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SUBEPITHELIAL COLLAGEN CONTENT IN THE PERIPHERAL AIRWAYS OF HEAVES-AFFECTED AND CONTROL HORSES

par

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Résumé

Chez les patients asthmatiques, on retrouve un remodelage de la matrice extracellulaire des poumons, caractérisé par une augmentation du collagène ou fibrose de la couche sous-épithéliale des voies respiratoires. Le souffle, maladie inflammatoire chronique des voies respiratoires inférieures des chevaux matures, présente des similarités physiopathologiques avec l'asthme humain, incluant le remodelage. Ceci nous conduit à l'hypothèse que la fibrose de la couche sous-épithéliale pourrait être une composante des lésions pulmonaires chez les chevaux affectés, ce que notre étude avait pour objectif d'évaluer.

Des biopsies pulmonaires périphériques réalisées par voie thoracoscopique ont été obtenues chez 5 chevaux témoins et 6 chevaux atteints du souffle, avant (T0) et après une stimulation antigénique de 30 jours avec du foin moisi et de la paille. Avant le début de l'étude, les sujets étaient en rémission clinique et ne démontraient aucun signe clinique de maladie. Un examen microscopique des échantillons prélevés a été réalisé après traitement au picrosirius-rouge, colorant spécifique des fibres de collagène. La surface du collagène de la couche sous-épithéliale a été mesurée et corrigée en fonction de la taille de la voie respiratoire en utilisant des techniques morphométriques standards.

Les chevaux atteints de souffle ont une surface de collagène plus grande dans la couche sous-épithéliale ($p<0.1$) en comparaison avec les chevaux témoins. La fibrose de la couche sous-épithéliale demeure inchangée chez les chevaux malades après la stimulation antigénique de 30 jours. À T0, la fibrose de la couche sous-épithéliale est associée positivement aux variations maximales de pression pleurale et à la résistance pulmonaire chez les chevaux atteints de souffle.

Les résultats de cette étude suggèrent qu'une fibrose de la couche sous-épithéliale est présente dans les voies respiratoires périphériques des chevaux atteints de souffle et contribue au déficit de fonction résiduel pulmonaire observé lors de rémission clinique.

Mots-clés: Souffle, obstruction récurrente des voies respiratoires, modèle animal, asthme, chevaux, poumons, fibrose de la couche sous-épithéliale, remodelage

Abstract

Extracellular matrix remodelling is present in the human asthmatic lung, and is characterized by increased collagen or fibrosis of the subepithelial area of the airway. Heaves, a naturally occurring chronic lower airway inflammatory condition in horses shares aspects of pathophysiology with asthma, including features of airway remodelling. We thus hypothesize that airway fibrosis is a characteristic of remodelling in heaves. The aim of this study was to evaluate the presence of fibrosis in the subepithelial area of the peripheral airways of heaves-affected horses.

Peripheral lung biopsies acquired under thoracoscopic guidance were obtained from 5 control and 6 heaves-affected horses, both before (T0) and after a 30 day antigenic challenge with mouldy hay and straw. Prior to the study, diseased horses were in clinical remission and exhibited no clinical signs of disease. Obtained samples were microscopically examined using picrosirius-red, a collagen specific histological staining technique. Collagen area in the subepithelial layer, e.g. the region between the airway smooth muscle and the epithelial layer was measured, and corrected for airway size using standard morphometric techniques.

In comparison with controls, heaves-affected horses had an increased collagen content in the airway subepithelial area ($p<0.1$). No change in fibrosis of the subepithelial area was observed in diseased horses after the 30 day antigenic challenge. Peripheral airway subepithelial collagen at baseline was positively associated with maximal changes in transpulmonary pressure and pulmonary resistance in horses with heaves but not in controls.

Results of this study indicate that fibrosis of the subepithelial area is present in the peripheral airways of heaves-affected horses, and may play a role in residual lung function deficits observed in diseased horses even while in clinical remission.

Keywords: Heaves, recurrent airway obstruction, animal model, asthma, horses, lungs, fibrosis of the subepithelial area, remodelling.

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List of abbreviations

AHR: *Airway hyperresponsiveness*

ASM: *Airway smooth muscle*

BAL: *Bronchoalveolar lavage*

col1A2: *collagen type 1 alpha 2*

PL: *Maximal changes in transpulmonary pressure*

ECM: *Extracellular matrix*

EIPH: *Exercise-induced pulmonary hemorrhage*

EL: *Pulmonary elastance*

EMTU: *Epithelial-mesenchymal trophic unit*

FEV1: *Forced expiratory volume in one second*

GAG: *Glycosaminoglycans*

IAD: *Inflammatory airway disease*

Ig: *Immunoglobulin*

IL: *Interleukin*

K: *end expiration transpulmonary pressure*

LPS*: Lipopolysaccharides*

mm*: millimeter*

mRNA: *Messenger ribonucleic acid*

OVA: *Aerosolized ovalbumin*

PEV: *Peak expiratory volume*

Pi: *Epithelial membrane internal perimeter*

P_L: *Pulmonary resistance*

qPCR: *Quantitative polymerase chain reaction*

PLM: *Polarized light microscopy*

RAO: *Recurrent airway obstruction*

RBM: *Reticular basement membrane*

SEM: *Standard error of mean*

SSH: *Suppression subtractive hybridization*

TGF: *Transforming growth factor*

Th2-type: *T helper2-type*

TNF: *Tumor necrosis factor*

T0: *Time point before the start of a thirty day antigenic challenge with mouldy hay and straw*

T30: *Time point at the end of a thirty day antigenic challenge with mouldy hay and straw*

um: *Micrometer*

$\dot{V}$: *Flow rate*

VEGF: *Vascular endothelial growth factor*

V_T: *Tidal volume*

$\bar{x}$: *arithmetic mean*

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INTRODUCTION

Since the antique times, a chronically recurring, debilitating respiratory disease affecting the lower airways of equines has been described in stabled animals. Characterized by symptoms of respiratory distress during exacerbation, horses can nonetheless appear normal at pasture. The disease has been associated with a progressive loss of respiratory function and worsening of symptoms over years. More recently, a better understanding of heaves pathophysiology has revealed similarities with that of human asthma.

Studies at the molecular and cellular level have led to the discovery of genes involved in disease manifestation, further justifying the use of the horse as a model for human asthma. In asthmatics, the architectural changes which occur in the airway structures, collectively known as remodelling, limit the success of therapy. Such remodelling changes are not believed to be reversible and a better characterization of the latter in heaves may help target more appropriate therapies. Evidence suggests that airway remodelling may impair lung function, and lead to permanent deficits in baseline parameters.

Remodelling of the different connective tissues of the lung, defined as the extracellular matrix, has been extensively studied in rodent models of human asthma. An increase in collagen content is one of the well accepted features of extracellular matrix remodelling in these models. However, no studies to date have attempted to quantify these changes morphometrically in a naturally occurring model such as the horse. The objective of our study was the quantification of collagen content in the subepithelial region of the airways in heaves-affected horses and healthy controls. The relationship between collagen content in the subepithelial region of the airway and lung function was also explored.

The study of equine heaves thus serves a dual purpose in advancing scientific knowledge in both the veterinary and human medicine fields.

LITERATURE REVIEW

1. Morphology and histology of the normal equine lung

   a. Introduction

The equine lung is unique in its anatomic and functional characteristics. As opposed to other domestic species, in equines, both the right and left lung are composed of poorly divided cranial and caudal lobes, and the right lung also possesses a small accessory lobe (Parent, 1992). The lobes are divided into lobules, which in horses are well-defined but partially separated by abundant interlobular connective tissue (Tyler, Gillespie, & Nowell, 1971). The lung is protected by thick connective tissue covering its surface called the pulmonary or visceral pleura. This layer is in continuation with interlobular tissue. Interestingly, human lungs share these same morphological characteristics (Magno, 1990; McLaughlin, 1983).

Three portions make up the respiratory system: the air-conducting bronchi and bronchioles, followed by a transitional portion, the respiratory bronchiole, and finally the gas exchange portion, comprised of the alveolar ducts, alveolar sacs and alveoli (Banks, 1986).

   b. Bronchi, bronchioles and the alveolar structure

The airway branching pattern in horses is considered monopodial, i.e. the parent bronchus yields asymmetrical daughter bronchi at different branching angles (Smith, et al., 1994). This is in contrast to human airways, which tend to have a dichotomous branching system, where dividing bronchi are approximately symmetrical is size (Horsfield, Dart, Olson, Filley, & Cumming, 1971).

Air enters the trachea, which at its bifurcation is called the carina, and yields the primary extrapulmonary bronchi (main bronchi). Once inside the lung parenchyma, these airways become the primary intrapulmonary bronchi, which then divide into secondary and tertiary bronchi. Bronchi are airways greater than 2-mm in diameter and are surrounded by cartilage (McGorum, 2007). The bronchus is composed of a pseudostratified columnar

ciliated epithelium interspersed with the mucus-producing goblet cells (Pirie, Pirie, Cranston, & Wright, 1990). The number of goblet cells decreases, and the epithelium gradually changes to simple columnar as the bronchi become smaller. Adjacent to the epithelium and its basement membrane is the lamina propria, a layer of loose connective tissue composed of collagen, elastic fibers, other structural components and leucocytes. Together with the epithelium and its basement membrane, we refer to this part of the airway as the mucosa (Parent, 1992). Adjacent to the lamina propria, bundles of smooth muscle (lamina muscularis mucosa) are discernable and delimitate the beginning of the submucosa. The submucosa is a vast region containing connective tissue, seromucous bronchial glands, blood vessels and nerves. In equines, bronchial glands are not present in the smallest bronchi. Cartilaginous plates can be observed surrounding the submucosa, but are less evident as the bronchi branch and become smaller. The adventia is exterior to this hyaline cartilage and is an array of connective tissue that extends and blends into the lung parenchyma.

Bronchioles are smaller airways devoid of cartilage (less than 2-mm in diameter), which are divided into primary, secondary, tertiary and respiratory bronchioles. This terminology is based on morphological changes which occur as the bronchioles branch and decrease in size. In contrast to the bronchi, bronchioles have no surrounding cartilage and do not possess submucosal glands. A simple columnar epithelium is present in the primary and secondary bronchioles. In the tertiary bronchioles, the epithelium progressively shifts to a cuboidal configuration. Goblet cells are usually not present in the bronchioles of normal horses. Furthermore, ciliated cells become scarce in more distal airways and are absent in tertiary bronchioles. However, non-ciliated cells, also known as Clara cells, increase in the more distal airways (Pirie, et al., 1990). It has been proposed that these cells play an important role in secreting protective substances for the airway epithelium and in detoxification of contaminants (Plopper, Mariassy, & Hill, 1980; Wong, Keating, & Waddell, 2009). The bronchiole epithelium and basement membrane are seated on the lamina propria. This area is composed of a network of collagens, elastic fibers and other constituents which will be detailed further. The lamina propria is then encircled by a smooth muscle layer which contributes to greater airway wall thickness than in the bronchi.

The submucosa and adventia merge together and are difficult to discern due to the absence of dividing cartilage. The submucosa and adventia of the bronchiole are composed of less collagen but contain a greater amount of elastic fibers than the bronchi. They surround the small airways, and are of similar composition to the lamina propria. Respiratory bronchioles are not very developed, if at all, in horses (McLaughlin, Tyler, & Canada, 1961; Tyler, et al., 1971). An ultrastructural study of the distal equine airway describes a sudden junction between the tertiary bronchioles and the alveolar duct, with the absence of a connecting respiratory bronchiole (Pirie, et al., 1990). For the purpose of this paper, the region between the airway epithelium and smooth muscle will be subsequently referred to as the subepithelial layer or area.

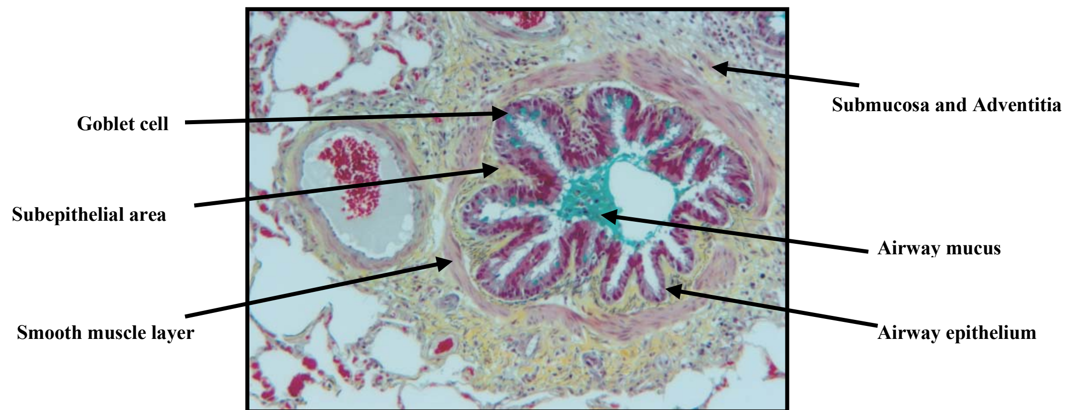


Figure 1 *Equine bronchiole stained with Movat pentachrome histological dye, illustrating the different airway regions*

The alveolar duct is the extension of the respiratory bronchiole, or as previously mentioned, is continuous with the tertiary bronchiole in horses. It is of the same cellular composition as the alveolus and serves as a canal into which the alveoli empty (Tyler, et al., 1971). It subdivides into the alveolar sacs, structures into which alveoli open. Alveoli are hollow structures responsible for gas exchange; they are separated from one another by the alveolar septum, a network of capillaries and some connective tissue. The cells which make up the alveoli are squamous epithelial cells, designated as type 1 pneumocytes, and cuboidal alveolar cells, designated as type II pneumocytes. Finally, alveolar macrophages

can also be found in between squamous epithelial cells and in the alveolar lumen. Pores of Kohn are small holes sometimes present in the alveolar septa; their role is to allow air movement between alveoli. The blood/air barrier is comprised of a thin layer of endothelium which lines the capillary, the basal lamina, and the type I pneumocyte. Type I cells comprise the medium for gas exchange while type II cells have a secretory and regenerative function, including the production of lung surfactant. Type I and II cells lie on the basal lamina and a network of collagen, reticular and elastic fibers (Banks, 1986).

c. Blood supply and innervation of the airway

Blood supply to the bronchioles is provided by bronchial blood vessels which are distributed along the branches of the respiratory tree; upon reaching the end of the tertiary bronchioles, they become the capillary network communicating with alveoli. Arteries closely follow the bronchial passages until the most peripheral airways. However, particular to the equine species, while veins mostly follow the trajectory of the larger airways, they can also be found in the interlobular tissue (Dellaman & Brown, 1976; McGorum, 2007). Similarly to humans, the bronchial artery also nourishes the pulmonary pleura and certain interalveolar septa (Tyler, et al., 1971). It has been proposed that this particularity is to palliate perfusion problems of the alveoli in species with considerable lung parenchyma dorsal to the beginning of the pulmonary artery (Tyler, et al., 1971).

Airway smooth muscle is innervated by visceral efferent fibers which follow the airway passages. These motor nerve fibers arise from thoracic and vagus segments of the sympathetic trunk. The sympathetic nerves are responsible for bronchodilation while parasympathetic nerves cause bronchoconstriction (Dellaman, 1976).

