0.1085	0.6682	0.1013	0.6892
NIGROSPORA						
PERICONIA						
PITHOMYCES	-0.09364	0.7117	0.2559	0.3053	0.7622	0.0002
POLYTHRINCIUM	0.3512	0.1530	0.3043	0.2195	0.3043	0.2195
STEMPHYLIUM	0.09364	0.7117			0.4778	0.0449
TETRAPLOA						
TORULA	0.5119	0.0299	0.1639	0.5159	-0.1706	0.4984
ULOCLADIUM						
TOTAL MYXOMYCETES			-0.3980	0.1019	-0.3583	0.1443
Misc. Myxomycetes			-0.3980	0.1019	-0.3583	0.1443
TOTAL ZYGOMYCETES						
Misc. Zygomycetes						
Misc. Phycomycetes						
Unidentified spores	-0.05678	0.8229	-0.09068	0.7205	-0.1532	0.5440
Algae	0.3885	0.1111	0.2990	0.2281	0.4566	0.0568
LYCOPODIUM (Club Moss)	-0.3994	0.1005	0.1449	0.5662	0.5347	0.0222

Analyses were performed using Spearman correlation test on data observed from 05/01/2014 to 23/05/2014. Red: statistically significant after Bonferroni correction.

ONLINE SUPPLEMENTARY ITEM 2

Methods

Enthalpy calculation

Enthalpy (h, expressed in kJ/kg) was calculated using the formula:

$$h = T + x\,(2500 + 1.9T),$$

where: T is temperature [°C] and x is the specific humidity of the air [kg/kg]. The specific humidity of the air is defined as the ratio of the mass of water vapor in an air parcel to the mass of dry air for the same parcel, and derived in turn with the formula:

$$x = 0.622\,(RH*P_{sat})/(P-RH*P_{sat}).$$

P_{sat} is saturation pressure and P is the atmospheric pressure (101325 Pa). P_{sat} corresponds to $610.5e^{A}$. A is a temperature-dependent variable, which corresponds to (17.269T)/(T+237.3) when temperature is ≥0°C, and to (21.875T)/(T+265.5) when temperature is <0°C.

General discussion

The aims of this thesis were 1) to develop and validate a tool for the reliable assessment of central airway remodeling in equine asthma, with a particular attention towards the ASM mass, and 2) to assess and compare the dynamics of central and peripheral airway remodeling reversal following long-term treatment with inhaled corticosteroids in the presence or absence of add-on bronchodilator administration (long-acting β_2-agonist). We hypothesized that 1) central airways – like peripheral airways – undergo structural remodeling processes in severe equine asthma, that 2) airway remodeling reversibility occurs more rapidly in the central airways than in the peripheral airways with ICS monotherapy, and that 3) the combination of ICS/LABA enhances peripheral airway remodeling reversal via an enhanced control of inflammation. Overall, the objectives of the thesis have been achieved, and our hypotheses were either confirmed (hypotheses 1 and 2) or rejected (hypothesis 3). The main findings of this thesis are summarized and briefly discussed hereafter.

1. Endobronchial ultrasound but not endobronchial biopsy sampling allows to estimate the airway smooth muscle mass in the equine central airways

Endobronchial biopsies provide valuable samples for studying qualitative but not quantitative changes of the ASM in the central airways of asthmatic horses.

In the context of this thesis, we initially investigated the potential application of endobronchial biopsy samples for the evaluation of central airway remodeling and inflammation in equine asthma, with particular attention towards alterations of the bronchial smooth muscle layer. Overall, our results (Article 1 and Article 2) support the use of endobronchial biopsies for the assessment of inflammatory infiltrate and qualitative structural changes of most bronchial structures, with the exception of cartilages and adventitia, which are rarely sampled. They provide valuable samples also for the quantitative analyses of the epithelium and lamina propria components (vessels, lymphatics, nerves, ECM proteins). The underlying smooth muscle layer is often harvested only partially however, which prevents an accurate assessment of its thickness by endobronchial biopsy in the equine species. The uncorrected ASM area is

the only morphometric parameter found to be increased in asthmatic horses compared to healthy controls, but it is strongly influenced by the age of the horse and by disease status. The percentage of the biopsy occupied by ASM (ASM%) is similar in asthmatic and healthy animals, and it negatively correlates with lung resistance in asthmatic horses. Nevertheless, as biopsies were harvested after bronchodilator administration in our study, a thickened lamina propria rather than an increased ASM mass is more likely the cause of the reduced ASM% (**Figure 13**). Significant correlations between the lamina propria thickness (epithelium-ASM distance) and pulmonary resistance, or between the lamina propria thickness and ASM%, in support of this hypothesis were not observed however (**Figure 14**, in Annex I).

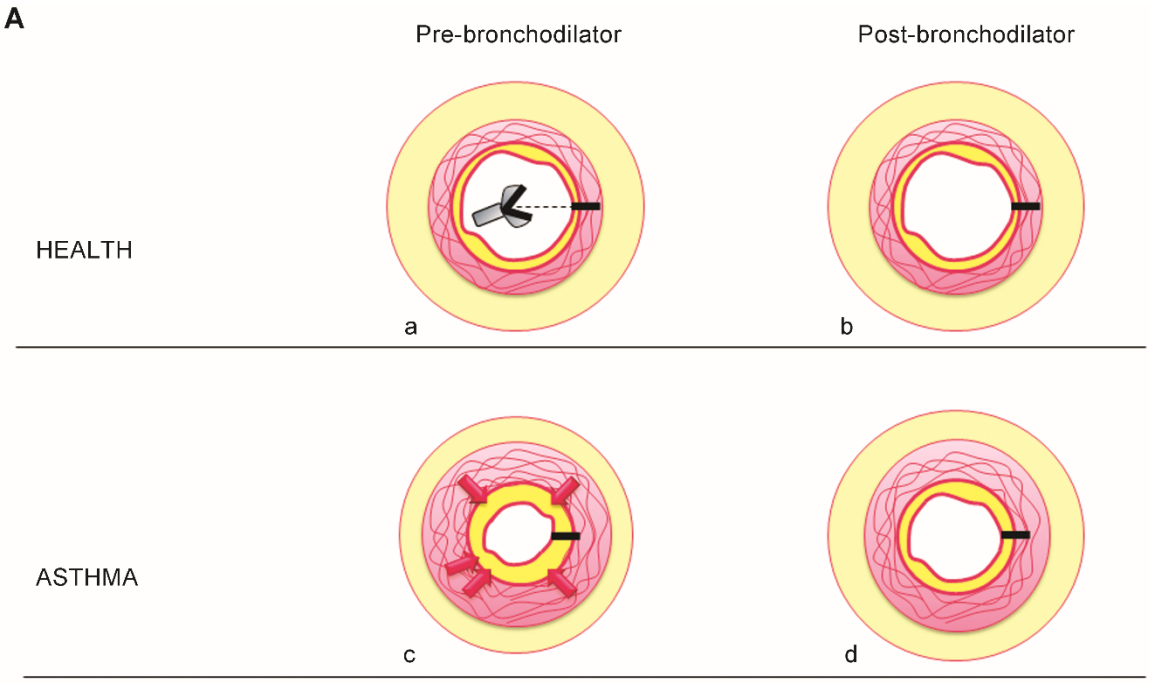

Figure 13. Effect of bronchoconstriction and lamina propria thickness on endobronchial biopsy sampling. A) Effect of remodeling and bronchoconstriction on the sampling ability of endobronchial biopsy forceps. The black horizontal line indicates the theoretical sampling ability of the endobronchial biopsy forceps in different situations. In healthy airways (a-b), the administration of a bronchodilator has no effect on airway dimension and the forceps grasp a

constant proportion of the ASM layer. In the asthmatic airways, bronchoconstriction induces a dynamic thickening of the lamina propria which distances the ASM layer from the airway lumen, proportionally reducing the relative quantity of ASM sampled (c). When bronchodilation is induced in asthmatic airways (d), edema and increased deposition of ECM within the lamina propria of equine asthmatic airways remain the only determinants of the amount of ASM within the biopsy sample.

A possible explanation of these findings could be related to the increased fragility of inflamed and edematous tissues. That is, in equine asthma the lamina propria is thickened, more likely in response to an increased deposition of ECM proteins and the presence of tissue edema and inflammation. Either of these processes can alter tissue firmness and elastic properties, and could cause regions of the lamina propria externally adjacent to the biopsy instrument to be torn off more easily. A similar phenomenon would affect ASM% but not the thickness of the lamina propria of the biopsy sample, which could explain why these two values were not correlated in our study.

Endobronchial biopsy findings correlate with lung function

In order to fully investigate the possible applications of endobronchial biopsy samples in equine asthma research, we developed a semi-quantitative histological score to evaluate endobronchial biopsy samples. We sought to verify whether inflammation or remodeling of the central airways could differentiate horses with asthma in clinical remission from controls. The score considered alterations occurring at the level of bronchial epithelium, lamina propria, and smooth muscle. Assessment of remodeling and inflammation of the outer airway wall (adventitia) was not attempted as this anatomical region is rarely sampled. As an example, among the endobronchial biopsy samples collected during the experimental protocol of Article 4, cartilage and parenchymal tissue were present, respectively, in 292 and 45 out of 942 samples (31% and 5%).

The histological parameters evaluated with the score could not identify the presence of asthma-associated tissue remodeling at any level of the bronchial wall, whereas they clearly indicated that, in the central airways, a severe inflammation of the bronchial epithelium and submucosa occurs during disease exacerbations. We also found increased mucus cells in horses with heaves. Overall, the score was a good indicator of the degree of central airway obstruction in asthmatic horses, as it significantly correlated with lung function in this group (**Figure 2** of Article 2, page 158, and **Figure 16** in Annex I).

We are aware of a single previous study which applied a similar score in endobronchial biopsy samples of human asthmatic patients (Gordon, Husain et al. 2013). In agreement with our results, the authors found that goblet cell hyperplasia and inflammation of the epithelial and submucosal layers are important discriminants of asthma. However, they were also able to demonstrate that ASM mass is increased in endobronchial biopsy samples of asthmatic subjects while we could not. Interestingly, while they looked at the presence of "prominent ASM" (which I interpret as jutting out toward the epithelium), we assessed whether the abaxial border of the ASM layer was visible as an indirect indicator of ASM growth. This observation is in agreement with the different degree of remodeling observed in the lamina propria thickness in asthmatic men and horses. The thinner lamina propria observed in asthmatic patients would permit to assess the presence of "prominent ASM" in endobronchial biopsy samples. Contrarily, the thickened lamina propria observed in asthmatic horses may contribute to our inability to identify any increase in ASM mass in endobronchial biopsy samples of horses.

The presence of central airway inflammation during asthma exacerbations in horses is in agreement with what is described in human asthma. However, contrarily to what is observed in people (Djukanovic, Roche et al. 1990), central airway inflammation decreased to normal levels during periods of disease remission, even when antigen exposure was protracted and remission was induced by corticosteroid administration (Article 2 and Article 4). Of note, our score was semi-quantitative, and may have masked qualitative or minimal but quantitatively significant changes.

Endobronchial ultrasound (EBUS) reliably estimates the ASM mass in central airways of asthmatic horses.

Because of the anatomical differences existing between equine and human bronchi, especially the greater ASM mass in equine compared to human airways, a thorough validation protocol was required for EBUS before it could be used as a tool for bronchial remodeling quantification in horses, which is described in Article 3. Our goal was to define which portion of the bronchial submucosa (identified as a unique hypoechoic layer at EBUS, L2) consisted of smooth muscle in the equine airways. As expected, a certain degree of variation was observed among the multiple airways studied, which is in agreement with histological findings in human specimens (Jones, Elliot et al. 2014). However, analyzing 8-10 airways per animal allowed us to correct for such variability. The finding that overall 75% of L2 (the echographic layer representing the bronchial submucosa) consists of smooth muscle both in healthy and asthmatic horses indicates that a thickened submucosa visualized at EBUS reflects a histological increase of the ASM in the equine airways. The ability of EBUS to reliably estimate ASM mass is a new finding in respiratory research, as previous studies only showed a thickening of the submucosal layer using EBUS in asthmatic patients (Soja, Grzanka et al. 2009), which was correlated with the degree of bronchial hyperresponsiveness (Kita, Fujimura et al. 2010). Endobronchial ultrasound is a non-invasive and repeatable procedure. It allows performing studies during which airway remodeling can be followed over time at the same bronchial site in both horses and humans, which is impossible by means of endobronchial biopsies. Nevertheless, how much of L2 consists of ASM vs. ECM in human bronchi remains to be determined.

In conclusion, our results suggest that endobronchial biopsies are not valuable samples for the quantitative evaluation of central ASM remodeling in horses, although they provide enough ASM tissue to assess its composition and qualitative alterations. Also, endobronchial biopsy samples are useful in the assessment of epithelial and lamina propria remodeling and inflammation. Contrarily, EBUS allows a reliable estimation of the ASM mass in central airways. The advantages of this procedure are mainly linked to its non-invasive nature and to

the possibility to study the same site over time, because tissue harvesting is not required. Performing EBUS and endobronchial ultrasound during the same experimental design would allow a thorough assessment of airway remodeling in both quantitative and qualitative terms, and should be implemented in future studies on this subject.

2. Equine asthma is characterized by central airway remodeling and inflammation

Central ASM mass is increased in equine asthma

Using endobronchial ultrasonography, we have shown for the first time that the submucosal thickness is increased in the central airways of asthmatic horses (Article 3). Furthermore, we have characterized the histological structures causing such increase. In asthmatic horses, ASM was increased compared to controls while it was unclear whether a thickened lamina propria also occurred. When corrected by airway size (Pi^2), a similar quantity of ECM was observed in the bronchial submucosa of asthmatic and control horses. Nonetheless, horses with asthma presented an increased thickness of the lamina propria assessed in airways of similar size. These findings are in agreement with the increased percentage of smooth muscle shortening reported in bronchial specimens of asthmatic patients (Elliot, Jones et al. 2015), and suggest that the airways of asthmatic horses may have been fixed in a (more) constricted state compared to those of healthy horses, which were fixed in a (more) dilated state. Whether this histologic bronchoconstriction reflects the presence of bronchospasm *in vivo* or it is a fixation artifact linked to the structural alterations observed in asthmatic airways (increased ASM mass, altered ASM or ECM composition with altered mechanical properties) remains to be ascertained. Also, there are no data specifically addressing the effect of fixation on smooth muscle and/or ECM morphology in bronchial specimens, or whether the mechanical properties of ASM and ECM may play a role in this process, which precludes further discussion on this subject.

Several mechanisms likely contribute to the increased ASM mass observed in equine central airways. Although we did not compare asthmatic and control horses, the decrease of ASM cell size and ECM fraction observed after pharmacological treatment in Article 4 suggests that myocyte hypertrophy and increased collagen deposition are important determinants of central ASM remodeling in equine asthma. We also observed a reduced number of proliferating myocytes with all treatments. However, due to the impossibility to obtain complete bronchial sections *in vivo*, it was not possible to determine whether it translated into a decreased number of myocytes around the airway. For this reason, the relative contribution of ASM hyperplasia to central airway remodeling in equine asthma could not be assessed and remains to be determined.

In the perspective of human asthma research, reporting an increased smooth muscle mass in the central airways of asthmatic horses is meaningful for two reasons. First, central ASM remodeling is a known feature of human asthma (James, Elliot et al. 2012, Elliot, Jones et al. 2015, Girodet, Allard et al. 2015), and the finding that it is also present in asthmatic horses strengthens its suitability as a model for the disease. Secondly, it permits comparing the responses of different bronchial sites to inflammatory insults and pharmacological interventions within the same subject. Besides central airway involvement, peripheral airway dysfunction is increasingly recognized in human asthma (Postma, Brightling et al. 2015). A recent study reports an overall prevalence of small airways disease of 50 to 60% among asthma cases (Usmani, Singh et al. 2016). However, given the physical/anatomical inaccessibility of peripheral bronchi, their remodeling and inflammation can hardly be assessed *in vivo* in asthmatic patients. A growing interest is now directed towards the implementation of transbronchial biopsies in asthma research, which however is challenging and not free of drawbacks (Balzar, Wenzel et al. 2002). Furthermore, harvested samples are often very small and contain incomplete sections of the small airways, which prevent accurate morphometrical studies. Peripheral lung sampling is instead well described in asthmatic horses (Lugo, Stick et al. 2002, Relave, David et al. 2008, Relave, David et al. 2010), and provides high quality samples for the assessment of airway remodeling and inflammation at this level of the lung. Simultaneous characterization of central and peripheral airway responses during

periods of equine asthma remission and exacerbation could lead to the identification of central markers of peripheral (dys)function.

Equine asthma exacerbations induce lamina propria thickening in central airways

We have observed that the ECM present in the lamina propria of the central airways undergoes remodeling in asthmatic horses. The distance between the epithelium and smooth muscle layer (corresponding to the lamina propria thickness) is increased in endobronchial biopsies obtained from asthmatic horses experiencing disease exacerbation compared to healthy animals. Of note, the lamina propria thickness in endobronchial biopsies is not influenced by ASM contraction, as the values measured in the biopsies of asthmatic horses experiencing disease exacerbation were similar when obtained after the administration of either bronchodilator or placebo (**Figure 15** in Annex I).

The increased lamina propria thickness observed in asthmatic horses compared to controls is in contradiction with what is described in human asthma. Indeed, two studies investigating this parameter in asthmatic patients have found a decreased epithelium-smooth muscle distance in occupational asthma compared to controls (Sumi, Foley et al. 2007), and in severe compared to moderate asthmatics (Pepe, Foley et al. 2005). In the first study, asthmatic patients were in remission from occupational asthma (not symptomatic and not taking any treatment) for more than 14 years on average. In the second study, severe asthmatics were on higher doses of corticosteroids treatment compared to moderate asthmatics. The hypothesis that this could have caused a greater decrease of the lamina propria thickness in that group is not supported by *in vitro* data (Jacques, Semlali et al. 2010). There are no obvious reasons explaining this discrepancy between asthmatic men and horses. A selective inward vs. outward growth of the ASM cells might be involved. In support of a preferential outward expansion of the smooth muscle layer in equine asthma, we have noticed that proliferating myocytes ($PCNA^+$/α-smooth muscle $actin^+$ cells) are often located proximally to the outer margin of the ASM layer in peripheral airways in asthmatic horses (see **Figure 4** of Article 4, page 230). However, myocyte proliferation density was not assessed on complete sections of central bronchi in the

studies presented in this thesis and no data are available concerning human asthma. Endobronchial biopsy morphology often did not permit to draw clear conclusions about the preferential location of proliferating myocytes within the central airways of asthmatic horses. Future studies on isolated lung specimens may help to elucidate this aspect of ASM remodeling. In addition, a thinner lamina propria represents a decreased load opposing central ASM contraction. As it is observed in human asthmatics in remission of the disease and in severely affected patients, it is tempting to speculate that a thinner lamina propria could possibly facilitate bronchospasm and asthma development. Conversely, asthmatics developing a thicker lamina propria in response to inflammatory and/or mechanical stimuli could benefit from it and present milder forms of the disease. The decreased epithelium-smooth muscle distance observed in asthmatic children with persistent obstructive pattern compared to those without obstructive pattern (Tillie-Leblond, de Blic et al. 2008) suggests that, if such a mechanism exists, it might be implemented very early in disease development. However, no other data support this theory, which remains purely speculative at this time.

In conclusion, we have reported for the first time that ASM mass and lamina propria thickness are increased in the central airways of asthmatic horses. While central ASM mass increase is also a feature of human asthma, the lamina propria is thinner in asthmatics than in healthy patients. The mechanisms involved in such bronchial remodeling remain to be elucidated.

3. Peripheral airway is the site of major dysfunction in equine asthma

Physiological variability of the airway structures along the bronchial tree is not well-described in horses. Article 4 of this thesis has shed light upon interesting aspects of ASM structure in equine asthma, namely the uneven increase in ASM mass and the different composition of the ECM surrounding smooth muscle cells at the central and peripheral levels of the bronchial tree. Also, the finding that peripheral ASM remodeling is associated with disease severity is

new in the equine species. In asthmatic horses, ASM increase is more marked peripherally (+200-300% compared to healthy animals) than centrally (+50%). The ECM fraction of the ASM layer is mainly made up of elastin peripherally and of collagen centrally, which reflects the mechanical properties of the lungs. Also, it is in agreement with a human study showing that the fractional area of the elastic fibers within the ASM was higher in the small airways than in the large airways (Araujo, Dolhnikoff et al. 2008). Unfortunately, the lack of healthy control horses in the same study did not permit to establish whether the different composition of ECM along the bronchial tree is a consequence of the disease or is observed also in healthy animals. Further studies will need to clarify this aspect.

In human asthma, ASM was reported to be increased 30% to 60% in peripheral airways and 50% to 100% in the medium to large airways of nonfatal and fatal cases, respectively (Carroll, Elliot et al. 1993, James, Elliot et al. 2012). The composition of the ASM layer and its variability along the bronchial tree has been investigated in a limited number of human studies. James and colleagues reported that, on average, 18-20% of the ASM layer was occupied by ECM both in central and peripheral airways of asthmatics and healthy patients (James, Elliot et al. 2012), which is in agreement with our results. In that study, a significant decrease of ECM fraction was observed only in the central airways of fatal asthmatics compared to control subjects, in the presence of an increased ASM mass (James, Elliot et al. 2012). Yick and colleagues reported similar values of elastin and collagen fraction in endobronchial biopsy samples of mild asthmatics and healthy subjects (Yick, Ferreira et al. 2012). On average, elastin and collagen fraction of the central ASM were 1-3% and 10-30%, respectively. These values closely resemble those we observed in the central airways of asthmatic horses. Finally, Araujo and colleagues reported a similar elastin fraction in central and peripheral airways of the same subjects, which was however increased in fatal asthmatics (14-17%) compared to non-fatal asthmatics (2-3%) and healthy patients (7-8%) (Araujo, Dolhnikoff et al. 2008). Although apparently contrasting, the findings of James and Araujo are not in disagreement as they used staining methods that differed for their ability to identify elastic fibers. Overall, asthmatic horses appear to have elastin fractions similar to those reported in peripheral airways of fatal asthmatics and in central airways of mild asthmatics. Similarly, the collagen fraction observed in equine central airways is comparable to that

observed in mild to moderate asthmatics. Concerning collagen fraction in peripheral airways, it appears to be significantly less in horses compared to that of human bronchioles. However, as we do not know the peripheral ASM composition in normal horses, it is hard to conclude whether it is physiological rather than an effect of the disease. A recent study investigating collagen I and III deposition in equine membranous bronchi reported that no immunolabeling was noted in the ASM layer of either control or asthmatic horses (Furness, Bienzle et al. 2010). This suggests that even if a difference exists in the collagen fraction of peripheral ASM of healthy and diseased horses, its magnitude is likely to be minimal.

The lamina propria of peripheral airways also undergoes remodeling in equine asthma, which is characterized by increased collagen and elastic fiber deposition (Setlakwe, Lemos et al. 2014). It is unclear whether the same changes are sustained by central airways of asthmatic horses. When measured on whole bronchial sections and corrected by the basal membrane length, the lamina propria of asthmatic horses was not greater than that of healthy animals, although it was thicker (Article 3). However, few horses were studied and control lungs were harvested from an abattoir. Lungs were included in the study only if macroscopically normal, which could have introduced a bias in the results. Future studies should ascertain these results using an improved experimental design. In asthmatic patients, the extent of thickening of the lamina propria of membranous airways, if any, remains to be determined. An increased deposition of collagen I and a decreased deposition of collagen III have been reported in fatal asthmatics compared to control patients, which are indicative of a pro-fibrotic process (Dolhnikoff, da Silva et al. 2009). As a consequence, even in the presence of an unvaried total quantity of collagen in the airways, its mechanical properties are likely to be altered.

In conclusion, asthmatic horses appear to present ASM changes similar to those observed in fatal asthma in their peripheral airways, whereas their central airways resemble those of mild asthmatic patients in terms of ASM quantity and composition. As for the ECM within the lamina propria, it is increased in peripheral airways of asthmatic horses. Although conflicting data have been obtained, it is likely that the lamina propria of central airways also displays a significant degree of remodeling in equine asthma. Further studies are needed to clarify this

point, to investigate epithelial alterations, and to characterize the type of bronchial and bronchiolar inflammation observed in equine asthma.

4. ASM remodeling is maximally but incompletely reversed by ICS treatment

Previous studies performed by our group have shown that peripheral ASM remodeling remains partly irreversible after up to 1-year ICS monotherapy in asthmatic horses (Leclere, Lavoie-Lamoureux et al. 2012). In that study, the decrease of ASM induced by ICS was already observed after 6 months of treatment, with no further improvement at 1 year. However, no information was provided concerning the kinetics of ASM remodeling reversal before 6 months of treatment. In this perspective, the experimental procedures described in Article 4 of this thesis were designed to accurately identify the amount of time required by ICS to induce the maximal degree of reversibility of ASM remodeling in equine asthma. Furthermore, we speculated that adding a bronchodilator to the ICS treatment (ICS/LABA combination) would facilitate peripheral ICS deposition and enhance the reversibility of ASM remodeling at the most distal bronchial sites in the same interval of time.

Reversibility of peripheral ASM remodeling requires at least 3 months of ICS treatment in asthmatic horses, and it is not enhanced by LABA add-on treatment

Article 4 confirms that peripheral ASM remodeling is only partly reversible, and that the maximal degree of reversibility is attained by the third month of ICS monotherapy when the offending antigens are not removed from the environment in which the horses were kept. Importantly, combining ICS with LABA did not enhance the proportion of ASM remodeling reversed with treatment. ASM mass was reduced on average by 30% in the horses studied, which corresponds to what has previously been observed after 6 months and 1 year of ICS treatment in the horse (Leclere, Lavoie-Lamoureux et al. 2012). These findings confirm the previously observed partial irreversibility of ASM remodeling in equine asthma, and are in

agreement with human studies reporting an increased ASM mass in controlled non-fatal asthma (James, Elliot et al. 2012). The awareness of an incomplete or at least insufficient effect of ICS monotherapy on peripheral airways is growing (Anderson, Zajda et al. 2012), but the reason because peripheral remodeling cannot be thoroughly reversed by this treatment remains unclear. Their inability at decreasing peripheral airway inflammation could be a reason (Martin 2002, Leclere, Lavoie-Lamoureux et al. 2012). Given the significant anti-inflammatory effect they exert in the proximal airways (Djukanovic, Wilson et al. 1992), the inability of ICS monotherapy to reach the most distal lung sites has been proposed as a possible explanation for its ineffectiveness. However, even when combined with LABA (which should facilitate distal deposition of ICS and also act synergistically with ICS to reduce inflammation (Barnes 2002)), a similar degree of ASM remodeling reversal was attained in our study. Although a precise assessment of tissue inflammation was beyond the aims of this thesis, we investigated the effect of treatment on submucosal peripheral inflammation using the semi-quantitative score described for endobronchial biopsy samples in Article 2. The results obtained are shown in Annex I (**Figure 17**), and suggest that either ICS or ICS/LABA were ineffective at reducing peripheral airway inflammation in the submucosa. However, the effect might have been limited to a single cell type, as observed in BALF, or also it might have been greater at the epithelial or adventitial level, which we did not assess.

In asthmatic patients, the administration of hydrofluoroalkane-flunisolide for 6 weeks was judged successful at controlling peripheral inflammation (Hauber, Gotfried et al. 2003), as it decreased eosinophilia while increasing the number of neutrophils and macrophages in transbronchial biopsy samples. This suggests that total cell number may not adequately reflect treatment efficacy. Future studies investigating this aspect of the disease in comparison to healthy subjects and its response to treatment would be required before concluding about the effectiveness of ICS with/without LABA in the peripheral airways of asthmatic horses.