2. The extracellular matrix of the lung

a. Introduction

The connective tissues of the lung are important in maintaining the architecture and function of the bronchi and bronchioles, the pulmonary arteries and veins, the pleura, as well as the interalveolar septum. This connective tissue is composed of cellular elements in addition to extracellular matrix (ECM). The extracellular matrix can be defined as a

complex network of structural proteins intertwined together. Its main components are fibrillar collagens and elastic fibers; however, proteoglycans and structural glycoproteins are also constituents of this complex network. Adhesive molecules such as fibronectin and tenascin can also be temporarily present in the ECM during development or regenerative processes (Chiquet-Ehrismann & Chiquet 2003). Its many important roles include structural support, tissue compartmentalization, and influencing cellular shape, function and interactions. Recent evidence suggests the presence of growth factors, proteinases and proteinase inhibitors in the ECM (Greenlee, Werb & Kheradmand, 2007; Dunsmore & Rannels, 1996). The altered presence and function of these factors could potentially have a role in ECM remodelling observed in respiratory diseases in various species. In the bronchi and bronchioles, the matrix can be divided into the subepithelial matrix, the submucosal matrix, the smooth muscle matrix, the adventitial matrix as well as the cartilage matrix (bronchi only). Finally, the alveolar wall possesses an interstitial matrix located between the fused epithelium and endothelium basement membranes (Parent, 1992).

b. Basement membrane

The basement membrane is a particular form of ECM that is placed between a cellular element, such as the epithelium, and adjacent connective tissue. In the airway, it underlies and supports the epithelium, mediates attachment of cells, and modulates the differentiation of epithelial cells. It is composed of three layers: the lamina lucida, the lamina densa and the lamina fibroreticularis. The term basal lamina, or "true basement membrane", is often used to encompass both the lamina lucida and densa (Payne, et al., 2003). The lamina lucida is the layer closest to the epithelium and is of low electron density; it is bordered by the lamina densa, an electron-dense region. The true basement membrane in humans is composed mostly of collagen type IV, laminin, enactin and proteoglycans (Dunsmore & Rannels, 1996). The lamina fibroreticularis is the layer in between the basal lamina and the adjacent lamina propria. In human asthma, the term subepithelial area or layer is used to designate this particular region of the airway (Roche, et al., 1989).

c. Collagen

Collagen is a vital constituent of the extracellular matrix and is the most abundant fibrous protein present in mammals. It represents approximately 25% of total proteins and is secreted mainly by connective tissue cells such as fibroblasts (Alberts, 2008). Additionally, it can be synthesized by other cell types including smooth muscle cells, pneumocytes and endothelial cells (Parent, 1992). This structural protein is primarily composed of three homologous polypeptide chains, known as α-chains, which are grouped together in a helical fashion. A typical chain is composed of approximately one thousand amino acid residues which are aligned in a unique order. Important amino acids include proline, which serves to stabilize the helix because of its ring configuration, and glycine, a molecule which can inhabit the tight interior of the triple helix because of its small size and thus allow the α-chains to pack firmly together (Alberts, 2008; Last & Reiser, 1984; Minor, 1980).

Single α-chains are produced intracellularly as vast precursors termed pro-α-chains. These pro-α-chains have a collagenous central region and terminal amino and carboxy pro-peptide units. These precursor molecules then undergo transformation; they are submitted to hydroxylation and glycosylation of end chain residues in the rough endoplasmic reticulum of the cell. Subsequently, three single α-chains assemble together to form a triple helical molecule and become procollagen. The procollagen is then secreted via exocytosis outside the cell, where the amino and carboxy propeptides are enzymatically cleaved to yield the collagen molecule, which will assemble with others to form a fibril. Finally, such fibrils may further combine to yield a collagen fiber (Alberts, 2008; Last & Reiser, 1984; Minor, 1980).

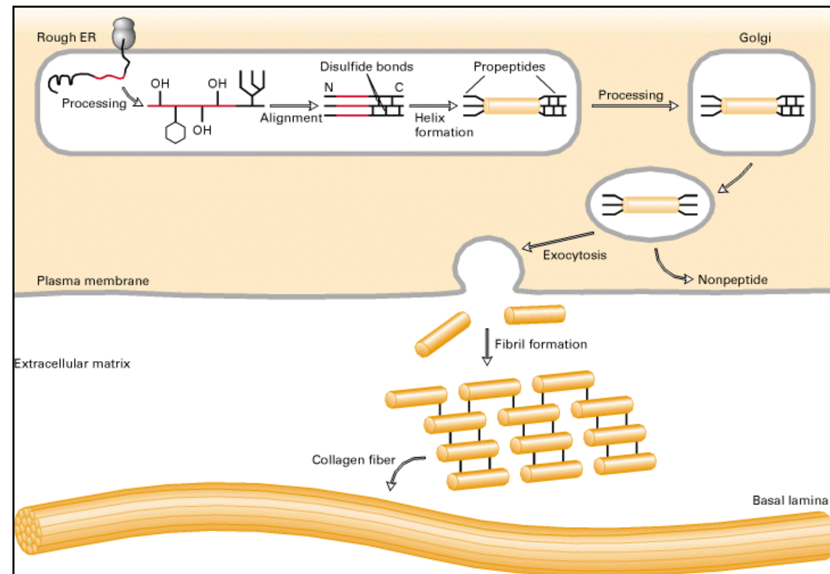


Figure 2 *Schematic representation of collagen fiber synthesis*[1]

Thus far, more than a dozen types of collagen have been identified in connective tissues, in addition to numerous different α-chain configurations (Dunsmore & Rannels, 1996). Collagen types differ according to their charge, hydrophobic nature, the size and location of helical and non-helical domains and other structural properties (Last & Reiser, 1984; Parent, 1992). Collagen fibers can further be divided into fibrillar and non-fibrillar collagens. Fibrillar collagens, such as types I, II and III, correspond to structural proteins initially assembled in a procollagen form as described above. Conversely, non-fibrillar collagens are assembled differently; some types are resistant to proteolytic cleaving and others may be configured as small-chain collagens (Dunsmore & Rannels, 1996).

The amount of collagen in a matrix is finely controlled and rests upon balanced mechanisms of production and degradation. Collagen degradation is performed by

[1] Taken from and authorized by *Berk et al. Molecular Cell Biology "Integrating Cells into Tissues" 22.3 Collagen: The Fibrous Proteins of the Matrix*

activated collagenases, collectively known as metalloproteinases, which are secreted by multiple cell types including fibroblasts and phagocytic cells (Parent, 1992). These specific enzymes cause a disruption of the normal α-chain and allow consequent denaturation of the collagen triple helix by other proteolytic enzymes. Changes in the immediate environment of the cellular ECM constituents can influence collagen degradation in the matrix and lead to abnormal synthesis of a different collagen type (Minor, 1980).

Collagen is a structural protein which provides support to adjacent tissues and acts as a barrier to limit movement of other ECM components. It has the ability to resist tensile forces and limit excessive expansion, and provides a framework for cellular communication (Dunsmore & Rannels, 1996; Minor, 1980). The collagen molecule's tensile strength can be attributed in part to the cross-linking architecture of the fibrils and the physical and chemical interactions with other ECM constituents (Minor, 1980). Collagen breakdown products have also been shown to influence cellular interactions in the ECM. These degradation peptide fragments result from proteolytic cleaving of collagen and can have similar structural features to chemokines and thus imitate their activity (Henson & Vandivier, 2006). A study by Weathington et al. (2006) has demonstrated that the acetylated tripeptide (N-acetyl Pro-Gly-Pro) is chemotactic for neutrophils and can recruit these inflammatory cells in the airways of mice. Furthermore, the peptide could induce remodelling changes in the lungs and heart of these mice if administered chronically by means of airway instillation. Furthermore, the collagen fragment's actions were found to be mediated through a neutrophil CXC chemokine receptor (Henson & Vandivier, 2006; Weathington, et al., 2006).

Collagen in the lung represents a minimum of 60% of the entire connective tissue protein (Parent, 1992). Type I and III collagen are the most abundant collagen types in the lungs of all mammals (Last & Reiser, 1984). These collagen types are profuse in the interstitium of the lung; they are present in the subepithelial, submucosal and advential matrices, as well as the adventia of blood vessels, the alveolar septa, the pulmonary pleura and interlobular tissue (Parent, 1992). An early study on collagen distribution in the lung described type III collagen as being irregularly prominent in the alveolar septa and in the perivascular region of the lung. In this same study, type I collagen fibers were irregularly

dispersed in the interstitium of the alveolar septa (Madri & Furthmayr, 1980). Type II collagen is present in hyaline cartilage, such as found in tracheal and bronchi tissue and is normally absent in other parts of the lung matrix (Parent, 1992). The following table illustrates the anatomic distribution of collagen types in the lung. The subepithelial matrix is thought to possess a similar composition to that of the bronchial submucosa and adventia.

Lung matrices	**Collagen Types**
Bronchial submucosa	I, III,IV, V, VI
Bronchial adventia	I, III, VI
Bronchial hyaline cartilage	II, IX, XI?, VI?
Airway smooth muscle	I, III, IV, V, VI
Alveolar interstitium	I, III, V, VI, IV?

Figure 3 *Distribution of collagen types in the different lung matrices*[2]

In equines, little is known about collagen distribution, type and quantity in the lung, and such information is inferred from other species. One study performed in clinically normal adult racing Thoroughbred racehorses qualitatively assessed collagen content in sections from the caudal region of the lung. Picrosirius-red stain, a histological dye used to color collagen which will be detailed further in this paper, was used to assess the distribution, thickness and relative abundance of collagen in the lung parenchyma and small airways. Results suggested that in clinically normal racing horses, airway collagen in the caudal lung correlated with histological and radiographic alterations compatible with equine exercise-induced pulmonary hemorrhage (Lakritz, et al., 1995). Nevertheless, these findings do not allow us to appreciate regional differences in collagen type or changes pertaining to other respiratory conditions in equines. However, a recent study further assessed collagen distribution in the equine lung (Furness, et al., 2010). Using an immunolabeling method,

[2] *Adapted from and authorized by (Parent, 1992)*

the presence of collagen types I and III was demonstrated in the lamina propria and adventia of small airways in the lung tissue sections of normal horses.

d. Elastin

Elastin is another fibrous component of the ECM. This important structural hydrophobic protein is found in the lung and is present in the airway wall, alveolar wall, and blood vessels. Amino acids that make up elastin assemble intracellularly; they are then secreted into the interstitial space where they further cross-link to one another. Elastic fibers are composed of elastin and microfibrils (fibrillin and micro-fibril associated glycoprotein) (Dunsmore & Rannels, 1996). Owing to their mechanical qualities, elastic fibers have the capacity to stretch and recoil the matrix of the lung (Dunsmore & Rannels, 1996; Starcher, 2000). Elastic fibers located in the alveolar septum allow considerable tissue elasticity in healthy lungs (Pelosi, Rocco, Negrini, & Passi, 2007).

e. Proteoglycans and other ECM constituents

Proteoglycans are part of the non-fibrillar component of the ECM. These macromolecules form a hydrated, gel-like film for the fibrous ECM proteins discussed above. Proteoglycans are comprised of a protein core (polypeptide chain) attached to glycosaminoglycan (GAG) side chains (Iozzo & Murdoch, 1996; Pelosi, et al., 2007). These glycosaminoglycans are heterogeneous polysaccharides and consist of repeating disaccharide molecules. The GAG side chains of proteoglycans can be divided into different classes based on molecular configuration properties such as sugar composition. There are four subclasses: hyaluronan, chondroitin and dermatan sulphate, heparin sulphate, and keratin sulphate (Willams, 2008). Hyaluronan, a small GAG, differs from the other types in that its sugars are not sulphated (Pelosi, et al., 2007; Willams, 2008). Proteoglycans serve various functions in the matrix such as determining water content, resisting compressive forces and influencing matrix assembly. Proteoglycans can bind to TGF-ß, a growth factor, and modulate its capacity to affect cellular proliferation and matrix deposition. Furthermore, proteoglycans such as decorin and biglycan can bind to collagen and thus alter fibril assembly (Pelosi, et al., 2007; Willams, 2008).

It is noteworthy that other adhesive-type molecules, such as fibronectin and laminin, are present in the matrix. These molecules facilitate cellular attachment to the matrix.

3. Heaves (recurrent airway obstruction)

a. Introduction

Heaves, also known as recurrent airway obstruction (RAO) is a lower airway respiratory disease occurring in mature horses which results in chronic airway inflammation and obstruction following exposure to environmental antigens (Robinson, 2001). It is a frequent cause of loss of performance in the equine population. Although horses of various breeds and gender can be affected, a genetic predisposition for developing heaves has been demonstrated in Lipizzaners and Warmbloods (Gerber, 1989). Heaves is a condition typical of the Northern hemisphere, where the cold winter climate limits the use of outdoor pastures year round. Heaves is more likely to be diagnosed during the winter months when horses are housed inside (Couetil & Ward, 2003).

b. Clinical manifestations

In periods of clinical remission, while at pasture for example, heaves-affected horses can appear normal at rest. However, they may still show signs of exercise intolerance and cough while ingesting food or exercising. During the acute phase of the disease, horses with heaves develop varying degrees of respiratory distress. Affected animals may exhibit signs of dry cough, serous to mucopurulent nasal discharge, increased breathing effort and intolerance to exercise. In severe cases, horses can become emaciated and develop a "heave line" due to external abdominal oblique muscle hypertrophy. They may show signs of tachypnea, flaring of nostrils and extension of the head and neck, and perform a double effort at expiration (Beech, 2001). During an acute manifestation of heaves, horses will show a decrease in dynamic compliance and an increase in pulmonary resistance and arterial hypoxemia, and may develop airway hyperresponsiveness (Willoughby & McDonell, 1979). These changes result from periods of acute airway obstruction. It has been repeatedly demonstrated that horses with clinical signs of heaves tend to improve when housed in a low dust environment, such as on outdoor pasture most of the day, and worsen when housed indoors on straw and hay (Grunig, et al., 1989; Lowell, 1964). The

clinical signs of heaves are thus reversible if appropriate environmental modifications and/or pharmacological therapy are used. Heaves needs to be differentiated from inflammatory airway disease (IAD), a condition involving inflammation of the lower airways, but with absence of strenuous breathing while at rest (Couetil, et al., 2007).

c. Etiology and pathogenesis

Chronic airway inflammation is central to the disease process in heaves. It is characterized by the presence of large amounts of neutrophils in the airway (Robinson, 2001). However, the exact relationship between chronic distal airway inflammation and the clinical symptoms observed in heaves is still unclear. Numerous mechanisms can lead to the airway neutrophilia observed in heaves and have a contributing role in disease manifestation.