Decreased ASM cell size and ECM fraction are the main contributors to ASM remodeling reversal in the first 3 months of treatment

Multiple mechanisms contributed to the decreased ASM mass in peripheral bronchi. Although myocyte proliferation was reduced by antigen avoidance and salmeterol treatments, the number of myocytes per basal membrane length remained unvaried in all the groups studied. It is possible that myocytes undergo a more rapid turnover during disease exacerbations, or that 12 weeks is an insufficient amount of time to reduce their number even when their proliferating density has decreased. Interestingly, the number of myocytes per basal membrane length was reduced after one year of ICS treatment (Leclere, Lavoie-Lamoureux et al. 2012). In that case, a consistent but statistically non-significant reduction of myocyte proliferation was observed at 6 months. Summarizing, up to one year is required for ASM hyperplasia to be significantly reduced after a burden of asthma exacerbation, as predicted by a recently-developed mathematical model of ASM growth in asthma (Chernyavsky, Croisier et al. 2014). Contrarily, ASM hypertrophy was rapidly reversed by antigen avoidance and fluticasone/salmeterol, although it was accompanied by a significant reduction of ASM mass only in ICS/LABA-treated horses. It is counterintuitive to observe an unvaried ASM mass in the presence of a reduced myocyte size and proliferation density, as it occurred after 4 weeks of antigen avoidance. Although statistically non-significant, the number of myocytes per basal membrane length increased by 30% on average in this group, and the hypothesis that it might have been caused by dramatically reduced apoptosis is not supported by recently published data obtained *in vitro* (Price, Shao et al. 2015). The effect of ECM composition was however disregarded in that study, and since many matrix proteins can regulate ASM proliferation and apoptosis, it also might have played a role.

Extracellular matrix elements are synthetized by ASM cells, and could contribute to the observed increase of ASM mass in asthma (Araujo, Dolhnikoff et al. 2008, Yick, Ferreira et al. 2012). In Article 4, 12 weeks of fluticasone/salmeterol significantly decreased the ECM fraction within the peripheral ASM of asthmatic, indicating a synergistic effect of the ICS/LABA combination on ECM deposition or turnover, and possibly on myocyte phenotype or its synthetic response. Unexpectedly, it was the elastin fraction that was significantly

decreased. As ASM composition of healthy equine airways has not been established, it is impossible to comment on the effect of the treatment (normalization vs. worsening). Based on what is described in asthmatic patients (Araujo, Dolhnikoff et al. 2008), fluticasone/salmeterol seems to induce a normalization of the peripheral ASM layer composition in equine asthma. There is however scarce information about the role of pharmacological treatments on ASM elastin content in human asthma.

In summary, once lung function has normalized, myocyte hyperproliferation appears to be the more rapidly reversible aspect of peripheral ASM remodeling in equine asthma, but it does not result in a concomitant decrease in ASM mass. It is, however, followed by a decrease in the ECM fraction of the ASM layer, with a maximum delay of 2 months. Whether this reflects a phenotype switch of the ASM cells (more proliferative/synthetic to more contractile) will have to be determined. Based on previously published data, a minimum of 1-year treatment could be necessary to reduce ASM peripheral hyperplasia. The kinetics of reversibility of ASM hypertrophy remains unclear. In our study, a great variability was observed at baseline among different subjects, and cell size was significantly reduced by 1 month of antigen avoidance or 3 months of fluticasone/salmeterol, in concomitance with the decreased neutrophilia in BALF.

5. ICS/LABA combination synergistically decreased the deposition of collagen in central airways of asthmatic horses

Collagen deposition within the ASM layer is synergistically reversed by ICS/LABA treatment

The central ASM layer is made up of collagen in a greater proportion than in the peripheral ASM. Although we did not use immunohistolabelling, we were able to differentiate collagen as the yellow-staining structure using Russell-Movat pentachrome technique. This staining is commonly used to assess total collagen in morphometrical studies of cardiac remodeling (Stephens, Nguyen et al. 2008). Collagen and other ECM proteins not only act as a mechanical

support for ASM, but they profoundly impact the biological and immunological activities of ASM cells by means of integrin-mediated interactions (Burgess, Ceresa et al. 2009). Moreover, the synthesis of ECM proteins, such as collagen I, by asthmatic myocytes is increased (Johnson, Burgess et al. 2004). Collagen I induces a functionally hypocontractile, proliferative phenotype of human ASM (Dekkers, Schaafsma et al. 2007, D'Antoni, Torregiani et al. 2008, Dekkers, Bos et al. 2012), and it also impairs the anti-migratory effects of fluticasone propionate on ASM cells (Bonacci, Schuliga et al. 2006). Whether type I collagen is increased in central airways of asthmatic horses, and particularly in their ASM layer, is unknown and deserves to be investigated. Within the smooth muscle layer, collagen I and III are similarly expressed in asthmatics and control patients (Araujo, Dolhnikoff et al. 2008, Yick, Ferreira et al. 2012). However, this similarity could have been the result of the pharmacological treatment implemented. In asthmatic horses, total collagen within the smooth muscle layer of central airways was significantly reduced by fluticasone/salmeterol combination and also by salmeterol monotherapy. The latter finding is of particular interest in asthma. Indeed, due to its questionable safety profile, LABA monotherapy cannot be administered to asthmatic patients. Multiple studies and meta-analyses have been conducted and indicate that an increased incidence of adverse effects is associated with LABA administration (Sears 2011). For this reason, studies investigating specifically its clinical effects cannot be performed. Until now, the information concerning the efficacy of LABA at inhibiting ECM synthesis by ASM cells was limited to *in vitro* data but lacked an *in vivo* validation (Hirst, Twort et al. 2000, Hirst, Martin et al. 2004, Ammit, Burgess et al. 2009). Our results support the ability of long-term LABA treatment at decreasing ECM deposition within the ASM layer of central airways in asthma. This, however, occurred in the absence of reduction of central airway inflammation, ASM mass, myocyte proliferation density, and after myocyte size had decreased compared to baseline. In human patients, 12 weeks of salbutamol treatment reduced the tenascin but not the collagen content of endobronchial biopsy samples of mild asthmatic patients (Altraja, Laitinen et al. 1999). Taken together, these results suggest that decreasing the ECM deposition in the absence of a concomitant inhibition of airway inflammation and/or ASM remodeling could produce clinically deleterious effects. In the presence of bronchospasm, a “fibrotic” airway (with increased ECM deposition) may counteract airway obstruction.

It is interesting to note that the deposition of different ECM proteins is downregulated by fluticasone/salmeterol treatment at different levels of the bronchial tree. Differently from salmeterol, which induced a similar reduction of collagen in ASM of both central and peripheral airways, fluticasone/salmeterol decreased the collagen fraction centrally but the elastin fraction peripherally. There is no data regarding the role of elastin fraction of ASM and how it is affected by pharmacological treatments in asthma (Reddel, Weiss et al. 2012). A greater elastin fraction is observed in peripheral compared to central airways of healthy subjects, but this difference is lost in asthma (Araujo, Dolhnikoff et al. 2008). Elastin expression in endobronchial biopsy samples of asthmatic subjects is proportional to AHR (Slats, Janssen et al. 2008). An increased fraction of elastin is observed after fatal asthma attacks (Araujo, Dolhnikoff et al. 2008). Overall, an increased quantity of elastin within the ASM layer seems to be detrimental for the development and severity of AHR, while its reversibility in response to treatment remains to be established.

Lamina propria thickening of the central airways is completely reversible by ICS/LABA

Expression of types I and III collagens is higher in the lamina propria of patients with moderate-to-severe asthma compared to mild asthmatics and controls (Chakir, Shannon et al. 2003). However, the same difference was not observed in another study (Dolhnikoff, da Silva et al. 2009) and the lamina propria of asthmatic bronchi appears to be thinner than that of healthy bronchi (Sumi, Foley et al. 2007). This occurs in spite of angiogenesis and impaired lymphangiogenesis which, together with other inflammatory insults, can contribute to edema development within the submucosal layer (Chetta, Zanini et al. 2007, Ebina 2008). As stated earlier, the lamina propria of central airways of asthmatic horses is thicker compared to that of healthy animals. This is true both when it is measured in complete bronchial sections and in endobronchial biopsy samples (Article 1 and 3, respectively). We have considered and previously discussed the possibility of this finding to be a technical artifact. However, the complete normalization of the lamina propria thickness observed after 3 months of treatment (while pulmonary function already normalized after 1 week) suggests that this finding is real, and not a technical consequence of bronchoconstriction. Secondly, a significant decrease of

ECM deposition was observed simultaneously within the ASM layer in the same animals, indicating that fluticasone/salmeterol produces a similar effect at both levels of the bronchial wall.

Mechanical stress *per se* increases ECM deposition in asthmatic airways (Grainge, Lau et al. 2011). To the same extent, our data suggest that bronchodilation *per se* can reverse ECM remodeling. Indeed, a reduced thickness of the lamina propria was observed with fluticasone/salmeterol and also with salmeterol monotherapy, at least until the 8th week, which is the period during which salmeterol was effective at inducing bronchodilation in asthmatic horses. However, fluticasone and antigen avoidance treatments were also effective at normalizing lung function (reducing airway obstruction, and thus reversing bronchoconstriction). These findings suggest that it is a β_2-adrenoceptor-related mechanism rather than the simple non-contraction of ASM which is responsible for the decreased deposition of ECM proteins in asthmatic airways.

Using EBUS, we have shown that the submucosal thickness of central airways of asthmatic horses had returned to almost normal values after 3 months of fluticasone/salmeterol administration. However, no change was observed after only 1 month of treatment. This reduction coincided with the normalization of lamina propria thickness and also with a reduction of uncorrected ASM area within endobronchial biopsy samples. As EBUS reliably estimates the ASM mass in asthmatic and control horses (Article 3), our findings suggest that fluticasone/salmeterol combination is effective at decreasing the central ASM mass in equine asthma. The constant proportion between lamina propria and ASM in central airway submucosa appears to be caused by the amount of ECM laying between the ASM fibers. The possibility that bronchoconstriction may have mimicked an increased ASM mass at EBUS at baseline has been considered. In fact, given the inability to accurately trace the airway epithelium on sonograms, a contracted airway would appear as a smaller one with a thicker bronchial wall. However, EBUS-assessed remodeling was unchanged at 1 month, when the lung function of horses had already normalized, which validates our results. That said, pretreating horses undergoing EBUS with a bronchodilator would guarantee that this type of bias is prevented, and we strongly suggest this precaution should be implemented in the future.

ECM remodeling reversal occurs faster in central than in peripheral airways of asthmatic horses

In our study, we did not observe a significant reduction of ECM deposition within the lamina propria of peripheral airways after 3 months of either treatment, while it was observed after one year of ICS treatment in asthmatic horses (Leclere, Lavoie-Lamoureux et al. 2012). Based on these data, it appears that ECM remodeling reversal occurs more rapidly in central than in peripheral bronchi. The hypothesis that it is linked to inflammation control is not supported by available data. In fact, both peripherally and centrally, a reduced deposition of collagen within the lamina propria was observed in the presence of an unvaried degree of inflammation (semi-quantitative score evaluating all cell types together). Furthermore, human studies have shown decreased inflammation in the absence of reduction of submucosal fibrosis (Hoshino, Takahashi et al. 1999). The specific contribution of each inflammatory cell type in equine asthma deserves however to be assessed in future studies. Alternatively, the possibility that LABA deposition is higher in central than in peripheral airways has to be considered, and could have accounted for the delayed peripheral effect. Studies showing a residual bronchoconstriction in small airways of asthmatic subjects in spite of a normal FEV_1 support this scenario (Pisi, Tzani et al. 2013). In our study, we did not perform experiments to assess the bronchial/pulmonary deposition of the drugs we administered. *In vitro* studies using primary horse-derived fibroblasts support a synergic role of ICS/β_2-agonists on cell proliferation, differentiation, and collagen synthesis (Franke and Abraham 2014). These results, contrarily to ours, do not support an anti-fibrotic effect of β_2-agonists alone. However, serum-induced collagen synthesis *in vitro* may not reflect the stimulus existing *in vivo*, and the molecules tested did not include salmeterol, which could explain the discrepancy with our results.

In conclusion, our results support the ability of long-term ICS/LABA treatment at decreasing ECM deposition within the ASM layer and lamina propria of central airways in horses. While an initial decrease of ECM deposition was also observed in LABA treated horses, it occurred in the absence of reduction of airway inflammation and finally led to worsen the lung function

in treated horses. These findings underline the importance to administer LABA only concomitantly with ICS. The blunted effect observed in the peripheral airways could be the consequence of a decreased drug deposition at this level of the bronchial tree that, in turn, did not reduce inflammation.

6. Environmental heat worsens bronchospasm in equine asthma

During the experimental protocol of Article 4, we noticed on two occasions that the clinical condition of asthmatic horses worsened in concomitance with episodes of sudden environmental heat (week 8 of both Studies I and II). Importantly, the recrudescence of clinical signs observed with heat seemed to negatively impact the effect of certain drugs to control central airway remodeling and inflammation. For example, bronchial inflammation increased following these episodes both in fluticasone and fluticasone/salmeterol-treated horses. In fluticasone-treated horses, the tendency of myocyte proliferation and lamina propria thickness to decrease was reversed. Salmeterol-induced inhibition of ECM deposition within the lamina propria of central airways also appeared to be affected. However, peripheral airway remodeling and inflammation did not appear to be altered by heat-associated acute bronchospasm. These findings are in agreement with previous work in which up to one month of antigen exposure did not increase peripheral ASM mass, myocyte number or size (Leclere, Lavoie-Lamoureux et al. 2011).

We then investigated whether and how environmental variables affect equine asthma presentation (Article 5). Our results are in agreement with observations made in human asthma indicating that high temperatures and humidity are associated with increased hospitalizations (Hayes, Collins et al. 2012, Kim, Lim et al. 2014). This is a new finding in asthmatic horses, for which the negative effect of cold temperatures had only been described up to now (Davis, Lockard et al. 2002, Davis, Malayer et al. 2005, Davis, Royer et al. 2006). However, we could not identify the relative contribution of heat, humidity, and airborne pollens to airway

obstruction. Prospective studies need to be performed to elucidate how these interconnected parameters can affect lung function, airway remodeling, and inflammation.

Conclusions and perspectives

A reliable protocol for the assessment of large airway remodeling has been developed, which comprises both EBUS and endobronchial biopsy sampling. With the samples provided by these two techniques, it is possible to obtain reliable data concerning quantitative and qualitative assessment of airway structure and inflammation in equine asthma.

Using this integrated approach, we have confirmed the hypothesis that central airways of asthmatic horses display ASM remodeling although its magnitude is milder compared to peripheral airways. Both hypertrophy and increased collagen deposition are likely to contribute to central ASM remodeling, while myocyte hyperplasia could not be confirmed in our studies. This is because endobronchial biopsy sampling provides only partial-thickness specimens of the bronchial wall, which prevents an accurate quantification of the number of myocytes within the ASM layer. Synthesis and deposition of ECM proteins within the lamina propria, which is thickened in equine asthma, also contributes to central airway remodeling. Lastly, epithelial and submucosal inflammation characterizes the large airways of asthmatic horses during periods of disease exacerbation.

Central airway remodeling and inflammation are reversible with clinical control of the disease, to the point that endobronchial biopsy samples of asthmatic horses in remission are indistinguishable from biopsy samples obtained from healthy horses. Conversely, differences have been consistently observed in previous studies between the membranous bronchioles of asthmatic horses and controls. Furthermore, the effect of pharmacological treatment on distal bronchioles appears to be limited to airway remodeling, while it does not reduce overall inflammation. These observations confirm that equine asthma is a disease primarily characterized (and possibly orchestrated) by peripheral airway dysfunction, although large airway involvement is common during exacerbations and contributes to the clinical manifestation of the disease. The blunted reversal of airway remodeling and inflammation observed in the peripheral compared to the central airway may be due to an inadequate drug deposition at this level of the lung or, alternatively, to an altered pulmonary environment which could foster corticoresistance.

Airway dysfunction is reversed with different dynamics and kinetics at the central and peripheral level of the bronchial tree. Also, the treatment strategy employed may differentially regulate the reversibility of specific aspects of airway remodeling and inflammation. Overall, the reversal of submucosal ECM remodeling (lamina propria thickening) and airway wall inflammation occur faster in central than peripheral airways. Fluticasone/salmeterol synergically inhibits ECM deposition within the lamina propria and ASM layer in central airways, while this effect is observed only in the ASM of peripheral airways. Also, BALF neutrophilia is reduced by fluticasone/salmeterol after 2 months, while the same drugs were ineffective when administered as monotherapies. Myocyte hypertrophy is rapidly reversed in central airways when disease is controlled, independently of the treatment strategy employed. Peripherally, myocyte hypertrophy cannot be reversed by fluticasone or salmeterol treatment given as monotherapy, but their combination is effective at reducing myocyte size after 3 months. The synergistic antinflammatory effect exerted by fluticasone/salmeterol on BALF neutrophils was not accompanied by a simultaneous enhancement of peripheral ASM remodeling reversal compared to fluticasone monotherapy.

In summary, our results suggest that the enhanced therapeutic effect of ICS/LABA over ICS monotherapy in asthmatic patients may be associated with a reduction of ECM deposition, mainly observed within the large airways, and possibly also with a decreased neutrophilia in the bronchoalveolar lavage fluid. However, ICS/LABA does not provide any additional benefit to ICS monotherapy in terms of peripheral airway smooth muscle remodeling and submucosal inflammation as both induce a 30% decrease of the airway smooth muscle mass in 3 months.

Lastly, in the equine asthma model, the reversibility of central airway remodeling does not mirror structural changes occurring in peripheral airways. Based on our data, it is impossible to estimate the dynamics of small airway remodeling or its reversibility based on central airway findings. Nevertheless, monitoring central airway remodeling over time or during equine asthma exacerbation may help to establish a prognosis or to predict the response to treatment.

Perspectives

This thesis has characterized central and peripheral airway remodeling in equine asthma, as well as their reversibility following several treatments. In order to gain a thorough picture of the dynamics of central ASM remodeling in equine asthma, it remains to be determined whether myocyte hyperplasia also contributes to the disease process. This cannot be performed on bronchial samples obtained by endobronchial biopsy samples. Post mortem lung would provide optimal specimens for this. Alternatively, bronchial biopsies obtained by means of cryoprobes could allow full-thickness sampling of the bronchial wall, which would permit to follow the effect of treatment longitudinally. We have described the ASM composition of equine asthmatic airways and how it is affected by several treatments, but ASM composition of healthy airways also needs to be determined in order to better understand the effect of such treatments. A more detailed description of ECM composition and mechanical properties could also reveal interesting aspects of the disease or of pharmacological interventions. Investigating the expression of MMPs and TIMPs could help to elucidate the mechanisms of ECM decrease we observed.

We have focused our attention on ASM and ECM, as they are recognized as important determinants of airway obstruction in asthma. However, a substantial body of evidence suggests that the bronchial epithelium may be the main orchestrator of the inflammatory response, which is another hallmark of asthma. Characterizing epithelial activation as well as bronchial inflammatory response in peripheral and central airways of asthmatic horses may provide interesting information and help to clarify why drug-induced control of inflammation differs at these two levels of the bronchial tree.

Finally, we have validated and optimized the use of endobronchial biopsy and EBUS as tools for the assessment of central remodeling in horses. Introducing these tools in equine clinical practice will allow to monitor bronchial remodeling over time in a large number of asthmatic subjects but also to investigate the presence of structural bronchial alterations in horses affected by inflammatory airway disease (IAD).

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« With a library [particularly if online – Ed.], it is easier to hope for serendipity than to look for a precise answer. »

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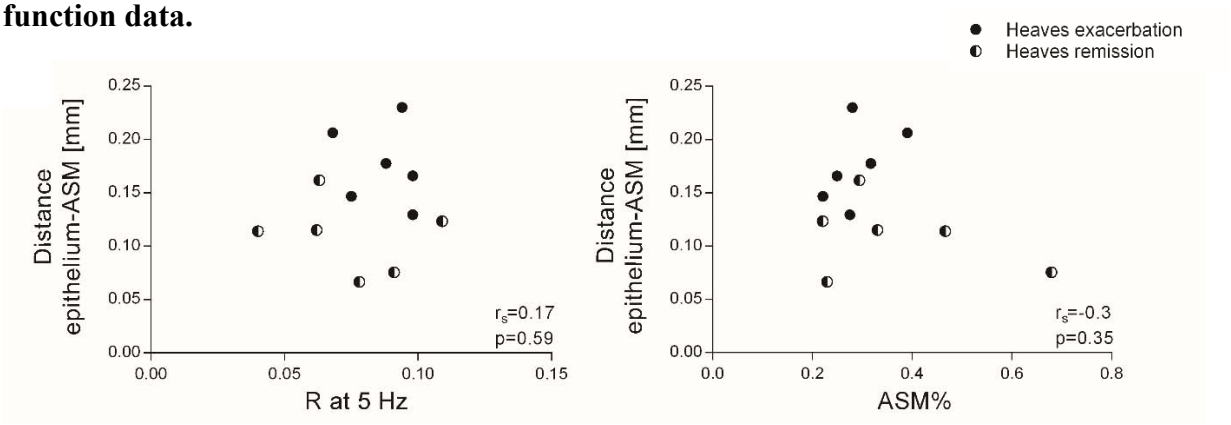
Annex I

Annex I comprises additional results pertinent to this thesis but which were not included in the articles presented due to editorial choices. These data may be helpful in the interpretation of our results.

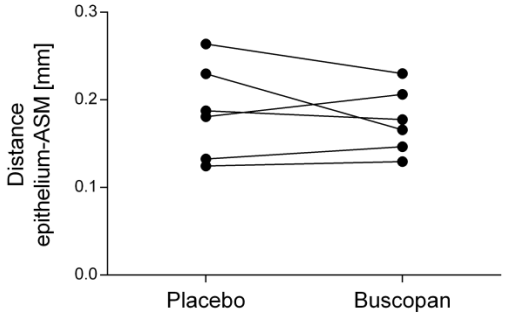
Additional results of Article 1

Figure 14 (Figure 1, Annex I). Correlation between lamina propria thickness and lung function data.



Left panel: correlation between the lamina propria thickness and pulmonary resistance at 5 Hz (R, expressed in kPa/L/s). Right panel: correlation between the lamina propria thickness and the percentage of the biopsy sample occupied by airway smooth muscle (ASM%). Of note, pulmonary resistance at 5 Hz was measured after the administration of a bronchodilator (Buscopan ® 0.3 mg/kg IV) in all horses studied.

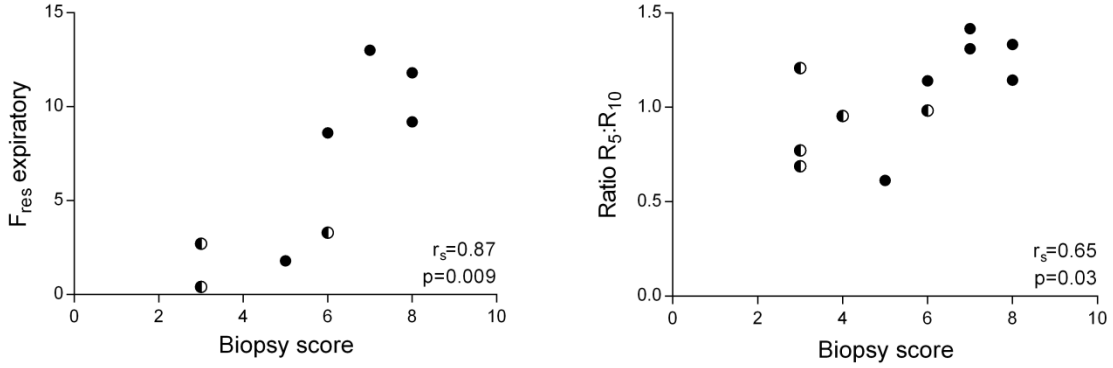
Figure 15 (Figure 2, Annex I). Effect of bronchoconstriction on lamina propria thickness.



Horses were studied during asthma exacerbation using a cross-sectional approach (see the methods of Article 1), Buscopan or placebo were administered 4 days apart. Each point represents one horse and corresponds to the mean values obtained by the analysis of 6 biopsy samples. Paired t-test was performed as statistical test (p=0.47).

Additional results of Article 2

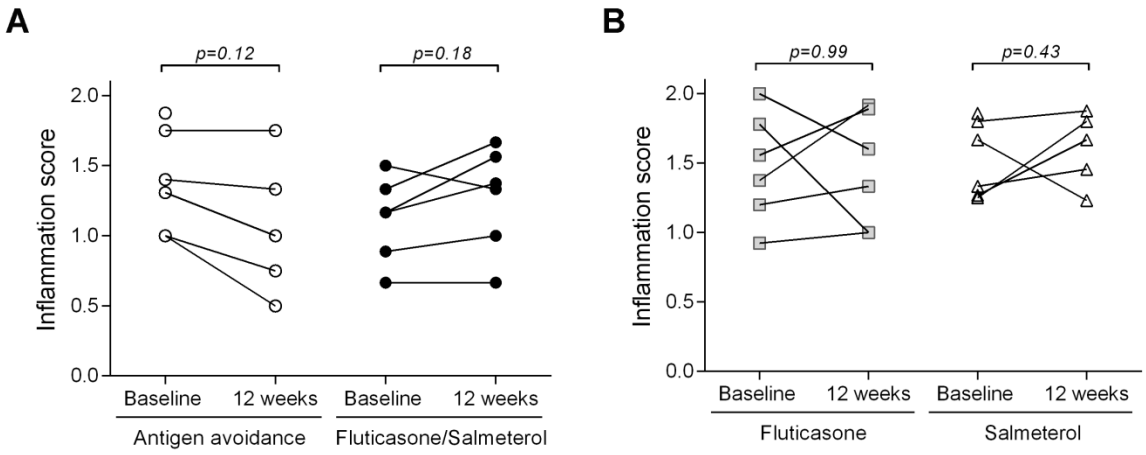
Figure 16 (Figure 3, Annex I). Relationship between biopsy score and lung function.



The biopsy score correlates with different parameters of airway obstruction measured by impulse oscillometry in asthmatic horses. Left panel: correlation with expiratory resonance frequency. Values of 4 horses were missing as their reactance curves did not cross the x axis. Right panel: correlation with the ratio between resistance at 5 Hz and 10 Hz, a previously validated parameter of airway obstruction in asthmatic horses (van Erck, Votion et al. 2003). Black circles: horses with asthma in exacerbation of the disease. Black and white circles: horses with asthma in remission of the disease.

Additional results of Article 4

Figure 17 (Figure 4, Annex I). Effect of pharmacological treatments and antigen avoidance on bronchiolar inflammation



A) Study I. B) Study II. Statistical analysis performed with non-parametric paired t-test (Wilcoxon). For one horse treated with antigen avoidance group (#383) and one treated with salmeterol (#391), assessment at 12 weeks was not possible due to low quality of the histological images.

Annex II

Annex II includes additional publications I have co-authored and pertinent to the subject of this thesis.

Article 6

Asthma "of horses and men" – how can equine heaves help us better understand human asthma immunopathology and its functional consequences?

Michela Bullone and Jean-Pierre Lavoie

Summary

This review summarizes the immunological aspects which support the use of the equine model of asthma. Similarities and differences between equine and human asthma are discussed.

Contribution

I have contributed to the literature review and preparation of the manuscript.

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Review

Asthma "of horses and men"—How can equine heaves help us better understand human asthma immunopathology and its functional consequences?

Michela Bullone, Jean-Pierre Lavoie*

Department of Clinical Sciences, Faculty of Veterinary Medicine, Université de Montréal, 3200 rue Sicotte, St-Hyacinthe, QC, Canada J2S 6C7

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ABSTRACT

Animal models have been studied to unravel etiological, immunopathological, and genetic attributes leading to asthma. However, while experiments in which the disease is artificially induced have helped discovering biological and molecular pathways leading to allergic airway inflammation, their contribution to the understanding of the causality of the disease has been more limited. Horses naturally suffer from an asthma-like condition called "heaves" which presents sticking similarities with human asthma. It is characterized by reversible airway obstruction, airway neutrophilic inflammation, and a predominant Th2 immune response. This model allows one to investigate the role of neutrophils in asthma, which remains contentious, the regulation of chronic neutrophilic inflammation, and their possible implication in pulmonary allergic responses. Furthermore, the pulmonary remodeling features in heaves closely resemble those of human asthma, which makes this model unique to investigate the kinetics, reversibility, as well as the physiological consequences of tissue remodeling. In conclusion, heaves and asthma share common clinical presentation and also important immunological and tissue remodeling features. This makes heaves an ideal model for the discovery of novel pathways implicated in the asthmatic inflammation and associated tissue remodeling.