A non-specific inflammatory response to inhaled moulds, yeasts, bacterial endotoxins, and noxious gases such as ammonia or methane are possible contributors to airway inflammation observed in heaves-affected horses. These pro-inflammatory agents can be found in the horse's immediate environment when stabled indoors. Pirie et al. (2002) showed that beta-glucan, one of the components of the cell wall of moulds and other organisms, could contribute to airway inflammation if present in high quantity in hay-dust suspensions. Heaves-affected horses receiving the hay-dust suspension developed airway neutrophilia and airway obstruction as opposed to controls which developed only mild airway neutrophilia (Pirie, Collie, Dixon, & McGorum, 2002). Furthermore, bacterial endotoxins (LPS) inhaled experimentally can cause a dose-dependent airway neutrophilia in both diseased and control horses; however, airway obstruction is only evident in heaves-affected horses. (Pirie, Dixon, Collie, & McGorum, 2001). Thus, these airborne irritants likely contribute to disease pathogenesis, but other mechanisms are also involved. They are unlikely to be the primary cause of the disease as inhalation of beta-glucan or endotoxins at concentrations normally present in mouldy hay causes only mild airway obstruction in heaves-affected horses.

It has long been proposed that an allergic-type reaction (hypersensitivity) to environmental antigens present in mouldy hay and straw is a more dominant contributor to

disease pathogenesis than the non-specific response briefly described above. It is suggested that inhaled moulds and fungi are the basis of the allergens. The two agents most often involved in the disease process are *Saccharopolyspora rectivirgula* and *Aspergillus fumigatus*, both components of mouldy hay. These agents have the capacity to cause airway obstruction in heaves-affected horses when inhaled, but not in healthy individuals (Derksen, Robinson, Scott, & Stick, 1988). The hypersensitivity reaction leads to airway inflammation which, as previously mentioned, is central to the disease process. In Type I allergic respiratory diseases in humans and other species, a biphasic response can be observed in sensitized individuals. The first is an early phase response which occurs minutes following antigenic exposure; it is mediated by the activation of cells bearing an allergen-specific IgE, such as mast cells. When these cells are activated, pro-inflammatory mediators are released, which causes various responses in the airway and can lead to airway obstruction. The second is a late phase response, occurring six to nine hours after antigenic exposure. It corresponds to the time it takes for neutrophil recruitment and airway obstruction to take place. An earlier study by Fairbairn et al. (1993) revealed that, following antigenic exposure, neutrophils accumulated in the lungs of heaves-affected horses after four to five hours, resembling the late phase allergic response. However, rapid development of airway obstruction is not characteristic of the disease, and a recent study showed that heaves-affected horses nebulized with a hay-straw dust suspension did not develop significant changes in respiratory resistance or bronchoalveolar fluid histamine concentrations in the first twenty minutes of challenge (Deaton, et al., 2007). Therefore, the traditional type I hypersensitivity is not a main feature in the pathogenesis of heaves. It is presently not known whether the late phase response can occur without the initial first phase response.

A type III hypersensitivity reaction follows the deposition of immune complexes in the lung and subsequent complement activation. This type of hypersensitivity reaction requires a period of presensitization to a specific antigen. Horses with heaves have been shown to possess serum antibodies to certain bacteria, such as *Saccharopolyspora rectivirgula* and to certain moulds, such as *Aspergillus fumigatus*. However, these antibodies seem to be associated with the dusty stabling conditions more so than to the disease (Lawson, et al.,

1979; Robinson, 2001). Thus, a type III reaction is unlikely an important part of the pathogenesis.

d. Pathophysiology

Clinical manifestations of heaves result from airway obstruction caused primarily by bronchospasm. Nonetheless, airway inflammation, mucus accumulation, and airway remodelling are also contributors to this phenomenon.

Bronchospasm can be defined as the abnormal contraction of the airway smooth muscle resulting in a narrowing of the lumen. Mediators of inflammation such as histamine (Derksen, et al. 1985), serotonin, and leucotriene D4 have been associated with acute manifestations of heaves (Olszewski, et al., 1999). These mediators act on the specific smooth muscle receptors in the airway and cause tension, contributing to bronchoconstriction (Robinson, 2001). However, they may not play a major role in bronchospasm in naturally occurring heaves per se, since antihistamines and leucotrienes antagonists are not effective at relieving clinical symptoms of heaves (Lavoie, et al., 2002). The bronchospasm observed in heaves-affected horses responds favorably to anticholinergic agents (Broadstone, Scott, Derksen, & Robinson, 1988) and beta 2-adrenergic drugs, (Scott, Berney, Derksen, & Robinson, 1991) strongly suggesting that most of the response must be mediated through the muscarinic receptor. It has also been suggested that a default in the inhibitory regulation of the airway smooth muscle contributes to the bronchospasm (Yu, Wang, Robinson, & Derksen, 1994). However, an altered beta-adrenergic receptor has been excluded as a possible culprit (Abraham, Kottke, & Ungemach, 2007).

As previously mentioned, airway inflammation in heaves involves the accumulation of neutrophils in the airway lumen. When neutrophils are activated, they may release damaging mediators (reactive oxygen species, proteases, nitric oxide etc.) and pro-inflammatory cytokines (TNF-alpha, IL-8 etc.) (Joubert, Silversides, & Lavoie, 2001). For example, the cytokine Interleukin 8 (IL-8) has been shown to be crucial to neutrophil recruitment and is overexpressed in the lungs of heaves-affected horses (Franchini, Gill, von Fellenberg, & Bracher, 2000; Franchini, et al., 1998). Therefore, persistent airway

neutrophilia and associated cytokine release is likely related to the airway obstruction observed, but the exact relationship between the two remains undetermined.

Recent studies also suggest that heaves is associated with a dominating Th2-type cytokine response, as is observed in human asthma. Interleukins IL-4 and IL-5, both Th2-type cytokines, were shown to be overexpressed in mRNA of heaves-affected horses in comparison with controls (Cordeau, et al., 2004; Lavoie, et al., 2001). Furthermore, a study by Dewachi et al. (2006) showed that horses suffering from heaves have a greater number of neutrophils expressing receptors for certain Th2-type cytokines. This suggests that Th2-type cytokines possibly modulate the neutrophilia observed in heaves and thus play an important role in overall pathogenesis. However, these findings are not clearly established as others have failed to associate a Th2-type response with heaves (Ainsworth, et al., 2003).

Cellular interactions being complex, other cell types likely play a role in pulmonary inflammation in heaves. There is increasing evidence that macrophages, epithelial cells, mast cells, smooth muscle cells and others are all potentially involved in modulating airway neutrophilia.

Mucus accumulation in the airways is another feature of the disease. Mucus is secreted by goblet cells that are interspersed in the superficial airway epithelium. It is a feature of most inflammatory lung diseases in horses caused by environmental irritants to the airway (Dixon, Railton, & McGorum, 1995). Interestingly, while airway neutrophilia and bronchospasm can be controlled through environment modification, mucus tends to persist at higher levels than in controls, even during clinical remission (Jefcoat, et al., 2001). Mucus buildup is thought to be caused by increased goblet cell number and/or activity as well as reduction in clearance due to increased viscoelastic properties (Robinson, 2001).

e. Gross pathology

Macroscopic lesions in heaves are inconsistent. Equine lungs affected with heaves may appear normal or pale due to hyperinflation. As a result of hyperinflation, rib imprinting may be visible on the lungs (Beech, 1991). Mucus often lines the trachea and major bronchi in varying amounts depending on disease severity. Tracheobronchial lymph nodes

can be enlarged, and fibrinous pleuritis is sometimes observed (Winder & von Fellenberg, 1988). Hypertrophied secondary respiratory muscles, more prominent bronchioles and an enlarged right ventricle, as a consequence of disease progression, are possible findings (Beech, 1991).

f. Diagnosis and therapeutics

Heaves is usually diagnosed based on signalement, history and clinical findings. Airway neutrophilia can be confirmed with bronchoalveolar lavage cytology (›25% neutrophils present during exacerbation) (Robinson, 2001). The reversibility of the condition can be established by removing horses from the stable and placing them on pasture or by the use of bronchodilators or corticosteroids (Leguillette, 2003; Robinson, 2001).

The most crucial part of a therapeutic or preventative program for a heaves-affected horse involves the control of the environment. Removing the horse from the stable and pasture-boarding it year round is the best way to control airway inflammation and clinical symptoms. Practically, it can take a few days to months before clinical signs at rest are resolved. When it is not feasible to pasture the horse year round, clinical improvement may be obtained by low dust feeding and bedding (Leguillette, 2003).

Corticosteroid therapy is considered the second line of treatment for heaves. Steroidal anti-inflammatory drugs act in many different ways on airway inflammation; they decrease gene transcription of pro-inflammatory cytokines known to be of relevance in human asthma, while also increasing transcription of anti-inflammatory genes (Alangari, 2010). They increase $ß_2$-adrenoreceptor expression, preventing downregulation of these receptors (Barnes, 1997). Thus, they probably allow prolonged efficacy of $ß_2$-agonists. Corticosteroid therapy can be administered systemically or by inhalation. Inhalation therapy reduces side effects; however, it is usually short acting, can become costly and can be difficult to manage for horse owners.

Finally, bronchodilators may be used during acute episodes of respiratory distress to improve pulmonary gas exchange by relaxing the airway smooth muscles. They do not act

on airway inflammation and are thus more of a diagnostic tool or emergency medication (Robinson, 2001) and should not be used as sole therapy.

4. Airway and alveolar changes in heaves

a. Structural alterations

i. Introduction

Airway remodelling can be defined as the altered structure of cells and tissues in the airways of diseased animals in comparison with healthy individuals (Elias, Zhu, Chupp, & Homer, 1999). It is a well-known feature of human asthma and now recognized as also present in the airways of heaves-affected horses, as will be detailed in this section. It has been repeatedly documented that lung sections of heaves-affected horses tend to have noticeably thicker airways involving all the tissue layers than control airways (Herszberg, et al., 2006; LeBlanc et al., 1991).

ii. Alveolar changes

Electron microscopy studies have demonstrated that heaves-affected horses have a reduced number of capillaries and vascular endothelial cells, as well as the presence of an increased number of pores of Kohn in the alveolar lining. Moreover, diseased horses have an increased percentage of type II pneumocytes and decreased type I pneumocytes in their interalveolar septum (Gillespie & Tyler, 1969).

iii. Airway epithelial and goblet cell alteration

Structural alterations in the airway epithelium of heaves-affected horses have been profusely documented in the literature. For clarification purposes, cellular hyperplasia and hypertrophy refer to an increase in cell number and in cell size, respectively. Metaplasia refers to a form of less specialized cell type differentiation in response to chronic tissue injury.

A number of studies have described hyperplasia of epithelial and goblet cells in the airways of heaves-affected horses (Breeze, 1979; Persson & Lindberg, 1991; Winder & von Fellenberg, 1987). Winder et al. (1987) reported that changes in epithelial and goblet cell

structure are already present in horses with mild disease. The hyperplastic and squamous metaplastic changes become more pronounced, occur in a greater number of airways, and are spread more diffusely throughout the lung in heaves-affected horses. In these same horses, goblet cell hyperplasia is highly prominent, affecting the entire bronchial tree, including some of the terminal bronchioles (Winder & von Fellenberg, 1988).

Concurrently, another study involving a post–mortem histological analysis of heaves-affected peripheral airways described hyperplastic changes in the epithelial layer, as well as increased apoptosis and desquamation (Kaup, Drommer, Damsch, & Deegen, 1990). In the same report, goblet cells had evidence of metaplasia, which again appears to be more marked in horses with severe heaves. Other observations included a decrease in the number of Clara cells in mildly affected horses while in more severely affected individuals, Clara cells tended to be substituted by vacuolated cells (Kaup, et al., 1990). This same report described epithelial and goblet cell changes as being more prominent in the caudo-dorsal region of the lung (Kaup, et al., 1990). However, while the above study suggests uneven distribution of remodelling changes in the lung, Lugo et al. (2006) recently showed, using histomorphometric techniques, that no regional differences could be found in relationship to airway inflammation or to remodelling of the epithelium and mucus producing cells in the airways of heaves-affected horses.

The exact mechanism by which these changes occur is unknown; however, as previously mentioned, non-specific airway inflammation can lead to epithelial/mucus cell remodelling. Irritants such as hay or stable dust are likely responsible for the increased number and secreting activity of goblet cells. Goblet cell metaplasia and the subsequent increase in mucus production contribute to a reduced lumen caliber in diseased horses and offer an explanation as to why lung function parameters are still slightly abnormal in horses in clinical remission.

iv. Airway smooth muscle

Airway smooth muscle hyperplasia and hypertrophy in heaves has been described qualitatively (Winder & von Fellenberg, 1987, 1988). Using quantitative morphometric techniques, a study by Herszberg et al. (2006) established that there is a nearly threefold

increase in airway smooth muscle mass (hyperplasia) in the airways of heaves-affected horses in comparison with healthy individuals. This change involved all airways but was most prominent in the smaller ones. The increase in airway smooth muscle mass is concurrent with augmented myocyte proliferation and apoptosis. It has been suggested that the increased apoptosis serves as a compensatory response to airway smooth muscle hyperplasia (Herszberg, et al., 2006). Hypertrophic changes are also probable, as observed in human asthma (Ebina, et al., 1990; Hirst, et al., 2004), yet no quantitative analysis has been performed in heavy horses to date. The modified smooth muscle mass in diseased equines could contribute to airway obstruction by increasing muscle contraction strength and consequently causing narrowing of the lumen.

v. Extracellular matrix

A limited amount of information exists in the literature regarding structural changes in the pulmonary extracellular matrix in heaves-affected horses. Available material on the subject is generally limited to descriptive histopathology reports on heaves-affected horses. Furthermore, changes described are mainly those associated with the alveolar matrix or to the advential matrix peripheral to the bronchioles.

A study reported that horses with generalized radiographic pulmonary patterns may have underlying structural and functional remodelling in the lung (Lakritz, et al., 1995). Some of the changes described are those of altered collagen deposition in the alveolar septa. As horses with heaves generally have a bronchointerstitial pattern (Robinson, 2001) on thoracic radiographs, the afore mentioned study suggests underlying remodelling could be at play. Kaup et al. (1990) reported a correlation between the degree of remodelling in the terminal airways and alveolar region of the lung with clinical severity of disease in heaves-affected horses, suggesting functional consequences of these changes.

An early report by the same author, using brightfield and electron microscopy, reported that fibrosis was present in the alveolar region of moderate to severely heaves-affected horses. There was a marked increase in the number of collagen fibers in regions where inflammatory cells predominated. Lung fibrosis was even seen in less severely diseased animals; it was characterized by focally enlarged alveolar septa, due to an increase in

collagen, elastic fibers, and other connective tissues (Kaup, et al., 1990). Interestingly, even in severely affected horses, some regions of the lung were completely devoid of structural alteration. Similarly, another study also denotes thickening of the alveolar septa in heavy horses due to cellular infiltrates and fibrosis (Winder & von Fellenberg, 1988).

An additional pathology report describes peribronchial and peribronchiolar fibrosis (fibrosis in periphery of the bronchial structure) as present in varying degrees of severity in five out of seven heaves-affected horses examined. Furthermore, in six out of seven controls, it was considered absent (Thurlbeck & Lowell, 1964). Unfortunately, none of these reports provided a quantitative assessment of ECM changes in the lung, and they did not differentiate between different components of the matrix.

b. Comparing heaves with asthma

Asthma is a chronic lower airway inflammatory condition in humans which resembles many aspects of disease etiology and physiopathology with heaves in equines. It is classically characterized as being an eosinophilic type inflammation of the airways associated with an upregulation of Th2-type cytokines (Murphy & O'Byrne, 2010). Airway eosinophilia is not observed in heaves-affected horses; however, as previously mentioned, there is increasing evidence indicating that Th2-type cytokines may contribute to the disease. Interestingly, it is now recognized that neutrophils are also significant contributors to airway inflammation in some subtypes of human asthma (Baines, Simpson, Scott, & Gibson, 2009).