1. Introduction

Major discoveries related to human diseases have been gained through animal experiments. It is undoubted that mice models have helped uncovering novel immunological mechanisms responsible for the development of different disease processes. Nevertheless, therapeutic strategies derived from these studies have been for the most part disappointing when translated to human diseases, including asthma (Clienti et al., 2011; Giembycz and Newton, 2011; Nair et al., 2012). This may be in part due to different transcriptional responses to acute inflammatory insults in mice and men (Seok et al., 2013).

Development of animal models better mimicking human diseases not only in their clinical presentation, but also taking into account genetic diversity and the complexity of immunopathological mechanisms leading to disease ontogeny, is considered crucial for the discovery of novel therapeutic approaches (Hein and Griebel, 2003). Domestic animal species spontaneously develop diseases having striking similarities with human conditions. Lifespan and size of large animals are more similar to men than to rodents, as is their developmental, innate, and mucosal immunity. For instance, mice lack the gene encoding for the interleukin-8 (Hol et al., 2010), a cytokine implicated in severe neutrophilic asthma and in respiratory virus-induced asthma exacerbations (Nakagome et al., 2012; Rohde et al., 2014), and also essential for neutrophil recruitment in men, cattle, and horses (Caswell et al., 1999, 2001; Cook et al., 2009; Douglass et al., 1996; Franchini et al., 1998; Kaur and Singh, 2013).

2. Equine heaves, as a naturally occurring model of asthma

Horses naturally develop an asthma-like condition currently known in the veterinary scientific community as "heaves" or RAO (recurrent airway obstruction) (Robinson, 2001). This condition has also been known in the past as chronic bronchiolitis, broken-wind, hay sickness, emphysema, small airway disease, allergic airway disease, and chronic obstructive pulmonary disease. As "heaves" was the term used to introduce the horse as an animal model for asthma,

* Corresponding author at: Department of Clinical Sciences, Faculty of Veterinary Medicine, Université de Montréal, 3200 rue Sicotte, St-Hyacinthe, QC, Canada J2S 6C7. Tel.: +1 450 773 8521.
E-mail address: Jean-Pierre.Lavoie@umontreal.ca (J.-P. Lavoie).

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we will employ this term in this review. The name "inflammatory airway disease" (IAD) has been coined to describe a milder form of equine respiratory inflammatory disease in which no respiratory effort is observable at rest. It is characterized by mild clinical signs (nasal discharge, cough, decreased athletic performance) detected in the presence of inflammatory abnormalities of the bronchoalveolar lavage fluid (BALF) cytology (Couetil et al., 2007). It has recently been proposed that heaves and IAD in all their clinical variants are grouped together under the definition of "equine asthma" (Lavoie, personal communication).

Both human asthma and equine heaves are heterogeneous diseases which might present in a variety of clinical forms depending upon the stage of the disease, the chronicity of the condition, and possibly upon different pathogenetic pathways leading to its development. We recognize that not all forms or stages of human asthma necessarily share the same attributes as equine heaves. Based on the definition of the most recent GINA guidelines (GSAMP, 2014), we believe that heaves represent an ideal animal model for the study of non-allergic asthma, late-onset asthma, and severe asthma.

2.1. "Heaves" and asthma

Heaves is a chronic obstructive respiratory condition naturally affecting 10–15% of adult horses living in temperate climates (Hotchkiss et al., 2007). It shares remarkable similarities with human asthma (Table 1). In heaves, disease exacerbations, during which horses suffer from respiratory distress episodes comparable to those affecting severe asthma patients, are triggered by inhalation of environmental antigens (Pirie et al., 2003). It had been postulated that heaves was analogous to allergic pneumonitis in man (Farmer's lung disease), as moldy hay is an important triggering factor for both diseases. However, these two conditions are otherwise different in their clinical presentation, lung pathology, and underlying immunopathological mechanisms. For instance, bronchiolitis and alveolitis with granuloma formation and extensive fibrosis leads to a restrictive respiratory pattern in allergic pneumonitis (Costabel et al., 2012), while in heaves these changes are not present.

The exposure to hay and dusts leading to heaves is rather a consequence of the human influence on horses' natural environment. Molds and fungi are indeed common antigens in the stables, suggesting that heaves is a disease of "domestication". However, horses can develop a similar condition while at pasture, with grass pollen being then the likely triggering factor (Dixon and McGorum, 1990; Seahorn and Beadle, 1993). Therefore, the antigens toward which horses develop an asthma-type response vary according to environmental exposure.

During clinical exacerbation of heaves, horses develop a pulmonary neutrophilic inflammation (Jean et al., 2011) (Fig. 1). While asthma is commonly described as an eosinophilic disease, it is now recognized that neutrophilic inflammation may be present in asthma of all severities, although it is more common in severe asthmatic patients and during acute disease exacerbations (Nakagome et al., 2012; Qiu et al., 2007; Wenzel, 2012). Eosinophils, metachromatic cells, or neutrophils may infiltrate the airway lumen when horses develop the mild-to-moderate asthmatic-type response seen in IAD.

Both heaves and asthma are characterized by reversible airflow obstruction, as a consequence of bronchospasms, increased mucus production, airway hyperresponsiveness, and pulmonary remodeling (Fig. 2). During periods of remission of the disease, when offending antigens are removed from the horses' environment,

Table 1
Similarities and differences between equine respiratory conditions and asthma.

	IAD	Heaves	Mild asthma	Severe asthma
Epidemiology and etiology				
Naturally occurring disease				
• Early in life	√	†	√	√
• Adult-onset	√	√	√	√
Environmental component	√	√	√	√
Genetic component		√	√	†
↑ Endotoxin sensitivity	√	√	√	√
Pathophysiology				
Airway hyperresponsiveness	√	√	√	√
• Early phase		–	√	√
• Late phase		√	√	√
Airway obstruction	Sub-clinical	Reversible*	Reversible	Partly reversible
High temperature/humidity induced exacerbations	?	√	√	√
Tissue remodeling				
• ↑ ASM mass	?	√	√	√
• ↑ ECM mass	?	√	√	√
• ↑ basal membrane thickness	?	†	√	√
• ↑ mucous producing cells	?	√	√	√
• Associated bronchiectasis	?	†	†	†
Hypercoagulability state	?	√	√	√
Immunology				
Airway neutrophilia	†	√	†	†
Airway eosinophilia	†	-	†	†
Airway mastocytosis	†	† (rare)	†	
Tissue inflammation	?	√	√	√
Th2-mediated inflammatory response	√	√	√	√
Th1-mediated inflammatory response	√	√	†	†
Th17-mediated inflammatory response	†	√	√	√
IgE mediated response	?	†	√	√
Associated atopy	?	†	†	†
Innate immune activation				
• Systemic inflammation	†	√	†	√
• ↑ Endotoxin sensitivity	†	√	√	√

√: present †: may be present. *: may be only partly reversible. ?: Not evaluated to our knowledge.

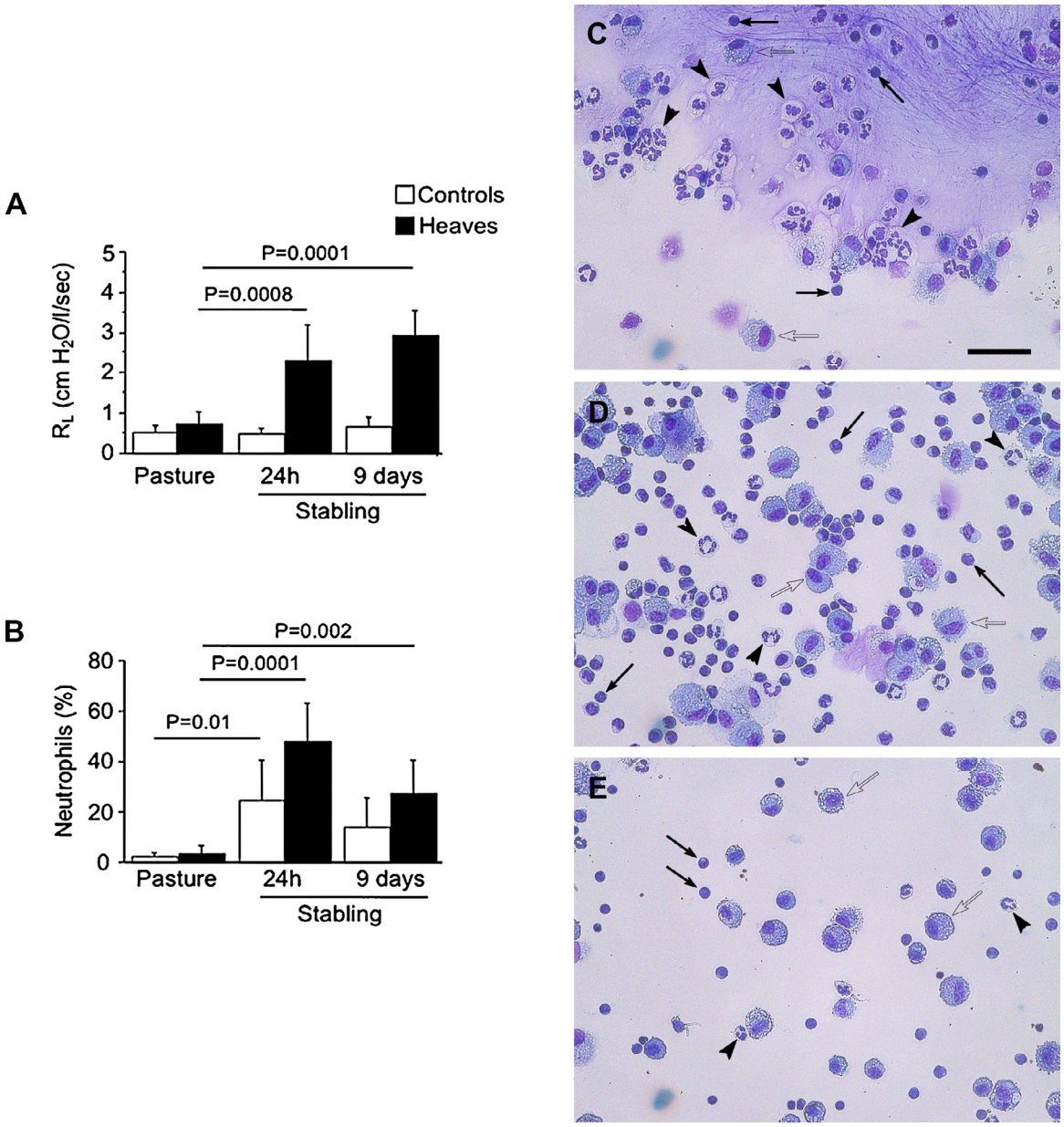

Fig. 1. (A, B) Horses with heaves during clinical remission of the disease have pulmonary resistance (R_L) values and neutrophil percentages in BALF similar to those of healthy controls. However, after antigen exposure, sustained increased in R_L and neutrophil percentages in BALF are present only in horses with heaves (reprinted from Joubert et al., 2011 with the permission of Elsevier, license number: 3423941179982). (C–E) BAL fluid cytology of a horse with heaves during exacerbation (C) and remission (D) of the disease, and of a healthy horse (E). Note the presence of mucus (upper right), the increased cellularity of BAL fluids and also the increase in neutrophil percentage in the horse suffering from heaves during exacerbation of the disease. Neutrophils are indicated by black arrowheads; lymphocytes by black arrows and macrophages by transparent arrows.

horses with heaves are clinically indistinguishable from healthy animals, and their airway function and bronchial cytology normalize (Leclere et al., 2011). However, as reported in neutrophilic human asthma (Wood et al., 2012), systemic inflammation (Lavoie-Lamoureux et al., 2012a,b) and subclinical airway obstruction (Leclere et al., 2012a; Van Erck et al., 2006) persist in these horses. The latter is explained, at least in part, by a persistent remodeling of the airways (Lanctot Setlakwe et al., 2014; Leclere et al., 2012a).

2.2. Advantages of the equine asthma model

Heaves is a naturally occurring disease in which pathogenetical mechanisms are likely to be similar to those observed in human asthma, possibly triggered by immunological "defects" rather than from external manipulations as it happens in rodent models. Also, similar to the natural history of human asthma, horses with heaves experience repeated episodes of airway obstruction occurring over periods of years or sometimes even decades. This contrasts with rodent models in which chronicity can rarely be achieved for more than a 3-month period (Nials and Uddin, 2008; Yang et al., 2013). Furthermore, as the environmental triggering conditions are known, disease status may be modulated as required by specific research needs, avoiding the use of drugs or antigens irrelevant to disease development to induce bronchoconstriction. This represents a unique strength of equine heaves for the study of human asthma.