Moreover, human asthma shares similarities in airway remodelling with heaves. Increase in airway smooth muscle (ASM) mass in asthmatics is well-established, and characterized by myocyte hyperplasia and hypertrophy (Pepe, et al., 2005; Tagaya & Tamaoki, 2007). The ASM mass seems to increase concurrently with the degree of asthma severity, and migration of myocytes towards the epithelium follows this same tendency. It is believed that this migration of smooth muscle cells influences airway remodelling (Pepe, et al., 2005). Myocyte migration towards the epithelium lining has not yet been described in heaves.

Epithelial and goblet cell alteration is another contributor to remodelling in asthma. Damaged epithelial cells produce pro-inflammatory mediators, such as TGF-ß, which are likely involved in the remodelling process (Tagaya & Tamaoki, 2007). Goblet cell metaplasia is seen in the airways of mild to severe asthmatics. The consequent increase in mucus production contributes to airway lumen narrowing and obstruction, as observed in heaves.

Reticular basement membrane remodelling or subepithelial fibrosis is a characteristic feature of human asthma (Bai & Knight, 2005; Roche, et al., 1989; Tagaya & Tamaoki, 2007). The "true basement membrane" is unchanged in asthmatics (Roche, et al., 1989), while the reticular basement membrane (RBM) is significantly thickened. This thickening is characterized by an increase deposition of collagens types I, III and V, as well as other ECM components such as tenascin, laminin and fibronectin (Payne, et al., 2003; Tran & Halayko, 2007; Wilson & Li, 1997). RBM thickening is not a feature described in heaves, as the basement membrane of equine airways cannot be properly visualized under brightfield microscopy. However, the lamina propria (area adjacent to and often merging with the reticular basement membrane) can be visualized in equine airways and evaluated with morphometric techniques.

In asthma, remodelling changes in the subepithelial area are thought to arise as a consequence of prolonged airway inflammation and are believed to be partially responsible for eventual irreversible obstruction in patients with severe asthma (Nakagawa T, 2004). Studies have demonstrated that an increase in the degree of subepithelial fibrosis correlates with an increase in the severity of asthma (Benayoun, et al., 2003; Chetta, et al., 1997). However, others failed to identify a significant correlation between gravity of disease presentation and remodelling (Chu, et al., 1998; Pepe, et al., 2005).

It has been proposed that RBM thickening serves as a protective mechanism for the airway by increasing the latter's capacity to resist bronchoconstriction and thus decrease excessive narrowing (Lambert, Codd, Alley, & Pack, 1994; Niimi, et al., 2003). Nonetheless, subepithelial remodelling could also have a detrimental effect by increasing

airway wall stiffness and disturbing the dynamic balance between compressive and tension forces on the airway, leading to its collapse (Bento & Hershenson, 1998).

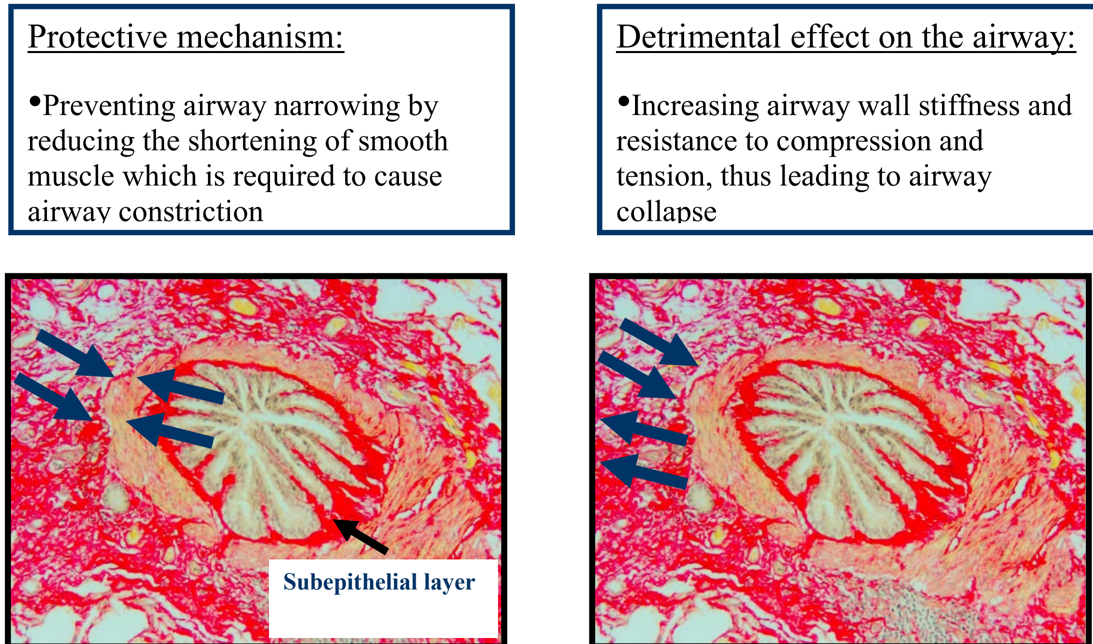


Figure 4 *Possible impacts of subepithelial remodelling on the airway lumen caliber*

Research further suggests that ECM has a role in modulating the functional properties of the airway smooth muscle cells. In an *in vitro* study, ASM cells were adhered to ECM constituents to assess the mechanical response of the ASM cells to these matrix constituents. Type I collagen caused an increase in the contractile strength of the ASM cell while response to other matrix constituents was different (Rodriguez, et al., 2009). This study corroborates that ECM remodelling influences other processes in the airway.

Many cytokines, growth factors, and other mediators have been shown to have fibrogenic properties and are likely linked to airway remodelling in asthma. TGF-ß, Th2-type cytokines such as IL-5, -9 and -13, and VEGF have been associated with remodelling changes (Doherty & Broide, 2007). TGF-ß, a cytokine secreted by mesenchymal cells (fibroblasts, vascular and airway smooth muscle cells) and inflammatory cells (eosinophils) has been shown to play a crucial role in human asthma (Halwani, Al-Muhsen, Al-Jahdali, & Hamid, 2011). Indeed, the TGF-ß1 isoform has an increased expression in the airways of

asthmatics compared to controls (Minshall, et al., 1997). Increased concentrations of the latter have been associated with subepithelial collagen deposition (Makinde, Murphy, & Agrawal, 2007). Among its various functions, TGF-ß can induce the proliferation of fibroblasts and subsequent production of ECM components (Makinde, et al., 2007). As well, TGF-ß has the capacity to reduce collagenase production, thus altering normal collagen degradation in a matrix (Doherty & Broide, 2007). Nonetheless, a study comparing total TGF-ß1 isoform concentrations in bronchoalveolar lavage fluid samples between heaves-affected and control horses yielded no appreciative difference (Desjardins, et al., 2004). However, until its lung tissue expression has been evaluated, the contribution of TGF-ß in airway remodelling in heaves will remain uncertain.

Since subepithelial changes in collagen content are important in overall pathogenesis of human asthma, we hypothesize that airway ECM remodelling will also be present in heaves. In support of this hypothesis, a study performed by our research group has shown, using suppression subtractive hybridization technique (SSH), that collagen type 1 alpha 2 (col1A2) is over expressed in the heaves-affected horses. Furthermore, upregulation of this gene was confirmed using qPCR (Lefebvre-Lavoie, Lussier, & Lavoie, 2008). An increased collagen content in the airway wall of heaves-affected horses would be a likely contributor to persistent airway function deficits in heaves, even after appropriate therapy with outdoor management and corticosteroid use.

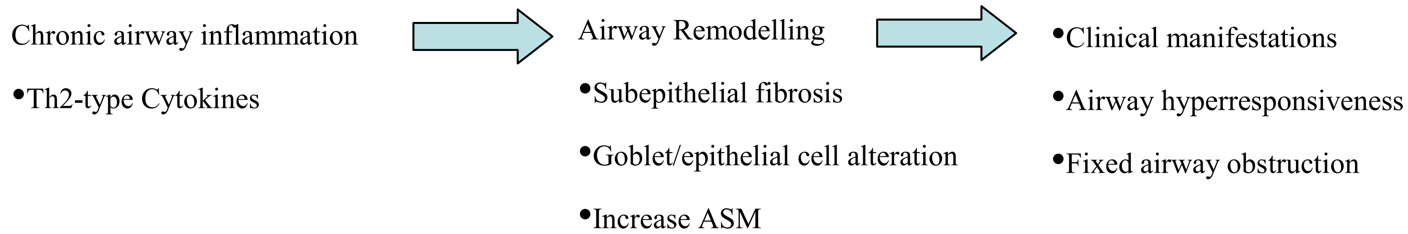


Figure 5 *Possible causative associations between airway inflammation, remodelling and disease manifestation in asthma*[3].

5. ECM histomorphometric techniques

a. Introduction

Histological sections can be qualitatively described for changes in patterns or structure of tissues. Such changes can be subjectively evaluated using a designed scoring system, based on severity of changes for example. However, spatial distribution and density of components are harder qualities for a person to subjectively assess. Quantitative measurements of histological sections are thus necessary to better appreciate the latter tissue characteristics. Such an evaluation has the advantage of being more objective and more easily reproducible by another evaluator. Quantitative measures can further be correlated to physiological and biochemical data, such as lung function parameters and inflammatory cell populations. The term "morphometry" can be used to designate a quantitative assessment of a given tissue component (Weiber, 1979).

For histomorphometric studies, lungs must be carefully removed from the cadaver during necropsy to avoid lacerations of the visceral pleura, which could result in tissue damage (Dunnill, 1962). Post-mortem lungs are partially collapsed and hold little air, resulting in an unnatural microscopic appearance. Thus, re-expanding the lungs to approximate ante-mortem dimensions is desirable; this will mimic the intrapleural pressures present when the animal was alive. This can be achieved by inflating the lungs with gas and fixing them in formalin. Inflation by liquid infusion is also described but

[3] *Adapted from and authorized by "Airway Remodelling in Asthma", Jack A. Elias, 2000*

results in greater artifacts than a gas instillation system (Dunnill, 1962). Once expanded and fixed, lung sections can then be cut processed and preserved in paraffin to be evaluated at a future date (Weibel & Vidone, 1961).

b. Staining protocols

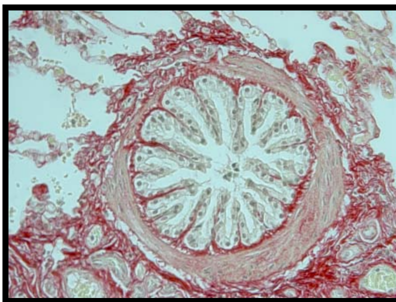
Numerous stains have been described in the literature for highlighting collagen and elastic fibers in various tissues. The considerable size of both these ECM proteins in addition to the weight of their molecular content allows a successful reaction to take place with the dye, leading to a useable scale for histological interpretation. These staining protocols are of use in the evaluation of fibrous ECM components. However, they are less efficient for non-fibrous ECM constituents, given that their components can be accidentally removed while processing the given tissue or are often in too small quantity to be detectable (Parent, 1992).

c. Collagen staining

i. Picrosirius-red

The need for more accurate collagen staining techniques resulted from the lack of specificity offered by traditional stains such as van Gieson and Masson's trichome. Both these stains rely on a poorly-understood mechanism of differential binding, determined in part by size and structure of molecules. In addition to having a lower specificity, these stains lack the ability to color thinner collagen fibers, resulting in an inaccurate estimation of collagen content in tissues (Whittaker, Kloner, Boughner, & Pickering, 1994). The discovery of the picrosirius-red stain resulted from the work of Sweat et al. (1964). This research group showed that the Sirius red F3BA (color index 35780) could dissolve in a saturated solution of picric acid and repeatedly stain thinner collagen fibers. As opposed to the van Gieson and trichome methods, the stain does not fade out and can be used under polarized light microscopy (Sweat, Puchtler, & Rosenthal, 1964). The picrosirius-red stain occurs through a reaction between the sulphonic acid groups of the dye and the basic amino groups of collagen at a low pH. Picric acid is required in order to obtain selectivity for collagen fibers although the mechanism resulting in the latter is unknown (Junqueira, Bignolas, & Brentani, 1979).

Picrosirius-red stain has been extensively used for histological analysis of collagen fibers in various organs and diseases. Myocardial collagen in rats (Whittaker, et al., 1994), dermal collagen in humans (Constantine & Mowry, 1968b), lung collagen in idiopathic interstitial pneumonia in people (Tzortzaki, et al., 2006) and thyroid neoplasms (Koren, et al., 2001) are examples of tissues or conditions for which the stain proved appropriate in histological evaluation. Furthermore, in lung tissue of asthmatics (Chu, et al., 1998) and in a murine model of the same disease (Corbel, et al., 2003), picrosirius-red was used successfully to quantitatively evaluate collagen content (subepithelial fibrosis). Finally, it has proved of use in evaluating lung collagen distribution (Lakritz, et al., 1995) and its morphometric assessment in horses with exercise induced pulmonary hemorrhage (Derksen, et al., 2009).



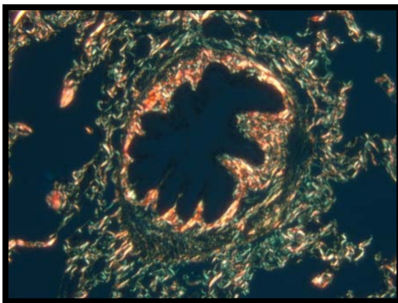
- Nuclei – BLACK if stained (may be brown or grey)
- Collagen – RED on pale yellow background
- Cytoplasm – YELLOW

Figure 6 *Picrosirius-red staining under brightfield microscopy (Kiernan, 2007)*

ii. Polarized microscopy

Collagen fibers can be visualized under polarized light due to their birefringence properties. Birefringence refers to the separation of light into two waves traveling at different speeds as it hits an object (Inoue, 2002). The colors (bright orange, yellow, red and green) observed under polarization are caused by a phenomenon called retardance. It corresponds to the "phase difference suffered by the two components of the orthogonally polarized light in passing through the crystal", or in the latter case, a collagen fiber (Inoue, 2002).

The combined use of the picrosirius-red staining method and polarized light microscopy for the visualization of collagen fibers was first mentioned in the literature by Mowry et al. (Constantine & Mowry, 1968a). In the late 1970s and early 1980s, two Brazilian research groups further suggested this coloration be considered collagen specific with the use of polarized light (Junqueira, et al., 1979; Vidal, Mello, & Pimentel, 1982). It was initially proposed that differences in color under polarized light corresponded to collagen types and thus allowed differentiation between types I, II and III (Junqueira, Cossermelli, & Brentani, 1978). However, the same author later determined that difference in polarization colors was due to fiber thickness rather than type (Junqueira, Montes, & Sanchez, 1982). Dayan et al. further established that the degree of packing of collagen molecules and fiber orientation also influenced the polarization colors microscopically observed. For example, this group demonstrated that more thickly-packed collagen molecules with better alignment exhibited a polarization color of a longer wavelength (Dayan, Hiss, Hirshberg, Bubis, & Wolman, 1989).



- Large fibers collagen – BRIGHT YELLOW or ORANGE (red) birefringence
- Thin fibers collagen (including reticular fibers) - GREEN birefringence
- Some materials including Type 4 collagen in basement membranes, keratohyaline granules, and some types of mucus are stained RED but are not birefringent

Figure 7 *Picrosirius-red staining under polarized light microscopy (Kiernan, 2007)*

d. Elastin and proteoglycan staining

Elastin, another important large structural protein of the extracellular matrix of the lung can be evaluated by use of several stains, including Movat pentachrome. This histological stain will color elastic fibers black or dark purple and allow their visualization under brightfield microscopy (Parent, 1992; Russell, 1972). Our research group has used the

Movat pentachrome stain successfully for elastic fiber visualization in the airways of heaves-affected horses (Lavoie, unpublished data).

Alcian blue can be used as a histological stain for the evaluation of proteoglycans, GAGs and mucosubstances in a matrix (Parent, 1992). Using this dye, proteoglycans will appear blue under brightfield microscopy while other tissue elements will appear red to pink. pH of the solution can be modified to allow more specificity for the polysaccharides put into evidence (Lev & Spicer, 1964). The reaction is based on an electrostatic attraction between the positive dye and the polyanionic glycosaminoglycan chain (GAG's) (Lev & Spicer, 1964).

MATERIALS AND METHODS

All experimental procedures were performed in accordance with the guidelines of the Canadian Council for Animal Protection. The project was further approved by the Animal Care Committee of the Faculté de Médecine Vétérinaire of the Université de Montréal. The experiment conducted for the subject of this paper was part of a larger research program pertaining to the roles of airway inflammation and remodelling in heaves and their possible reversibility. Procedures detailed in this section are those pertaining to this particular project on fibrosis of the subepithelial area.

1. Animals

Eleven horses belonging to the research herd of the University of Montreal's "Laboratoire de biologie cellulaire et moléculaire respiratoire" were used for this project. Six individuals were affected with heaves while the other five were healthy controls. The mean weight for all the horses was 486 ± 50.5 kg (445-555 kg). The median age for the controls and heaves-affected horses were, respectfully, 15.5 ± 0.8 yrs (11-22 yrs) and 16.8 ± 0.9 yrs (15-19 yrs). Repartition by sex was as follows: the five controls and four heaves-affected horses were mares, while the two others were geldings. Repartition by breed was as follows: five horses were Standardbreds, two were Quarter horses, one was an Arabian cross, and all others were mixed-breed. Horses affected with heaves had a history of chronic respiratory disease and were intermittently symptomatic for a minimum of three years prior to the study. Respiratory function test measurements were abnormal and bronchoalveolar lavage (BAL) fluid cytology revealed airway neutrophilia (›25%) in the diseased horses. Control horses had a history free of respiratory disease. All horses admitted to the project were vaccinated and dewormed on a regular basis. Prior to the start of the study, the subjects were trained to stand in stocks quietly and tolerate masks covering their noses and placement of naso-oesophageal catheters and balloons.

2. Clinical examination and respiratory scores

During the experimental phase of the project, horses were assessed daily for appetite, manure production and general comfort. A brief clinical exam was performed every

morning, and the following parameters were recorded: mucous membrane color and moisture, temperature, pulse and respiratory rate. Respiratory difficulty was evaluated by a clinical score based on a total score of 8 points, performed twice daily. The total score comprises two components: nasal flaring (1-4) and abdominal effort on respiration (1-4). The use of this clinical score has been validated in the literature (Robinson, et al., 2000).

Abdominal expiratory effort	**Score**
Clinically normal	1
Slight abdominal movement	2
Moderate abdominal movement	3
Severe and substantial abdominal movement	4

Nostril Flaring	**Score**
Clinically normal	1
Slight and infrequent nostril flaring	2
Moderate nostril flaring	3
Severe and continuous flaring for each breath	4

Total clinical score	**Score**
Clinically normal	2
Mild respiratory signs	3 or 4
Moderate respiratory signs	5 or 6
Severe respiratory signs	7 or 8

Table 1 *Description of the respiratory clinical scores performed on the subject horses during the experimental phase of the project*

3. Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed on sedated horses standing in stocks by a method previously described (Leclere, et al., 2010). A 2.5 meter bronchoscope (Olympus Medical Systems corp., Tokyo, Japan) was inserted through the medial nasal meatus to access the upper respiratory tract. The bronchoscope was oriented in the main bronchi where two, 250-mL boluses of isotonic saline were instilled. BAL fluid was then collected in siliconized glass vessels, and placed on ice. Following filtration through sterile gauze,

the BAL fluid was centrifuged (1500 rpm at 5 °C for 5 minutes) and cell pellets were re-suspended (RMPI 1640 culture medium). Analysis of fluid was performed within one hour of sampling. A hemocytometer was used to establish total cell counts on fresh lavage samples. Differential cell counts obtained with a cytospin slide were stained with Wright-giemsa and Toluidine blue. A minimum of 400 cells per specimen were counted under oil-immersion microscopy.

4. Lung function measurements

Respiratory mechanic function tests were performed on unsedated horses standing in stocks. A heated pneumotachograph[4] was used to obtain flow rates of inspired and expired air. The pneumotachograph was linked to a differential pressure transducer[5]. Electronic integration of the flow signal as a function of time yields the tidal volume (VT). Before recording measures, system calibration was performed by submitting the pneumotachograph to known flow rates. Difference in transpulmonary pressure (PL) is calculated using a differential pressure transducer[6] which allows esophageal pressure to be subtracted from atmospheric pressure. Esophageal pressure was measured by placing a tied balloon around a naso-esophageal polyethylene catheter which was then placed in the distal third of the esophagus. Before measures were taken, the balloon was distended with air to check for leaks. The placement of the esophageal balloon was adapted for each horse in order to avoid cardiac interference and to obtain the greatest possible variation of pressure between inspiration and expiration. The most favorable position was recorded and used for further tests. A water manometer was used for pressure transducer calibration prior to measurements.

Once equipment preparation was set, ninety second breathing sequences were recorded. If the horse moved or swallowed during the sequence, it was repeated to record at least ten good breaths. Amplified transducer signals cross a digital converter and are sent to a

[4] Fleisch No. 4, Oern Medical, Richmond, Virginia, USA
[5] Model 143PC03D, Micro switch, Honeywell, Scarborough, Ontario, Canada
[6] Model HCXPM005D6V, Sensor Technics, Newport News, Virginia, USA

computer system with data acquisition software[7] to obtain measurements. Signal frequency was 120 Hz during measurement recordings. Pulmonary resistance (R_L) and elastance (E_L) values were deduced from applying the following formula:

$$P_L = (E_L \times V) + (R_L \times \dot{V}) + K$$

$\dot{V}$: Flow rate

V : Volume

K : End expiration transpulmonary pressure

The determination coefficient of the above formula was calculated for each respiration.

5. Thoracoscopic guided peripheral lung biopsy procedure

Two thoracoscopic guided lung biopsy procedures were performed on each horse, with a thirty day interval, using a method previously described (Relave, et al., 2008). In brief, horses were secured in standing stocks and premedicated with intravenous detomidine HCl (6µg/Kg) and butorphanol (0.02mg/kg). Detomidine was administered throughout the procedure at a continuous infusion rate of 0.8 mg/kg/min for the first fifteen minutes and decreased by half every fifteen minutes from then on. On each procedure day, a thoracoscopy was performed on a control and heaves-affected horse. Biopsies were obtained from the same hemithorax, selected at random, on the given day. The other hemithorax was used for the second thoracoscopy thirty days later. Aseptical surgical preparation and local anesthetic infiltration (2% lidocaine) were completed prior to the thoracoscopic procedure using a pre-tied ligating loop. Post-operatively, intramuscular procaine penicillin (22,000U/kg) and intravenous phenylbutazone (2.2mg/kg) were administered to the horses; this was continued twice a day for two additional days. Horses were visually monitored for twenty four hours in their stalls before being returned to the pasture. Clinical exams were then performed as follows: at two and twelve hours post surgery, BID for the next two days, and SID for the following five days. Radiographic

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[7] Anadat and Labdat 5.1, RHT-Infodat, Montreal, Qc, Canada

examination of the thorax was also performed pre and post operatively to assess for the presence of pneumothorax or other pulmonary complications.

6. Experimental protocol

For three months before the study began, the horses were kept on pasture in an antigen poor environment to induce disease remission (antigen withdrawal period). In addition to access to grazing pasture, the subjects were fed a pelleted hay ration and sweet feed twice a day. During this time, the heaves-affected horses were in clinical remission and exhibited no signs of respiratory disease. Lung function tests and bronchoalveolar lavage were performed near the end of the three month antigen withdrawal period; these were followed, seven to ten days later, by the thoracoscopic guided lung biopsy procedure.

After recovery from the surgical procedure, a 30-day antigenic challenge was started. During this period, horses were housed in a dusty stable environment. They were bedded on straw, fed timothy hay, and received sweet feed twice a day. In addition, dusty and mouldy hay was shaken in front of the horses' faces for three minutes every morning. Heaves-affected horses developed signs of airway obstruction that were manifested by flaring of the nostrils and an abdominal component to respiration. Controls remained normal and showed no signs of respiratory disease during this period. Lung function tests, bronchoalveolar lavage and thoracoscopic biopsy procedures were repeated (seven to ten days apart) at the end of the thirty day antigenic challenge (T30). At T30, all heaves-affected horses had a clinical respiratory score $\geq$ 6. Histomorphometric assessment of subepithelial area collagen deposition of the T0 and T30 peripheral lung biopsies was then performed at an ulterior date.

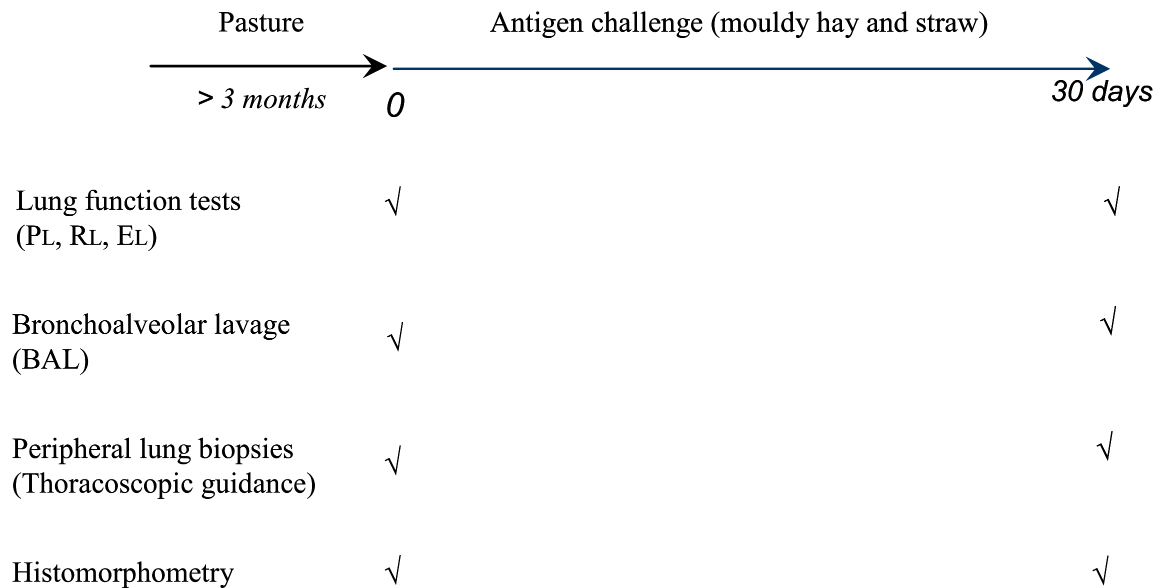


Figure 8 *Schematic representation of the experimental protocol*

7. Picrosirius-red staining procedure

Peripheral lung tissue biopsies were soaked in 10% neutral-buffered formalin for 24 hours before being embedded in paraffin and mounted in blocks according to standard procedures. Using a microtome, they were then cut to a five µm thickness and mounted on standard microscope slides. Slides were then incubated at 37°C overnight to allow proper tissue adhesion to the slide. The picrosirius-red coloration was performed to specifically stain collagen fibers (Sweat, et al., 1964). A pre-digestion step with 0.5% papain solution was performed in order to remove proteoglycans and permit the stain to better penetrate collagen fibers, following the protocol used by Lecocq et al. (2008) (Annexe A).

8. Histological examination

Assessment of all histological sections was done by the same observer, who was blinded as to which group the horses belonged to and the time period being evaluated. Picrosirius-red stained lung tissue sections were evaluated under brightfield microscopy, with the use of a Leica DM4000B polarized light microscope (PLM). All lung tissue on the slide was carefully screened under the microscope field at 5X objective magnification for

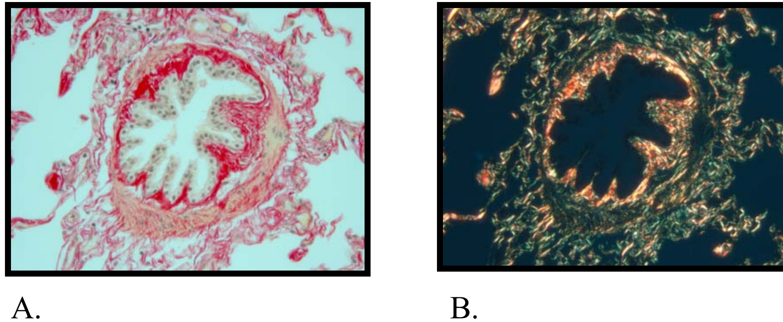
recognizable peripheral airways. Once located, the selected airway was centered and the microscope magnification strength was adjusted (10X or 20X), depending on the airway size. The airway was positioned to take up most of the microscope field. If the airways responded to the criteria described below, they were digitalized with a Leica DFC320 video camera, mounted on the microscope.

Stain coloration	Smooth muscle	Internal perimeter of epithelium	Airway size	Airway angle
Picrosirius-red stain had to be successful in that collagen fibers appeared red and contrast was apparent with other tissue elements	Minimum of 50% of airway smooth muscle had to be present around subepithelial layer	Intact enough to allow perimeter measurement using tracing tool	Airways that could be digitalized with a 10X or 20X objective magnification strength	Airways offering a close to perpendicular cross-section

Table 2 *Inclusion criteria for digitized airways used for morphometric analysis*

To exploit collagen birefringence properties, peripheral airways were further analyzed under polarized light. Prior to digitalizing images, extinction on a black background was performed with the two cross polarizer filters (polarizer and analyzer) equipped with the microscope. These two filters are placed at 90 degrees from each other. The polarizer polarizes light as it traverses the tissue, while the analyzer limits other interfering light sources. A digitized image of a stage micrometer[8] at 10X and 20X magnification was also taken in order to calibrate image size when using image analysis software.