The horse is especially well suited for prospective studies requiring multiple analyses repeated over time and on the same subjects. Indeed, additional advantages of horses are those linked to their size. Venipuncture and blood analysis can be performed

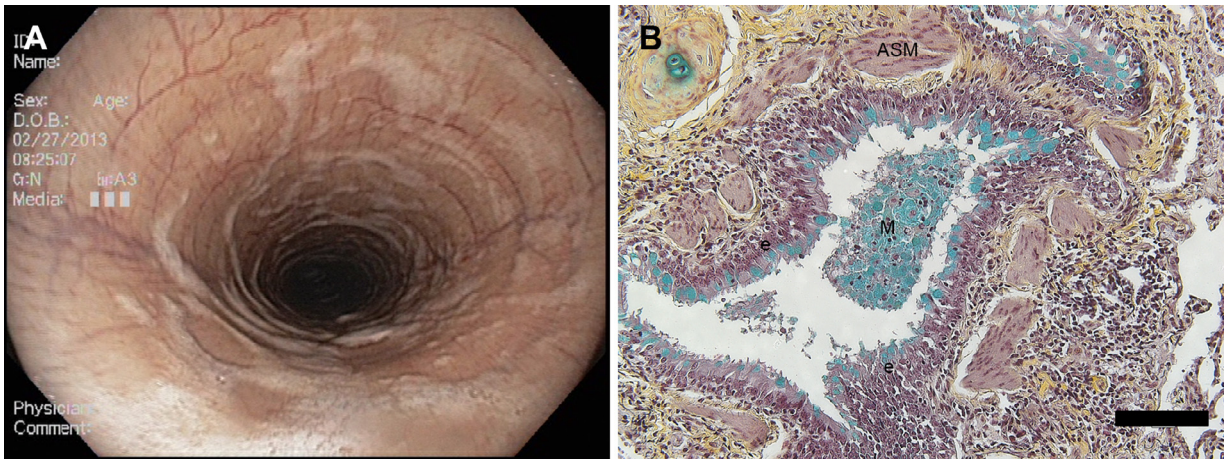

Fig. 2. (A) Endoscopic image of the trachea of a horse suffering from heaves during disease exacerbation. Note the mucus accumulation. (B) Histologic section of the lung parenchyma of a horse with heaves. The bronchial lumens of the smaller airways are filled with mucus (green) containing inflammatory and epithelial cells. Epithelial mucus-producing cells are increased in number. Sub-epithelial inflammatory infiltrate is also evident. Airway smooth muscle mass is increased compared to normal horses (scale bar = 100 μm). ASM: airway smooth muscle; M: mucus; e: bronchial epithelium.

recurrently allowing the non-invasive collection of large quantities of circulating cells without altering the animal immune response. Bronchoscopy is performed in standing sedated animals, allowing mucus collection, tracheal wash (TW) aspirates, bronchoalveolar lavage (BAL), and/or bronchial epithelial brushing. Furthermore, the equine tracheobronchial tree offers more than 40 reachable carinae for endobronchial biopsy collection (Fig. 3). Although endobronchial biopsies have been shown to be inadequate samples for quantitative studies of airway smooth muscle (ASM) mass (Bullone et al., 2014a), they provide valuable information regarding epithelial, extracellular matrix components, and ASM cell phenotypes (Jeffery et al., 2003; Leclere et al., 2011, 2012a; Leguillette et al., 2009; Pini et al., 2007). Horses' lungs also permit harvesting large peripheral lung biopsies by means of thoracoscopic surgery (Lugo et al., 2002; Relave et al., 2008, 2010), which makes heaves perhaps the only animal model allowing the study of small airways remodeling over time in the same subjects. Furthermore, techniques such as spirometry, impulse oscillometry, and endobronchial ultrasound among others allow studying lung function as well as structural remodeling in this species (Bullone et al., 2014b; Couetil et al., 2000; Van Erck et al., 2006).

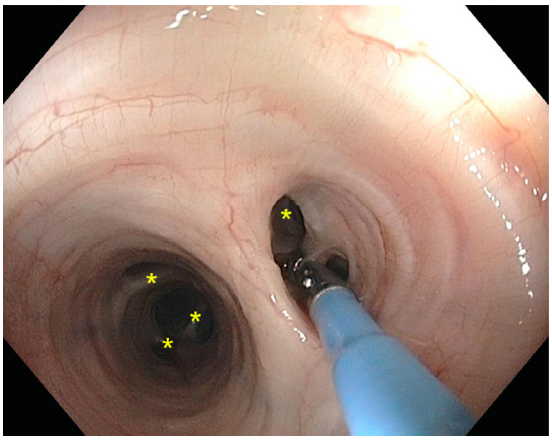

Fig. 3. Endobronchial biopsy procedure in a horse. Carinae from which endobronchial biopsies can be withdrawn are numerous (marked with yellow stars). Biopsies may be withdrawn from carinae of 1st, 2nd, 3rd, and 4th generation. This is due to the branching pattern of the equine bronchial tree (monopodial), in which several lateral ancillary bronchi stems from the main caudal bronchus before reaching the maximal caudal accessible sites where it is possible to lodge the endoscope.

Table 2
Efficacy of several treatments on asthma in equine and rodent animal models, as well as in man.

	Horse	Rodents	Man
Antinflammatory agents			
Inhaled corticosteroids	√	√	√
Systemic corticosteroids	√	√	√
Anti-leukotriens	—	√	(√)*
Cromones	(√)	√	(√)*
Non-steroidal antinflammatory drugs	—		—
Bronchodilators			
β-adrenergic agents	(√),*	(√)	(√),*
Anticholinergic agents	(√),*	√	(√),*
Phosphodiesterase (PDE) inhibitors			
Methylxanthine inhibitors	(√),*	√	(√),*
Selective PDE inhibitors	—	√	—
Antioxidants	(√)	√	(√),*
Expectorant, mucolytic and mucokinetic agents	—	(√)	—
Immunomodulators	?	√	(√)
Antibiotics			
Macrolides	?	√	(√),*
Cellular therapy			
Adoptive transfer of stem cells	—	√	?

√: treatment with demonstrated efficacy in that species. (√): treatment whose efficacy in that species is supported by a low level of scientific evidence. —: treatment with no demonstrated efficacy in that species. ?: treatment with unknown efficacy in that species. *: treatment suggested as possible add-on therapy in selected cases.

The effect of aging on immunological variables is often disregarded when rodent models are employed in experimental settings, but evidences support important immunological functions to be dependent on or to change with age (Busse and Mathur, 2010; Lee et al., 2012). Horses' lifespan (≈30 years) is undoubtedly more similar to that of man than those of rodents, cats, or dogs. Also, horses develop heaves in their adulthood, which is reminiscent of late-onset human asthma, with both conditions generally displaying neutrophilic pulmonary inflammation and a less pronounced allergic component (Brazil et al., 2005; Wenzel, 2012).

The treatments of choice for heaves are corticosteroids, either inhaled or systemically administered. Also, both adrenergic and anticholinergic bronchodilators have been proved effective at inhibiting the bronchospasm associated with heaves. Many molecules targeting specific intracellular pathways or mediators shown to be involved in murine asthma models were effective when tested in mice, but not in horses or in man (Table 2). For instance, p38 MAPK inhibitors (Bhavsar et al., 2010; Chopra et al., 2008; Lavoie et al., 2008), PDE4 inhibitors (Giembycz and Newton,

2011; Lavoie et al., 2006; Matera et al., 2014), as well as cysteinyl-leukotriene antagonists, were poorly effective as sole therapy in horses with heaves (Kolm et al., 2003; Lavoie et al., 2002) despite showing promising outcomes in rodent models (Bos et al., 2007; Pera et al., 2011). These drugs are now considered either ineffective or only as add-on therapies for uncontrolled asthma rather than as first-choice monotherapies in asthma international guidelines (Busse and Lemanske, 2007; Turner et al., 2011). Thus, equine heaves appears to be a valuable preclinical model for reliably testing the efficacy of new drugs for asthmatic patients.

2.3. Disadvantages of the equine model of asthma

Using horses as an asthma animal model is not free of drawbacks. A direct consequence of their large size is limited accessibility and higher cost for drugs, breeding facilities and equipment, procedure materials, and ordinary care. An equine tissue bank has been developed for respiratory research (http://www.ertb.ca), which makes this model available to researchers lacking the facilities or the technical expertise required for handling these animals. Also, few antibodies have been validated for this species (Schnabel et al., 2013) and newly discovered immune system cells may not yet be characterized in horses. However, the equine genome has been entirely sequenced (Wade et al., 2009), which facilitates the identification of homologous sequences among different species in order to improve cross-reactivity. Studying subjects of different breed, size, age, and origin increases intra-group variability and further complicates data analysis and interpretation. However, it provides a heterogeneous population as seen in human.

3. Heaves and immunology

3.1. Neutrophils and the Th2 paradigm in asthma

Asthma is generally considered as an eosinophilic disease, especially in its allergic form, driven by a Th2-type inflammatory response. However, the kinetic of inflammatory cell recruitment into the airway lumen of antigen-challenged asthmatics identified eosinophil accumulation to occur after the early asthmatic response (Lommatzsch et al., 2006). Interestingly, neutrophil recruitment to the airway lumen is common in acute asthma exacerbations (Fahy et al., 1995; Lopuhaa et al., 2002; Norzila et al., 2000; Ordonez et al., 2000) and occurs as early as 4 h after allergen challenge (Nocker et al., 1999). These findings support a role for neutrophils during uncontrolled phases of the disease (exacerbations or asthma attacks). However, while neutrophil recruitment in experimental settings is a clear consequence of antigenic challenge, it is not established whether during real-life asthma attacks, it precedes or follows the fall in lung function. Also, the immunologic mechanisms linking neutrophil activation/recruitment to the Th2-predominant immune response in asthma remain incompletely elucidated.

In horses with heaves, a predominant Th2-type immune response associated with an airway neutrophilia has been described both in the acute and chronic phases of the diseases (Beadle et al., 2002; Cordeau et al., 2004; Klukowska-Rotzler et al., 2012a; Lavoie et al., 2001). Pulmonary neutrophilia develops as early as 5–6 h after antigen exposure, preceding the development of airway obstruction (Brazil et al., 2005; Fairbairn et al., 1993; Franchini et al., 2000). Peripheral neutrophils are primed in heaves, also during periods of remission of the disease (Lavoie-Lamoureux et al., 2012a). An increased number of neutrophils express IL-5 and IL-9 receptors in heaves-affected horses compared to controls, which could link the observed Th2-type immune response to the neutrophilic chronic inflammatory phenotype (Dewachi et al., 2006). Also, recombinant equine IL-4 stimulation induced an increase in IL-8 and IL-4R expression in equine neutrophil *ex vivo*, suggesting that Th2 cytokines may contribute to the recruitment and activation of neutrophils during allergic inflammation (Lavoie-Lamoureux et al., 2010).

Disease chronicity has also been associated with induction/activation of Th17-mediate immunity in heaves (Ainsworth et al., 2006; Debrue et al., 2005). Th17 cytokines may therefore contribute to the sustained airway neutrophilic inflammation in this disease, as reported in human asthma (Linden and Dahlen, 2014). However, Th17 and Th2 responses may not be mutually exclusive but rather sequentially expressed in the airways (Lavoie-Lamoureux et al., 2010), hallmarks of consecutive phases of the inflammatory process. Also, and as reported in human asthma, multiple molecular phenotypes or endotypes possibly occur in heaves, as predominant Th1 and mixed Th1/Th2-type responses has been reported, suggesting complex immune processes contributing to the disease in some circumstances (Ainsworth et al., 2003, 2006; Beadle et al., 2002; Giguere et al., 2002). Clearly, and similar to human asthma, the pathways responsible for the asthmatic phenotype in heaves are likely to be complex and influenced by both genetic and environmental factors.

3.2. Unraveling the mechanisms linking asthma clinical signs, inflammation, and tissue remodeling

Asthma is a chronic obstructive disease characterized by airway hyperresponsiveness, inflammation, and remodeling. The identification of excessive and possibly inappropriate airway inflammation as a phenomenon underlying asthma pathophysiology was recognized in the late 1980s and led to the proposal of the "inflammatory paradigm", by which local chronic inflammation would trigger tissue remodeling and consequently amplify the constrictive effect of airway hyperresponsiveness (Walter and Holtzman, 2005). Remodeling has been documented to occur at all levels of the bronchial wall, with ASM being the structure whose architectural and phenotypical alterations more profoundly impact asthma clinical manifestations (Lambert et al., 1993; Oliver et al., 2007). Nevertheless, the mechanisms regulating remodeling and inflammation in asthma as well as their effect on airway hyperresponsiveness are far from clear and are subject of extensive research.

The study of remodeling features in asthmatic patients is complicated by both a difficulty in obtaining tissue samples (mainly small airways) and by technical impossibility of controlling for several variables linked to the environment in which the patient live (antigen exposure, alimentary habits, environmental temperature and humidity, medication adherence, and compliance), which can affect asthma clinical presentations and thus remodeling features. The horse is a model that, despite the biological/genetic variability among different subjects, allows removal of most of the "environmental noise". Furthermore, lung samples can be sequentially obtained from both central and peripheral airways from the same horses.

While studying equine heaves as an asthma model, our group has shown that, following corticosteroid inhalation therapy, clinical manifestations of the disease, and small ASM remodeling were dissociated from pulmonary neutrophilia (Leclere et al., 2012a). Conversely, when remission of the disease was achieved by long-term antigen avoidance strategies, normalization of airway neutrophilia preceded the decrease in ASM mass. Also, IL-8 and TNF-α mRNA expression in BAL cells remained elevated in horses receiving inhaled fluticasone when compared to horses treated with antigen avoidance strategies. These results indicate that a greater degree of activated neutrophils were indeed present within the bronchi of horses showing the better outcome in terms of ASM remodeling features. Alterations in the architecture of the airway wall are not limited to the ASM cells in asthma. The extracellular

matrix (ECM), a dynamic three-dimensional fibrous network essential to the mechanical properties of the airways, is also altered in quantity and composition in the asthmatic airways (Roche et al., 1989; Wilson and Li, 1997). We recently reported an increase in collagen and elastic fiber content in the peripheral airways of horses with heaves in remission, which was correlated with alterations in airway function (Lanctot Setlakwe et al., 2014). These findings indicate that increased collagen content contribute to the residual airway obstruction in asthmatic horses, while increased and disorganized elastic fiber content decreased the elastic properties (compliance) of the lung. Interestingly, while collagen remodeling is considered poorly responsive to intervention, we observed a reduction in airway collagen content after a 1-year treatment with either inhaled corticosteroids or antigen avoidance strategies (Leclere et al., 2012b). The reduced collagen content was not associated with decreased TGF-ß or inflammatory cytokine expression by BAL cells in these horses. Taken together, these findings suggest that BAL neutrophilic inflammation is not strictly associated with small airway remodeling or airway obstruction in chronic disease, but a direct link seems to exist between airway hyperreactivity and tissue remodeling.