[8] Fisher Micromaster Microscope Stage Micrometers; 2mm with 0.01mm division



A. B.

Figure 9 *A. Normal peripheral equine airway stained with picrosirius-red. The subepithelial collagen area is stained red and is discernable from the surrounding smooth muscle layer. B. Same airway under polarized light.*

9. Image analysis

Digitized airway images were transferred to the computer software program MBF Image J® (National Institutes Health)[9]. The threshold colour Plugin[10] function was added to the software to allow for more specific subepithelial area collagen selection. A known distance on the stage micrometer was recorded (2 mm) for the given magnification and set into the software for automated calibration in µm units. Using a designed protocol (Annexe B), subepithelial collagen (stained red) was subtracted from the rest of the image and its area (µm²) was measured and recorded. The internal perimeter of the airway epithelium (µm) was also measured using the software tracing tool. The collagen area in the subepithelial layer was then corrected for airway size using a standard correction factor (James, Pare, & Hogg, 1988).

Corrected collagen = subepithelial collagen area (µm²) /internal perimeter (µm) ²

The internal perimeter of the airway has been demonstrated as a reliable marker of airway size and stays constant regardless of changes in degree of smooth muscle shortening or relaxation (James, et al., 1988); it was thus used to adjust for airway size. The same background correction factor was also applied to all images to facilitate subepithelial area and smooth muscle differentiation.

[9] Image J®, McMaster Biophotonics Facility Image J for Microscopy version 12.41, National Health Institute
[10] Threshold_colour function Plugin,, designed by G. Landini

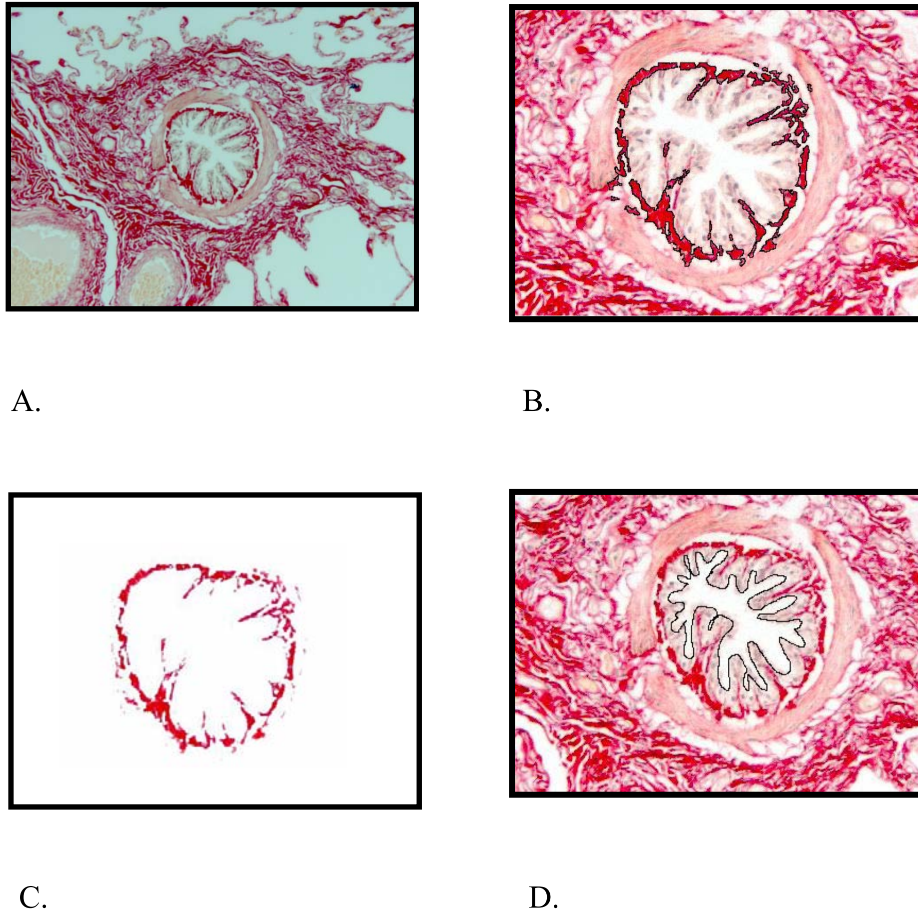
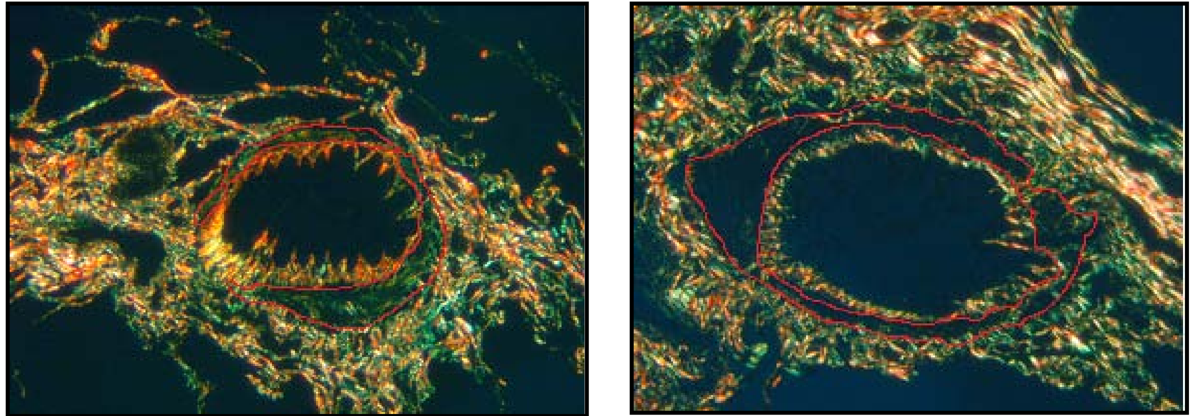


Figure 10 *Steps for image analysis of picrosirius-red stained peripheral airways. A. Picrosirius-red stained airway. B. Background correction factor applied and subepithelial area collagen selected with a black colored tracing tool. C. Subepithelial area collagen is extracted from the image and area can be measured. D. Internal airway epithelium perimeter is hand traced with a black colored tracing tool and measured.*

10. Polarized light microscopy assessment

All digitized picrosirius-red stained images under polarized light were printed on standard paper. They were visually sorted by an observer, who was blinded as to which group of horses the airways belonged to. Airways were subjectively assessed for differences pertaining to collagen fiber spatial distribution or regional changes in the polarized colors. Quantification of collagen fibers by color assortment was attempted using

the threshold color Plugin with MBF image J®. However, due to large varieties of shades in the different polarization colors, proportioning their distribution in the subepithelial layer of the airway was not possible. Subjective evaluation of the collagen fibers in the muscle layer was more feasible, as its delimitation from the other structures was easier to establish because of its dark appearance under polarized light. Images were divided in two categories based on presence or absence of collagen fibers in the smooth muscle layer. Presence of collagen fibers meant that the airway smooth muscle, which would normally appear black under polarized light (no birefringence properties), was interspersed with collagen fibers (bright green, yellow or orange). Absence of collagen fibers meant that the airway smooth muscle remained black or had very small amounts of collagen fibers interspersed within it.



A. B.

Figure 11 *Picrosirius-red stained airways under polarized light were divided into two categories based on presence or absence of collagen fibers in the airway smooth muscle. A. The ASM is encircled in red. The presence of green birefringent collagen fibers can be observed. B. The ASM is encircled in red. A dark appearance and absence of brightly colored collagen fibers is noted.*

11. Statistical analysis

A two way ANOVA was used for the statistical analysis of the respiratory function parameters, BAL total cell counts (%) and neutrophil cell count (%). Data were considered

significant if $p<0.05$. Statistical analysis was performed with Prism 5 (GraphPad software, La Jolla, Ca, USA) analysis software.

A repeated measures ANOVA was used for the statistical analysis of the airways' collagen content (Collagen area/ perimeter²). Mean collagen content was calculated for every horse with time (within-subject) and groups (between-subject) main effects. Data were considered significant if $p<0.1$. Statistical analysis was performed with the Super ANOVA (Abacus Concepts, Berkeley, Ca, USA) analysis software.

A two-sided Fisher exact test was used for analysis of the qualitatively assessed collagen content in the airway smooth muscle of picrosirius-red stained airways under polarized light in heaves-affected and control horses. Results were considered significant if $p<0.05$. Statistical analysis was performed with Prism 5 (GraphPad software, La Jolla, Ca, USA) analysis software.

Associations between measures of pulmonary function (R_L, E_L and P_L), BAL total cell counts (%), neutrophil cell count (%) and mean collagen content were measured by a least squares linear regression and the coefficient of determination R^2. A p value inferior to 0.1 was considered significant. Prism 5 (GraphPad software, La Jolla, Ca, USA) analysis software was used.

RESULTS

1. Picrosirius-red stained equine peripheral airways

The picrosirius-red histological dye was successful in staining peripheral equine airways obtained by the thoracoscopic guided lung biopsy procedure. Subepithelial area collagen stained red, could be extracted from the image and its area measured. For the controls (n=5) and heaves-affected horses (n=6), respectively, fifty six (range 6-15 airways per horse) and fifty eight (range 4-15 airways per horse) peripheral airways were assessed with histomorphometric techniques.

Horse	Group	Number of sampled airways at T0	Number of sampled airways at T30
1	Control	4	2
2	Control	9	6
3	Control	7	5
4	Control	7	7
5	Control	6	3
6	Heaves	10	5
7	Heaves	7	7
8	Heaves	3	1
9	Heaves	5	7
10	Heaves	5	4
11	Heaves	2	2

Table 3 *Peripheral equine airway repartition between heaves-affected horses and controls, divided between T0 (antigen withdrawal) and T30 (antigen exposure) assessment periods.*

2. Descriptive observations of picrosirius-red stained airways under brightfield microscopy

Subepithelial area collagen distribution was very heterogeneous among the digitized picrosirius-red stained airways in both groups of horses. Some airways had a very dense collagen fiber network, taking up most of the subepithelial area, while others had an uncondensed collagen network, with large areas of empty space between fibers, likely attributed to a processing artifact. Most airways' circumferential distribution of collagen fibers around the epithelium was symmetrical. However, a minority of airways had markedly uneven circumferential collagen distribution in the airway subepithelial area. Airway smooth muscle followed this same tendency. Important differences in collagen distribution could be observed within the airways of a given horse. A relatively constant observation was that airways possessing a denser and thicker subepithelial collagen area generally also possessed substantial airway smooth muscle surrounding the latter, and this independent of the degree of airway contraction. Most collagen fibers in the subepithelial area were heterogeneously dispersed and no specific pattern or fiber orientation was visualized. However, two airways had collagen fibers that appeared to be oriented perpendicular to smooth muscle fibers around the whole airway. The presence of collagen fibers in the airway smooth muscle was a feature of multiple airways.

Airway epithelium was generally well defined, intact and its internal perimeter easy to delimitate from the airway lumen. Occasional airways had a partly torn epithelium, which likely arose during tissue processing, but generally didn't affect the internal perimeter measurements. The degree of airway contraction varied greatly in all airways, independent of the group or time period evaluated.

Although not a focus of this study, mucus could be visualized in the airway lumen in a minority of horses.

3. Respiratory scores of heaves-affected horses and controls before and after a thirty day antigenic challenge with mouldy hay and straw

At T0, all five control horses had a total respiratory score of 2, which corresponds to clinical normalcy. In the heaves-affected horses, three individuals had a total respiratory score of 2. The remaining individuals of this group had a total respiratory score of 3, corresponding to mild respiratory symptoms.

At T30, the total respiratory scores of all the horses in the control group remained at 2. In the diseased horses, three individuals had a total respiratory score of 6, corresponding to moderate respiratory signs. The remaining individuals of this group had a total respiratory score of 8, corresponding to severe respiratory signs. All the heaves-affected horses showed clinical signs of airway obstruction, evidenced by marked increase in abdominal effort on respiration and flaring of the nostrils.

4. Respiratory mechanics of heaves-affected horses and controls before and after a thirty day antigenic challenge with mouldy hay and straw

After three months of low antigen exposure (T0), there is no difference in pulmonary function parameters between controls and heaves-affected horses. After thirty days of antigenic challenge (T30), only heaves-affected horses developed significant airway obstruction ($p<0.01$)[11].

[11] Results obtained from ″Leclere, M., A. Lavoie-Lamoureux, et al. (2010). "Effect of Antigen Exposure on Airway Smooth Muscle Remodeling in an Equine Model of Chronic Asthma." *Am J Respir Cell Mol Biol*. In Press.″

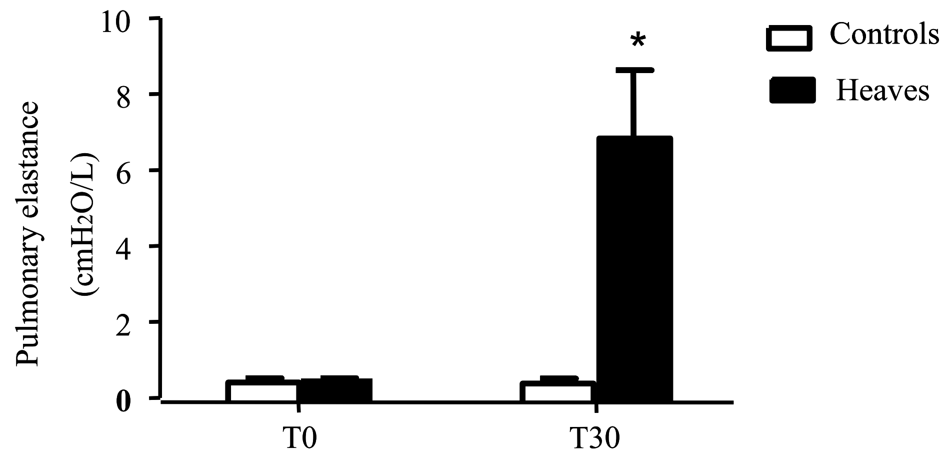


Figure 12 *Pulmonary elastance before (T0) and after (T30) a thirty day antigenic challenge in heaves-affected horses and controls (Mean ±SEM) *p<0.01: Different from T0 within the same group and controls at the same time point.*

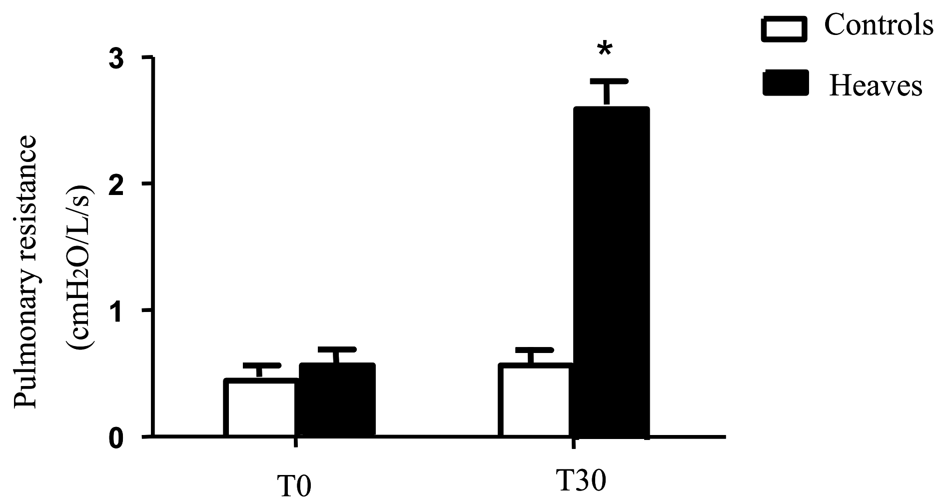


Figure 13 *Pulmonary resistance before (T0) and after (T30) a thirty day antigenic challenge in heaves-affected horses and controls (Mean ±SEM. *p<0.01: Different from T0 within the same group and controls at the same time point.*

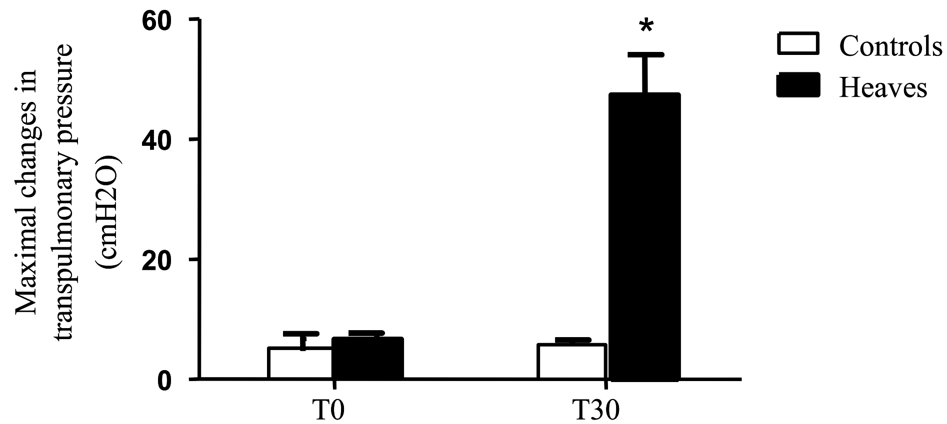


Figure 14 *Maximal changes in transpulmonary pressure before (T0) and after (T30) a thirty day antigenic challenge in heaves-affected horses and controls (Mean ±SEM). *p<0.01: Different from T0 within the same group and controls at the same time point.*

After three months of antigen exposure (T30), there was a significant increase in BAL neutrophil cell count (%) in heaves-affected horses and controls ($p<0.01$). No difference between groups could be appreciated. There were no significant group or time effects on the total cell counts (%).

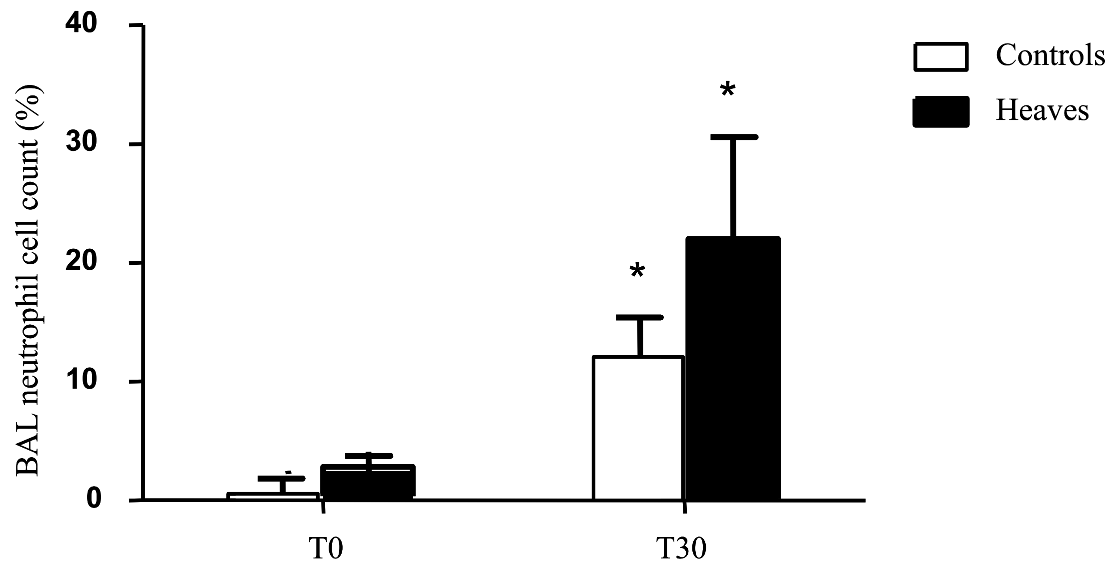


Figure 15 *BAL neutrophil cell count (%) before (T0) and after (T30) a thirty day antigenic challenge in heaves-affected horses and controls (Mean±SEM). * p<0.01: Different from T0 within the same group.*

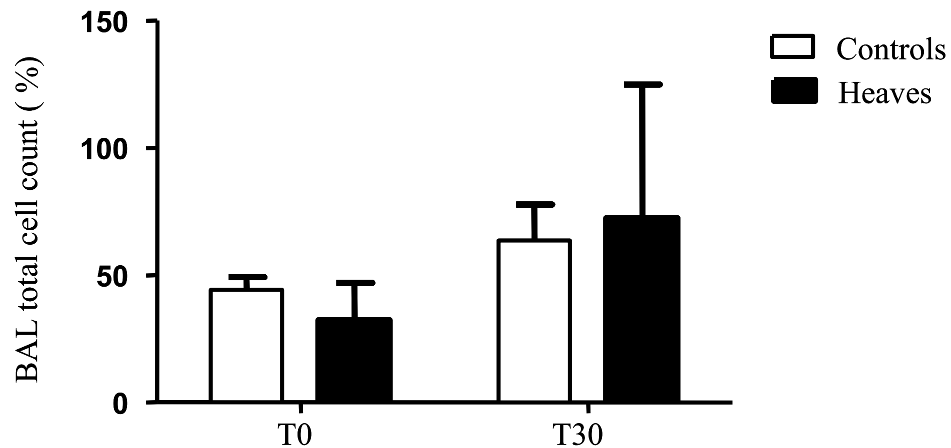


Figure 16 *BAL total cell counts (%) before (T0) and after (T30) a thirty day antigenic challenge in heaves-affected horses and controls (Mean±SEM).*

5. Subepithelial area collagen content in the airways of heaves-affected horses in comparison with controls

In comparison with controls, horses with heaves have an increased collagen content in the airway subepithelial area (p=0.0586). Collagen content is denoted as subepithelial area corrected for airway size (collagen area / Perimeter²). However, no time main effects or time or group-time interaction (p›0.1) were observed. The thirty day antigen challenge failed to alter subepithelial area collagen content in airways of control or heaves-affected horses. (c.f Figure 17 and 18).

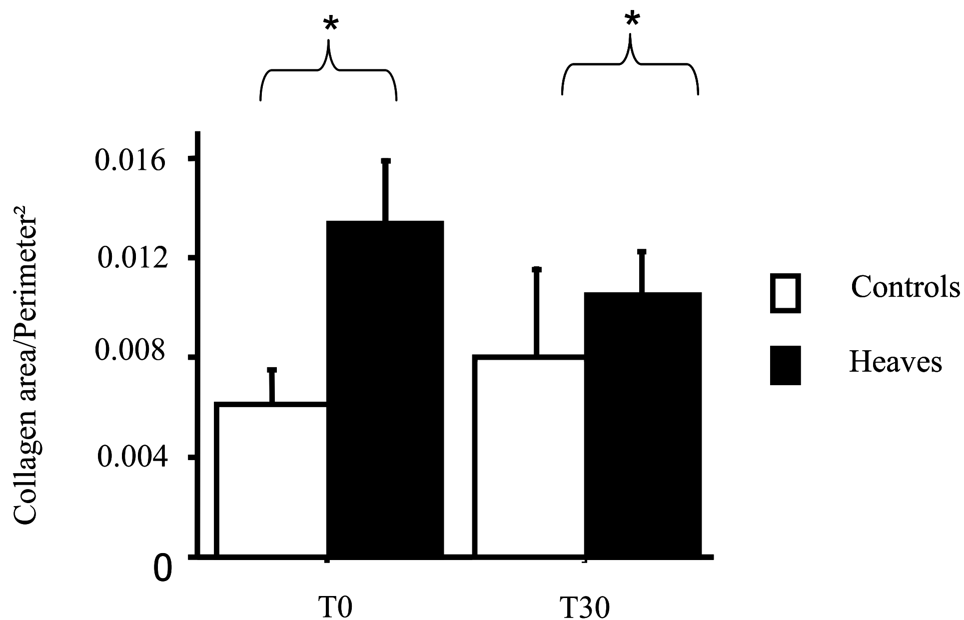


Figure 17 *Peripheral airway subepithelial area collagen content (mean per horse) before (T0) and after (T30) a thirty day antigenic challenge in controls and heaves-affected horses (mean±SEM) * p<0.1: Different between groups.*

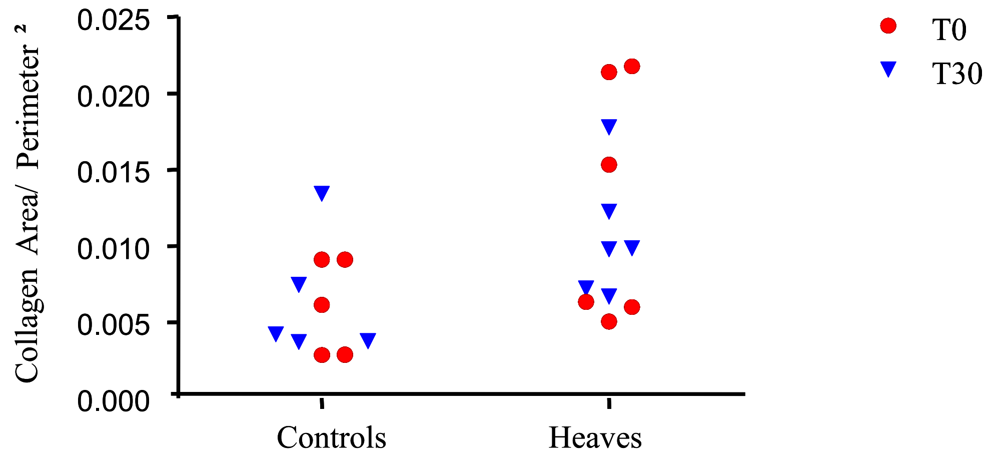


Figure 18 *Peripheral airway subepithelial area collagen content (mean per horse) divided by controls and heaves-affected horses. T0 and T30 peripheral airway subepithelial area collagen mean values per horse are shown.*

6. Airway distribution of heaves-affected horses and controls as a function of time

Subepithelial area collagen content in the airways of controls and heaves-affected horses before (T0) and after (T30) a thirty day antigenic challenge are distributed as a function of airway size, represented by the epithelial membrane internal perimeter (µm). The increase in subepithelial area collagen content in heaves in comparison with controls is especially present in airways of smaller diameter. The difference between both groups appears to normalize as airways increase in size (›1725µm Pi) (c.f figure 19).

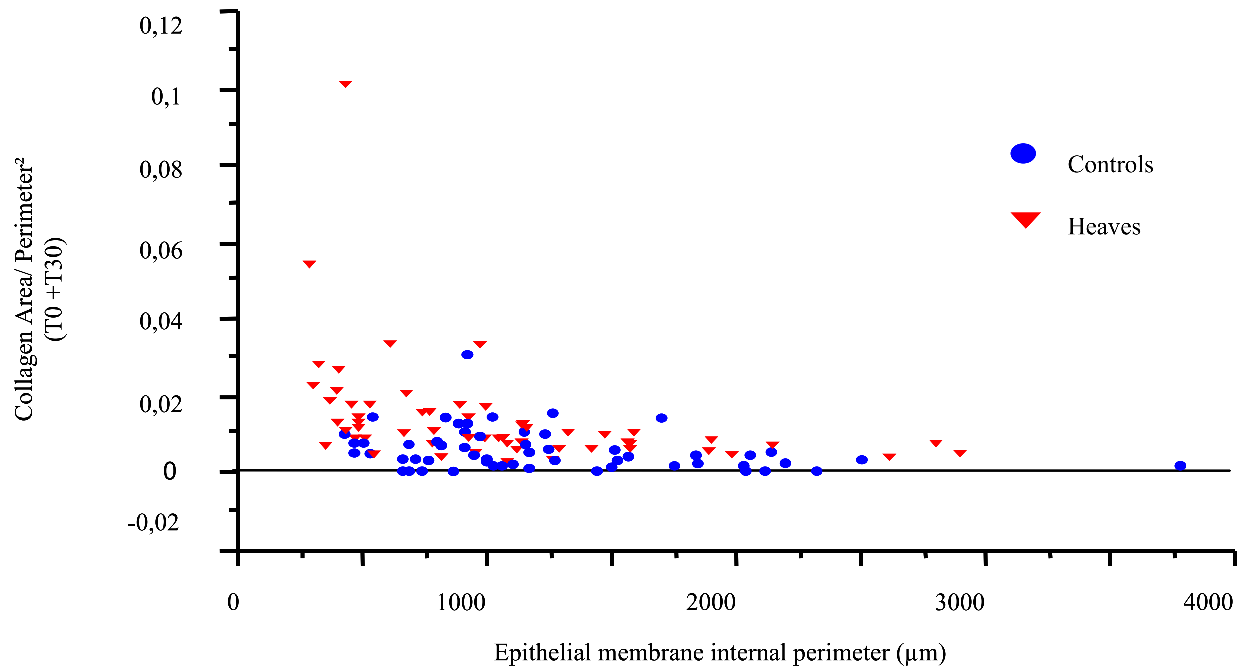


Figure 19 *Peripheral airway subepithelial area collagen content (T0 and T30) as a function of airway size (epithelium membrane internal perimeter (µm))*

When peripheral airways before (T0) and after (T30) a thirty day antigenic challenge are shown for every subject, interindividual variation in collagen content is high within the peripheral airways of a given horse. Individual variability is present in both controls and heaves-affected horses (c.f figure 20-21).