3.3. Mechanical regulation of the bronchial immune response

Increasing evidences suggest that mechanical stimulation of the airways prompts activation of several resident cells, with consequent upregulation of inflammatory gene expression and changes in phenotype of structural components (Le Bellego et al., 2009; Ludwig et al., 2004; Mohamed et al., 2010; Park et al., 2010; Park and Tschumperlin, 2009). Interestingly, smooth muscle cells can switch from their normal contractile phenotype to a more proliferative/synthetic one when chronic mechanical loads are imposed (DiSanto et al., 2003; Hirota et al., 2009). The (+)insert smooth muscle myosin heavy chain (SMMHC) isoform is a marker of the smooth muscle proliferative/hypercontractile phenotype and was found to be significantly increased in endobronchial biopsies from asthmatics compared to controls (Leguillette et al., 2009). To the same extent, (+)insert SMMHC is overexpressed in central and peripheral airways of horses with heaves compared to controls, indicating phenotype switching of ASM along the bronchial tree (Boivin et al., 2014). The relative contribution of mechanical load and inflammation to (+)insert SMMHC regulation is unknown. However, as corticosteroid administration and long-term antigen avoidance led to a significant reduction of the (+)insert expression in the airways of heaves-affected horses (Boivin et al., 2014), inflammation likely contributes to this process.

3.4. Innate immune activation is a feature of neutrophilic asthma

Chronic innate immune activation is present in neutrophilic human asthma as in equine heaves, which persists also during remission of the disease (Fu et al., 2013; Lavoie-Lamoureux et al., 2012a; Wood et al., 2012). It has been speculated that the chronic inflammatory response of the asthmatic airways could be the result of a defective innate immune system. An inappropriately developed or altered innate immune response could lead to an exaggerated reaction to normally no-noxious stimuli. Alternatively, there may be an inability of such system to be "switch off" after being activated. Indeed, chronic activation itself could prevent adequate negative feedback systems to act properly.

Neutrophils are first-line defense cells of the innate immune system. They are considered the hallmark of acute inflammatory processes, as they quickly congregate at sites of damaged or infected tissues in response of several chemotactic agents liberated during the initial insult. They directly fight the noxious agent by liberating antimicrobial and protease-rich granules, by producing extracellular traps (NETs; Fig. 4), and through phagocytosis. They also liberate several pro- and anti-inflammatory cytokines, thus possibly modulating the inflammatory response. Peripheral blood neutrophils are activated in human asthma and in equine heaves (Dewachi et al., 2006; Lavoie-Lamoureux et al., 2012a; Mann and Chung, 2006; Marr et al., 1997; Tremblay et al., 1993; Wood et al., 2012). Available data support an early neutrophilic wave to happen as early as 5 h after antigen exposure in both asthma and heaves (Brazil et al., 2005; Nocker et al., 1999). Interestingly, healthy human and equine subjects develop mild but significant neutrophilic pulmonary inflammation after allergen challenge, which spontaneously resolves within few days/hours despite protraction of the stimulus (Leclere et al., 2011; Nocker et al., 1999). Such natural clearance is not observed in asthma or in heaves. Furthermore, neutrophils recruited to the airways are activated in horses with heaves but not in control animals exposed to the same environment, as shown by the increased identification of NETs in the first group only (Cote et al., 2014). Whether systemic neutrophilic activation results from an intrinsic defect of the neutrophils, or neutrophils are activated secondary to the lung inflammation remains to be definitively determined. Basal peripheral neutrophil activation is not dissimilar between horses with heaves in remission and controls, but changes in response to specific stimuli, suggesting that alterations of the innate immune response to specific noxa may be associated with heaves pathology (Lavoie-Lamoureux et al., 2012a). These differences being found even in the absence of lung inflammation provides some evidence of an intrinsic defect of neutrophil activation in heaves.

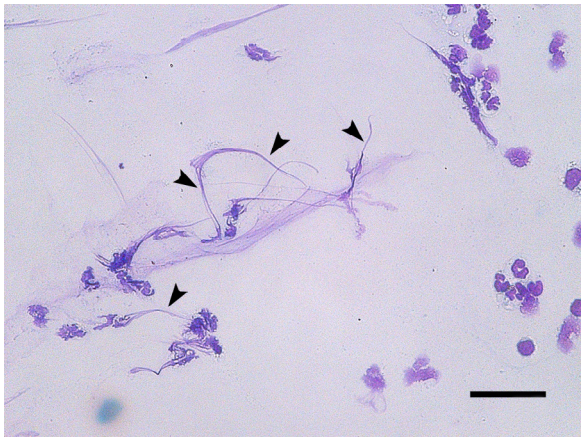

Fig. 4. BALF cytology of a horse with heaves during disease exacerbation. Note the presence of NETs, as indicated by the black arrow heads (40× magnification, scale bar = 50 μm).

3.5. Role of the bronchial epithelium in heaves

The ASM-centric paradigm leading asthma research in the last decades is slowly evolving toward asthma pathogenesis being driven by both the ASM and the bronchial epithelium (Erle and Sheppard, 2014). Bronchial epithelial cells obtained before and after antigen challenge showed an increased expression of transcription factors able to regulate the immunologic response (NF-Kβ, AP-1, and CREB) in horses with heaves compared to controls (Bureau et al., 2000; Couetil et al., 2006). Protein expression of IL-6, IL-10, and TNF-α and gene expression of CXCL1 and TLR4 were similar in bronchial epithelial cells of heaves and healthy horses early after antigen challenge (Ainsworth et al., 2006; Riihimaki et al., 2008). Bronchial epithelial cell cultured from horses with heaves and controls showed a similar increase in TLSP gene expression 6 h after hay dust suspension challenge

(Klukowska-Rotzler et al., 2012a). However, epithelial cells from horses with heaves showed an increased expression of IL-8 and TLR-4 compared to controls later on in the development of the disease (Ainsworth et al., 2006; Berndt et al., 2007), suggesting that these cells likely contribute to the persistent airway inflammation.

3.6. Heaves and immunity toward helminths

As suggested by the "hygiene hypothesis", regular use of modern antihelmintics and a decreased exposure to parasites could increase the risk for horses developing heaves and other allergic diseases (Strachan, 1989). Clearance of extracellular parasites, including helminths, is mediated by Th2-type immune response. Equine studies linking heaves to parasite immunity have been derived from data collected from 2 half-sibling families of Swiss horses affected with heaves (Ramseyer et al., 2007). Some of these horses (all belonging to the same family) have an increased resistance to intestinal parasite infestation when compared to the other family of heaves affected horses, or to control animals (Brundler et al., 2011; Neuhaus et al., 2010). These differences between the two heaves-affected families were associated with microsatellite markers near the gene of the IL-4 receptor alpha chain (IL4Rα), affecting its expression during disease exacerbations (Jost et al., 2007; Klukowska-Rotzler et al., 2012b; Racine et al., 2011). Also, hay dust and cyathostomin extract increased the expression of IL4Rα, IL-4, and IL-10 by isolated blood leukocytes only in the family showing increased parasite resistance (Lanz et al., 2013). Interestingly, IL4Rα is associated with defense against parasites in humans and animals, and a correlation between susceptibility to asthma and resistance to parasitic infections has been reported for asthmatics as well (Barnes et al., 2005; Hopkin, 2009). These findings suggest that a Th2-biased immune response is "genetically programmed" in some equine and human subjects, rather than being a consequence of an increased hygiene. While this genetic trait would be advantageous by providing increased parasite protection in the wild, it may be disadvantageous when the horse is moved into a domesticated milieu and exposed to high burdens of inhaled allergens, promoting an exaggerated and inappropriate pulmonary immune response.

3.7. Aging

Asthma incidence in the elderly population is growing and these patients are more likely to be underdiagnosed and undertreated than young asthmatic subjects (Hanania et al., 2011). Aging in healthy individuals is a physiological process associated with important changes in the immune function collectively known as immunosenescence or "inflamm-ageing". It results in a reduced capacity to cope with a variety of stressors and in a progressive pro-inflammatory status (Franceschi et al., 2000). Inflamm-aging has been demonstrated to occur in horses (Fermaglich and Horohov, 2002; Horohov et al., 2010), supporting the use of these animals for age-related immunological studies. Specifically, T-cells of aged horses (>20 years old) show a decreased proliferative response (Adams et al., 2008; Horohov et al., 2002) when compared to younger animals and an increased production of IFNγ and TNFα by lymphocytes and monocytes, respectively (Hansen et al., 2013, 2014; Katepalli et al., 2008). Furthermore, specific immune-aging processes appear to vary according to the anatomical locations. For instance, TNFα production increases with age in peripheral mononuclear cells but not in BALF cells. Also, while IL-1β mRNA expression increases with age in peripheral blood, they decrease in BALF cells (Adams et al., 2008; Hansen et al., 2013). Whether and how these findings correlate with the development of airway diseases and whether they are affected by common anti-asthma medications remains to be elucidated.

4. Conclusions

In conclusion, heaves and asthma share common clinical presentation but also important immunological basis. While equine heaves does not necessarily share the same attributes of all forms or stages of human asthma, the natural history of the disease and the similarities in the airway remodeling processes make heaves an ideal model to study the cellular and molecular pathways associated with the asthmatic airway response and its reversibility, especially regarding late-onset and severe asthma. Significant similarities in the therapeutic responses between horses suffering from heaves and asthmatic patients further support the study of equine heaves as a model for human asthma. Finally, the role of neutrophils in asthma (and in heaves) remains to be established. Progress in this area fostering the ability of regulating or re-programming the neutrophilic response in subjects with heaves could be important for elucidating the implication of such cells into asthma development.

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Article 7

Endoscopic evaluation of angiogenesis in the large airways of horses with heaves using narrow band imaging

Nicolas Herteman, Michela Bullone, Jean-Pierre Lavoie

Summary

The lamina propria of central airways is thickened in equine asthma. In order to assess the contribution of increased bronchial vascularity to this aspect of central airway remodeling, we have assessed airway angiogenesis using an endoscopic technique that facilitates the visualization of submucosal vessels. We observed an increased vascularity only in the tracheal mucosa of asthmatic horses.

Contribution

I participated in study design (50%), experimental procedures (100%), data analysis (30%), as well as preparation of the manuscript (20%).

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ENDOSCOPIC EVALUATION OF ANGIOGENESIS IN THE LARGE AIRWAYS OF HORSES WITH HEAVES USING NARROW BAND IMAGING

Nicolas Herteman, Michela Bullone, Jean-Pierre Lavoie

Département de Sciences Cliniques, Faculté de Médecine Vétérinaire, Université de Montréal

Running head: Bronchial angiogenesis in heaves

Key words: Blood vessels, Recurrent Airway Obstruction, Equine Asthma

Abbreviations: NBI: Narrow Band Imaging; ROI: Region of Interest.

Corresponding author : Jean-Pierre Lavoie, Département de sciences cliniques, 3200 rue Sicotte, Saint-Hyacinthe, QC, Canada, J2S 2M2, email: jean-pierre.lavoie@umontreal.ca.

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The videoendoscopies analyzed in this study originated from horses enrolled in a previous study that investigated the physiological and technical variables affecting the quality of equine endobronchial biopsy samples (see reference 3).

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Abstract

Background: Heaves is a severe debilitating condition of horses, characterized by lower airway inflammation and permanent structural changes of the bronchial wall. Chronic inflammation promotes the formation of new vessels, a phenomenon known as angiogenesis. Narrow band imaging (NBI) endoscopy is a non-invasive technique that enhances the visualization of submucosal vessels, and commonly is employed for the study of angiogenesis in human patients.

Objectives: Using NBI, we aimed to determine whether or not the central airways of horses with heaves undergo angiogenesis.

Animals: Horses with heaves during exacerbation of the disease (n=5) and healthy controls (n=6).

Methods: A library of NBI images was established from previously recorded videoendoscopies. Images were acquired by an operator blinded to horse ID. Images were obtained from 3 sites: 130 from the trachea (14±9.3 [mean±SD] images per horse with heaves and 10±5.4 from controls; p=0.45), 58 from the carina (5.4±3.2 from horses with heaves and 5.2±2.8 from controls; p>0.99) and 167 from the intermediate bronchi (17.8±6.7 from horses with heaves and 13±5.6 from controls; p=0.17). Using dedicated stereology software (NewCAST, Visiopharm; Denmark), the volume density of superficial and deep vessels was calculated blindly by point counting at each site for all horses.

Results: In the trachea, the volume density of superficial vessels was increased in horses with heaves compared to controls (p=0.02). No difference was found between groups for the volume density of both superficial and deep vessels at the carina or intermediate bronchi.

Conclusion and clinical relevance: NBI imaging of the airways was easily performed in standing sedated horses.

Introduction

Heaves, also known as recurrent airway obstruction, is a disease of horses characterized by episodes of respiratory distress at rest accompanied by neutrophilic pulmonary inflammation during exacerbations. This condition affects approximately 10-15% of adult horses >5-7 years of age in the northern hemisphere[1]. Exacerbations are triggered by exposure to environmental antigens, and can be reversed by enhancing environmental control or using steroids, bronchodilators or both.

Airway remodeling[1-3] leads to the thickening of the bronchial wall, contributing to airflow obstruction in heaves. Hyperplasia and hypertrophy of airway myocytes and fibrosis of the lamina propria are mainly involved in this process[2]. However, angiogenesis develops concurrently with inflammation and remodeling in human with asthma[4], increasing bronchial wall thickness, and therefore could be present in horses with heaves given the similarities between these conditions[1]. If present, targeting new blood vessel formation could represent a possible strategy to control inflammation and remodeling in heaves.

Angiogenesis is observed both in physiological (e.g. embryonic development, exercise, scarring) and pathological (e.g. tumor growth, inflammation) conditions[4-7]. It has been quantified using several methods. Among these, narrow band imaging (NBI) is a recently developed, non-invasive technique allowing an enhanced visualization of submucosal vessels during endoscopy. It employs filters producing light of specific blue and green wavelengths instead of the white light used during conventional endoscopy, which correspond to the peak light absorption of hemoglobin[5]. This technique facilitates visualizing blood vessels because they appear as contrasting structures (black or dark green, depending on their depth within the bronchial tissue) beneath the clear bronchial mucosa.

Despite the availability of new imaging techniques and the likelihood of angiogenesis contributing to remodeling in heaves, no study has attempted to quantify airway blood vessels in this condition. We hypothesized that NBI would allow identification of an increased density of blood vessels (i.e. angiogenesis) in the airways of horses with heaves compared to controls.

Material and methods

Animals and study design

A library of NBI images was established from videoendoscopies previously recorded. Additional details are provided elsewhere[3]. Eleven horses were studied (5 with heaves and 6 controls of similar age and weight) using an observational study design. Horses with heaves were part of our research herd and had a documented history of abnormal lung function when exposed to hay. The control horses were part of the teaching herd at our faculty. They were determined to be healthy based on history, physical examination and endoscopic evaluation of the respiratory tract. All horses were stabled and exposed to hay starting 2 weeks before the study period, in order to induce exacerbations of the disease in affected animals.

Bronchoscopy

Horses were sedated using detomidine (0.012 mg/kg IV) and butorphanol (0.01 mg/kg IV). An endoscope (13 mm Ø, CF-H180AL[a]) was passed through the ventral meatus of a nostril. Once past the larynx, lidocaine solution (0.5%) was instilled as needed to topically anesthetize the airways and prevent coughing episodes. Lower airway endoscopy of 1 randomly selected lung was performed in each horse. Endoscopies were performed using the NBI mode while advancing from the larynx towards the most distal bronchial site, and recorded on a computer in order to be analyzed retrospectively.

Selection of images

Images of the bronchial mucosa (tiff files, 1280x800 pixels) were acquired by an operator blinded to horse ID. Images were acquired when the submucosal vessels were clearly visible. Images in which mucus covered a considerable part of the bronchial mucosa were excluded. Images were obtained from 3 sites (Figure 1A): trachea, carina and between the 2nd and 9th branches of the main caudal bronchus. Each image was renamed and the order of analysis mixed by someone different from the person who conducted the morphometry using the stereology software.

Angiogenesis assessment

Vascularity was blindly assessed with NewCast software version 4.5.1.324[b]. A region of interest (ROI) was defined for each image by someone who was blinded to the group in order to exclude the darkest area of the image and the regions where accumulated mucus prevented a reliable assessment of submucosal vascularity. Superficial and deep vessels were differentiated based on their color. Superficial vessels appeared black whereas deep vessels appeared dark green during NBI endoscopy. Vascular density was assessed by point counting using grids with 256 crosses per screen, because this point density allowed reliable estimation of the vascular density in endoscopic images (data not shown). Blood vessel density was calculated for each horse as follow: $V_{vessel} = \Sigma P_{vessel} / \Sigma P_{bronchial\ wall}$, where ΣP_{vessel} indicates the sum of the points falling onto the vessels and $\Sigma P_{bronchial\ wall}$ the sum of the points falling onto the bronchial wall in each image.

Statistical analysis

Analyses were performed using Prism 5[c]. Non-parametric Mann-Whitney tests were used to compare the 2 groups. The level of statistical significance was set at $p<0.05$.

Results

A total of 130 images for the trachea (14±9.3 [mean±SD] images per horse with heaves and 10±5.4 for controls; p=0.45), 58 images for the carina (5.4±3.2 for horses with heaves and 5.2±2.8 for controls; p>0.99), and 167 images for intermediate bronchi (17.8±6.7 for horses with heaves and 13±5.6 for controls; p=0.17) were obtained. The number of images analyzed in the 2 groups was similar (mean±SD, 37±17.9 images per horse with heaves and 28±13.3 in the control group; p=0.62) and for each site.

No significant differences (Figure 1-B) were observed for the number of deep vessels in trachea (p=0.43), carina (p=0.93), and bronchi (p=0.53) between the 2 groups. Superficial vessel density was not different between the 2 groups at the level of the carina (p=0.93) and

the bronchi (p=0.53). Horses with heaves however had significantly increased tracheal superficial vessel density (p=0.02) compared to controls.

Discussion

Angiogenesis consists of the formation of new vessels (especially venules) from the pre-existing vascular tree, forming a capillary-like network. Many pathological conditions are associated with angiogenesis, presumably to provide adequate amounts of oxygen to tissues with increased metabolism, such as growing, inflamed, or neoplastic tissues. Contrary to what we hypothesized, no increase in vascular density of the bronchial tree was observed in heaves-affected horses compared to controls using NBI, whether evaluating superficial or deep vessels. The only significant difference observed was for superficial vessels of the trachea. These results suggest that either angiogenesis is only a tracheal feature in horses with heaves or that NBI is not sufficiently sensitive to evaluate angiogenesis occurring in the bronchi of affected horses.

Angiogenesis has been quantified using several methods. Unfortunately, most of these assays have limited clinical application because of their cost and invasiveness. Airway angiogenesis was first studied in endobronchial biopsies of asthmatic patients in research settings. It was shown to be associated with disease severity in children with asthma[4,6]. In recent years, several non-invasive imaging techniques have been developed and used as tools to evaluate angiogenesis[8]. Of particular interest, high magnification endoscopy provides an adequate assessment of the submucosal vascular network in asthmatic patients[7]. Narrow band imaging allows differentiation of blood vessels based on their depth, by making superficial blood vessels appear as brown or black and deep vessels as blue or green structures under the bronchial mucosa. It is used primarily in gastroenterology (e.g. adenomas in laparoscopy, inflammatory bowel disease, Barrett's esophagus), urology (e.g. bladder tumors, cystitis), and less frequently in pneumology [5] (during thoracoscopy for assessment of angiogenesis in lung tumors).

In this study, NBI was easily performed on sedated standing horses during routine bronchoscopy. Our results identified an increase in tracheal superficial vascularity in horses with heaves compared to controls. A similar finding has been reported in human asthmatic patients[7], but the clinical relevance of this observation is uncertain. The presence of luminal tracheal inflammatory mediators in heaves possibly could contribute to the increased vascularity we observed in these animals.

Administration of α2-agonists results in activation of peripheral receptors (α1 and α2) on the blood vessels leading to vasoconstriction, thus possibly affecting NBI measurements. However, although these effects were reported to be ≤ 8 minutes in duration[9], NBI was performed between 8 to 10 minutes after the first injection, thus likely minimizing this phenomenon. Also, differences between groups were observed only in the trachea, and we would have expected to see an effect at all sites if the sedation had an impact on blood vessel measurements.

An increase in the vascular network density in the bronchi of horses with heaves was not observed, unlike what is reported in human medicine[4,6,7]. Alternatively, angiogenesis might be present only during the establishment of the disease or a period of 2 weeks of antigen exposure may not have been long enough to generate angiogenesis. Finally, edema in the respiratory tract, bronchospasm, and mucus might have prevented optimal visualization of bronchial blood vessels in these horses.

Airways have a tubular shape, and endoscopic images were obtained with the camera oriented longitudinally to their axis. Some area of the tracheobronchial mucosal might have been analyzed twice on different images, introducing a bias in our results. However, this is unlikely to have occurred in our study for 2 reasons. First, when generating the library, care was taken to avoid selecting images in which overlapping mucosal fields could be identified (for trachea and bronchi). Second, during analysis, only the mucosal areas proximal to the endoscope had adequate conditions of brightness and contrast and therefore were included in the ROI. An additional bias could have resulted from the analysis of a 3-dimensional image using markers arranged in a bidimensional grid that does not take into account the image perspective. We

believe that the bias introduced by our approach, if it occurred, would have been similar in both groups because airway anatomy (dimensions) was very similar among the horses we studied.

In conclusion, evaluation of airway vascularity with NBI technology is easily performed in standing sedated horses. The clinical relevance of the increased tracheal vascularity observed in heaves-affected horses remains to be ascertained.

Footnotes

[a] Olympus; Richmond Hill, ON, Canada.

[b] NewCAST, Visiopharm; Denmark.

[c] GraphPad Software Inc; CA, USA.

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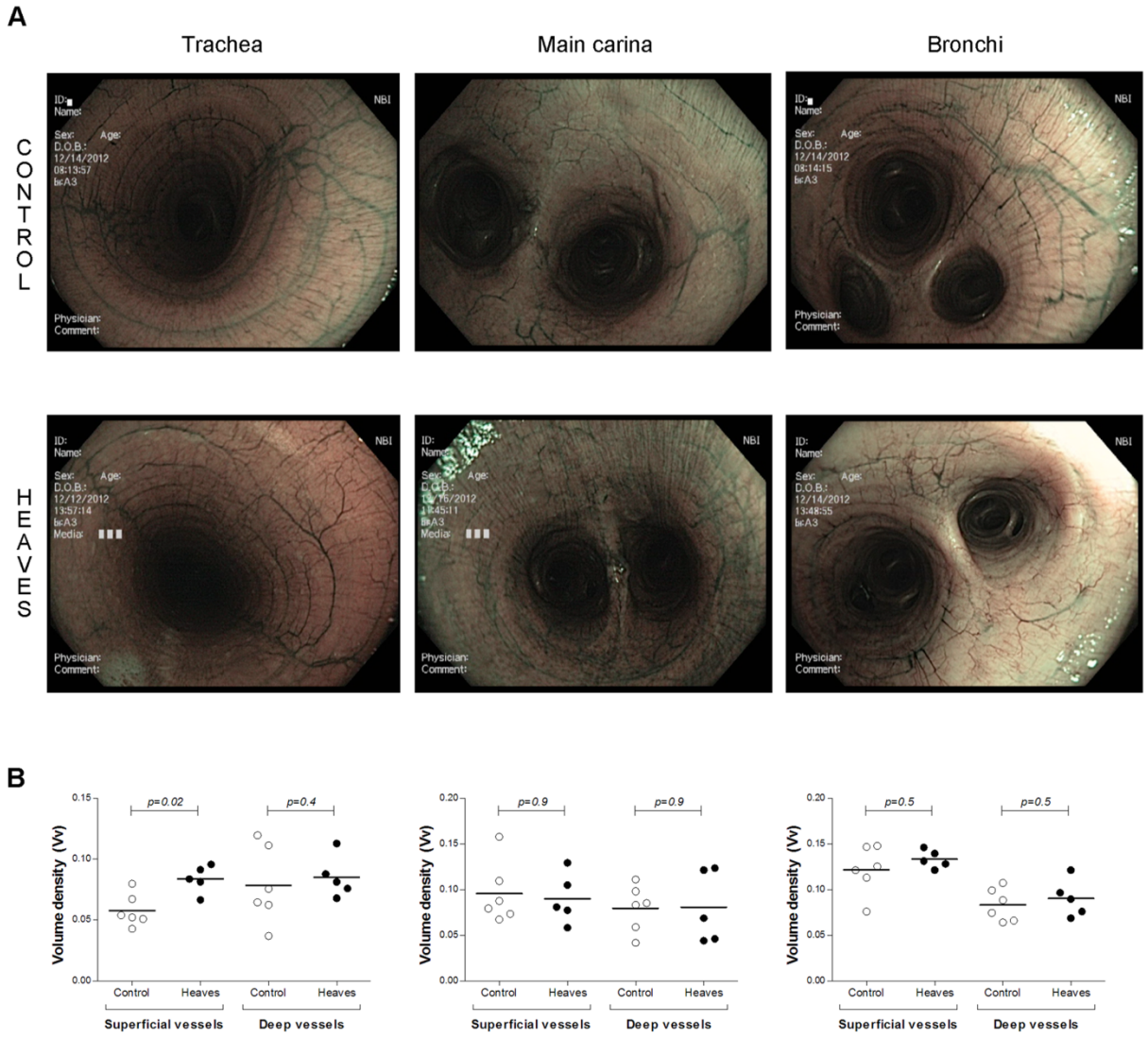

Figure 1: A- Narrow Band Imaging images of the trachea, the main carina and bronchi of control (upper panels) and affected horses (lower panels). B- Vessel area densities in the trachea (left), the main carina (center) and the bronchi (right). Each diagram has been divided in superficial and deep vessels.

Curriculum vitae

Education and training:

- **Université de Montréal** 2012-present
  Ph.D. in veterinary sciences

- **Université de Montréal** 2011-2012
  M.Sc. in veterinary sciences
  Accelerated passage to the Ph.D.

- **University of Liverpool** 2011-2015
  Certificate in Advanced Veterinary Practice (Equine Medicine)

- **Università degli Studi di Torino** 2004-2009
  Doctor in Veterinary Medicine

Stages:

- **Beaufort Cottage Equine Hospital** April 2011
  Externship
  Exning, Newmarket, UK

- **The Liphook Equine Hospital** June 2010
  Externship
  Forest Mere, Liphook, UK

- **University of Cambridge** Winter 2007
  ESOL lectures and examinations – CAE C1 (IELTS 7)
  Cambridge, UK

- **Colorado State University** Summer 2006
  Merck Summer Research Scholar
  Animal Cancer Center, Fort Collins, Colorado

Scholarships:

Université de Montréal Bourse de rédaction – Ph.D.	Fall 2015
Université de Montréal Mobility scholarship (FAPE Funds)	Fall 2105
Université de Montréal Mobility scholarship	Fall 2014
FESP, Université de Montréal Excellence scholarship Ph.D.	Fall 2014
Réseau en Santé Respiratoire du Québec Mobility scholarship	Summer 2014
Université de Montreal 2nd year Excellence scholarship - Ph.D.	Winter 2014
Université de Montréal Excellence scholarship - Ph.D.	Fall 2012
Université de Montréal Scholarship for accelerated passage to the Ph.D.	Fall 2012
FESP, Université de Montréal Excellence scholarship J.A. DeSève - M.Sc.	Fall 2012
Nature and Technology Research Funds, Government of Quebec, Canada Excellence scholarship for Foreign Students (PBEEE)	Summer 2012
Université de Montréal Recruitment scholarship - Ph.D.	Winter 2012
Université de Montréal Recruitment scholarship – M.Sc.	Fall 2011
Université de Montréal Excellence scholarship - M.Sc.	Winter 2011

INPDAP Italia — Fall 2011
Scholarship « Homo Sapiens Sapiens » - M.Sc.

Merck Merial Pharmaceuticals — Summer 2006
Summer Research School Program

EDISU Piemonte — 2004-2009
Excellence scholarship for undergraduate studies

Awards :

Best poster presentation Award - Ph.D. — Fall 2015
Congrès Québécois en Santé Respiratoire 2015
Levis, QC, Canada.

Joan A. O'Brien Research Award — Fall 2015
Veterinary Comparative Respiratory Society (VCRS), 33rd Symposium
Edinburgh, UK.

Joan A. O'Brien Research Award — Fall 2014
Veterinary Comparative Respiratory Society (VCRS), 32nd Symposium
Kennet Square, PA, USA.

« Premio di Laurea » for excellence in undergraduate studies — Summer 2009
EDISU Piemonte

Publications

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