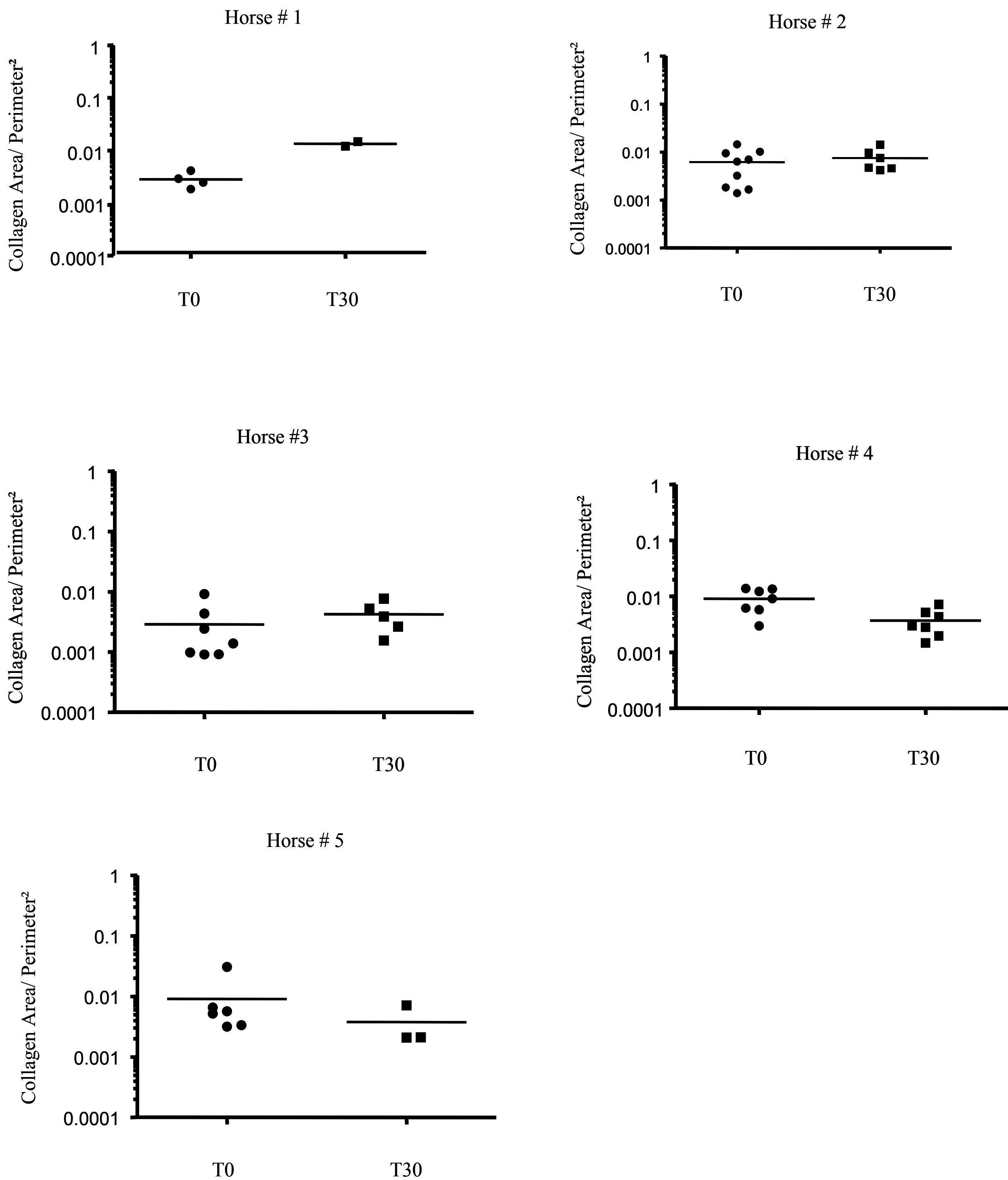


Figure 20 *T0 and T30 individual peripheral airway subepithelial area collagen content for each control horse (horses' # 1-5).*

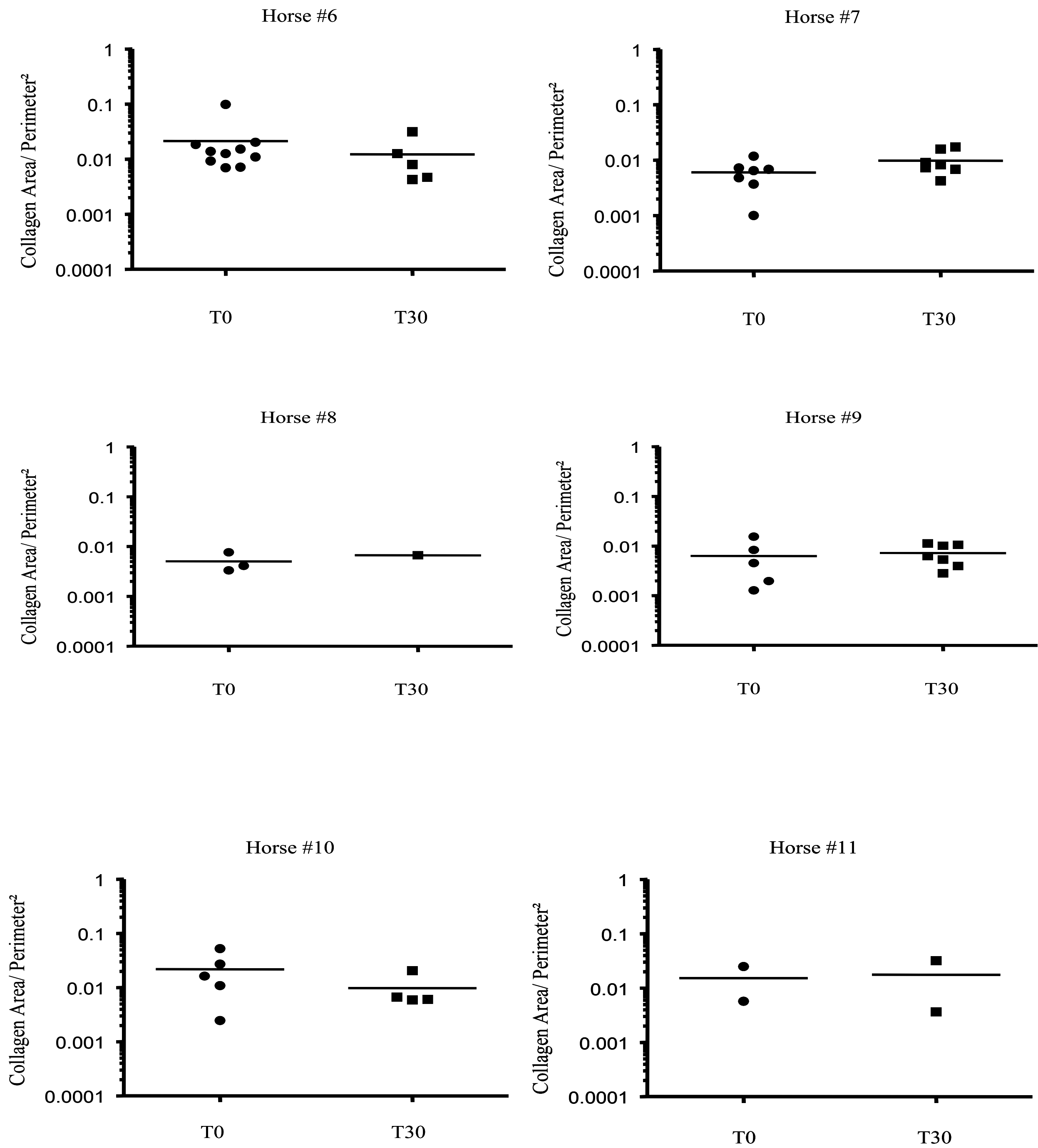


Figure 21 *T0 and T30 individual peripheral airway subepithelial area collagen content for each heaves-affected horse (horses' # 6-11).*

7. Peripheral airway subepithelial area collagen content as a function of pulmonary resistance, elastance and maximal changes in transpulmonary pressure

Peripheral airway subepithelial area collagen content was positively associated with maximal changes in transpulmonary pressure (PL) at T0 in heaves-affected horses, p=0.003, r^2=0.91. A similar tendency was observed with pulmonary resistance (RL), p=0.067, r^2=0.61. No association was observed with pulmonary elastance (EL) at T0 in heaves affected-horses, p›0.1. No association was present between peripheral airway subepithelial area collagen content and pulmonary resistance, elastance or maximal changes in transpulmonary pressure in control horses at T0 or at T30, p›0.1.

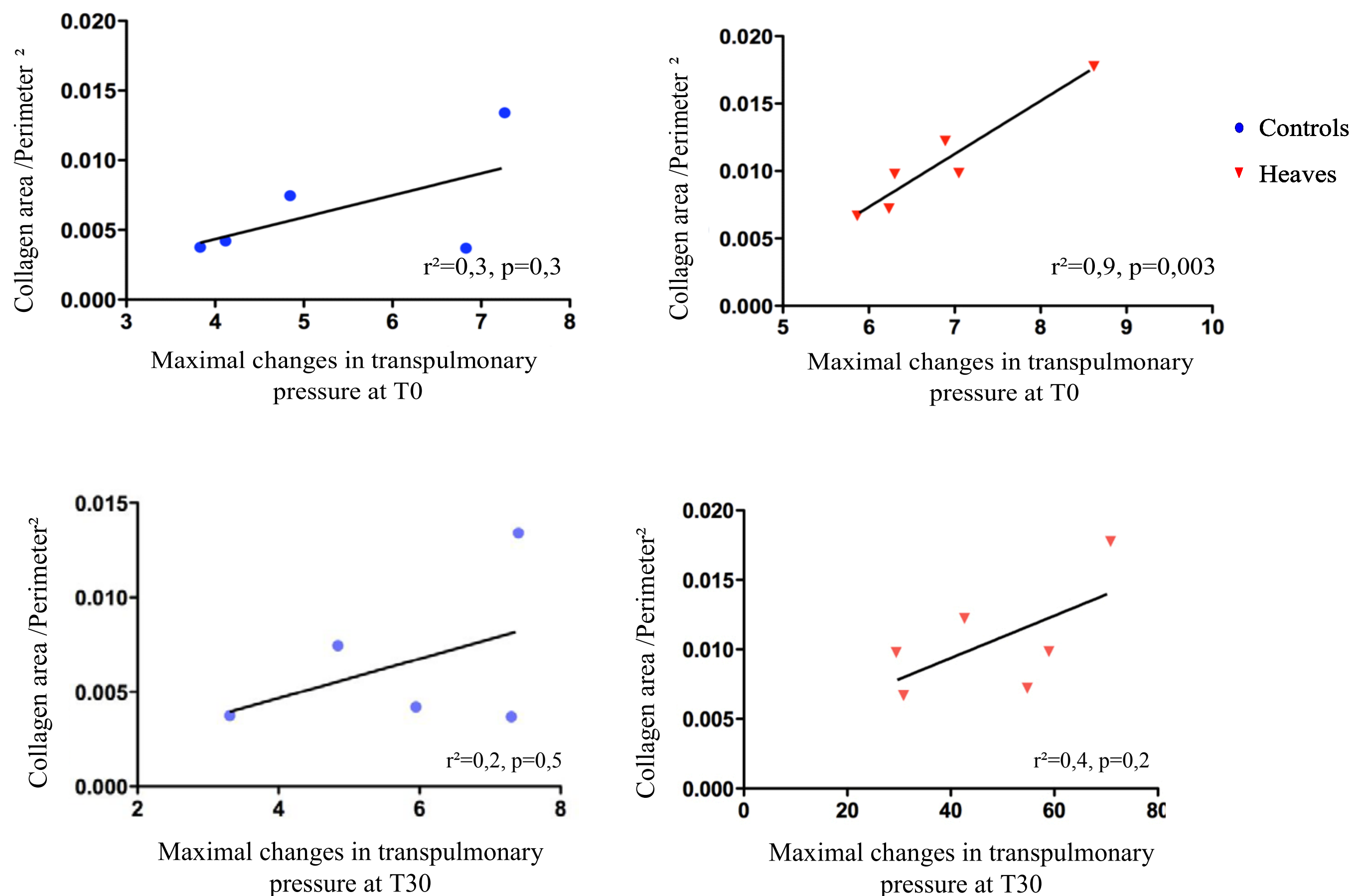


Figure 22 *Maximal changes in transpulmonary pressure as a function of peripheral airway subepithelial area collagen content of horses with heaves and controls after 3 months of antigen withdrawal (T0) and after 30 days of antigen exposure (T30).*

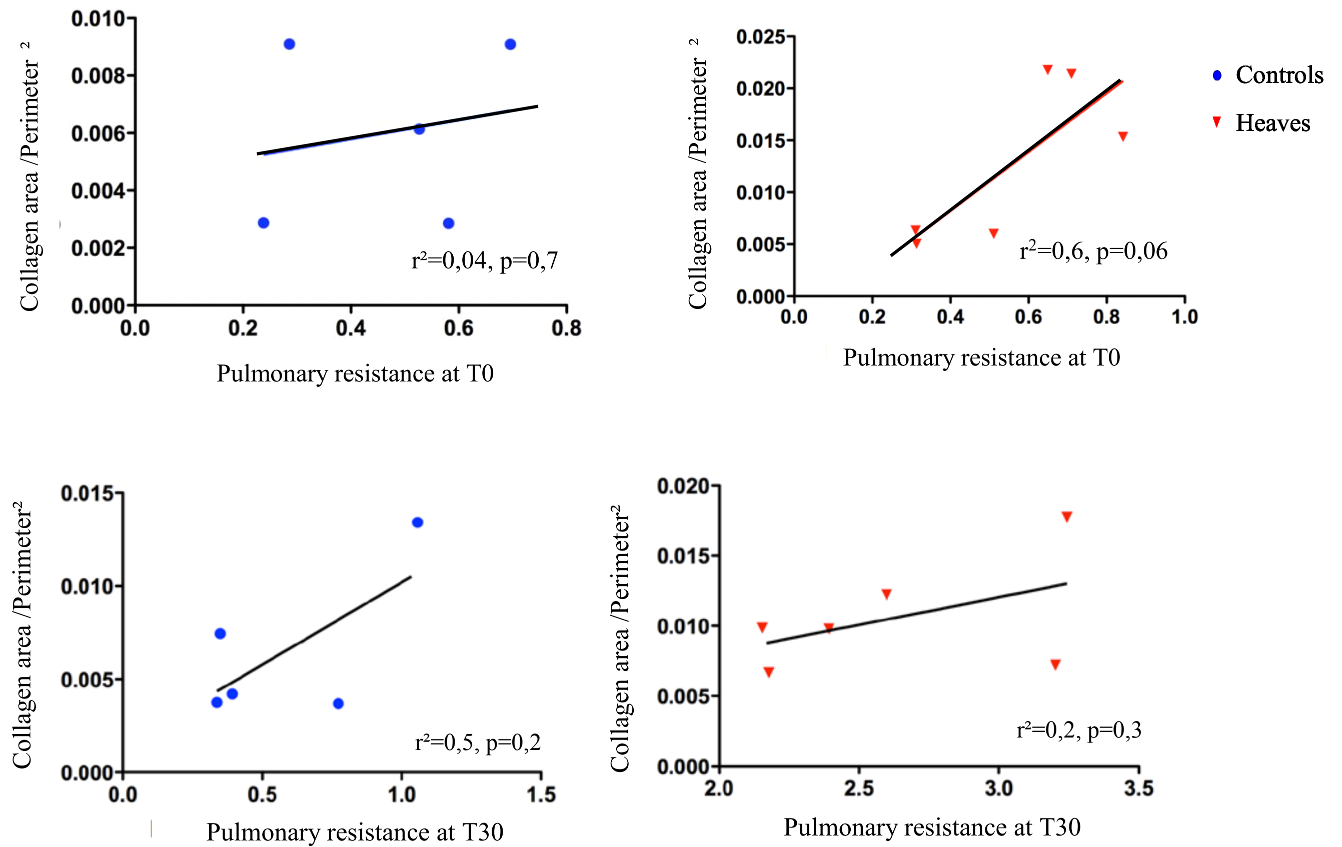


Figure 23 *Pulmonary resistance as a function of peripheral airway subepithelial area collagen content of horses with heaves and controls after 3 months of antigen withdrawal (T0) and after 30 days of antigen exposure (T30).*

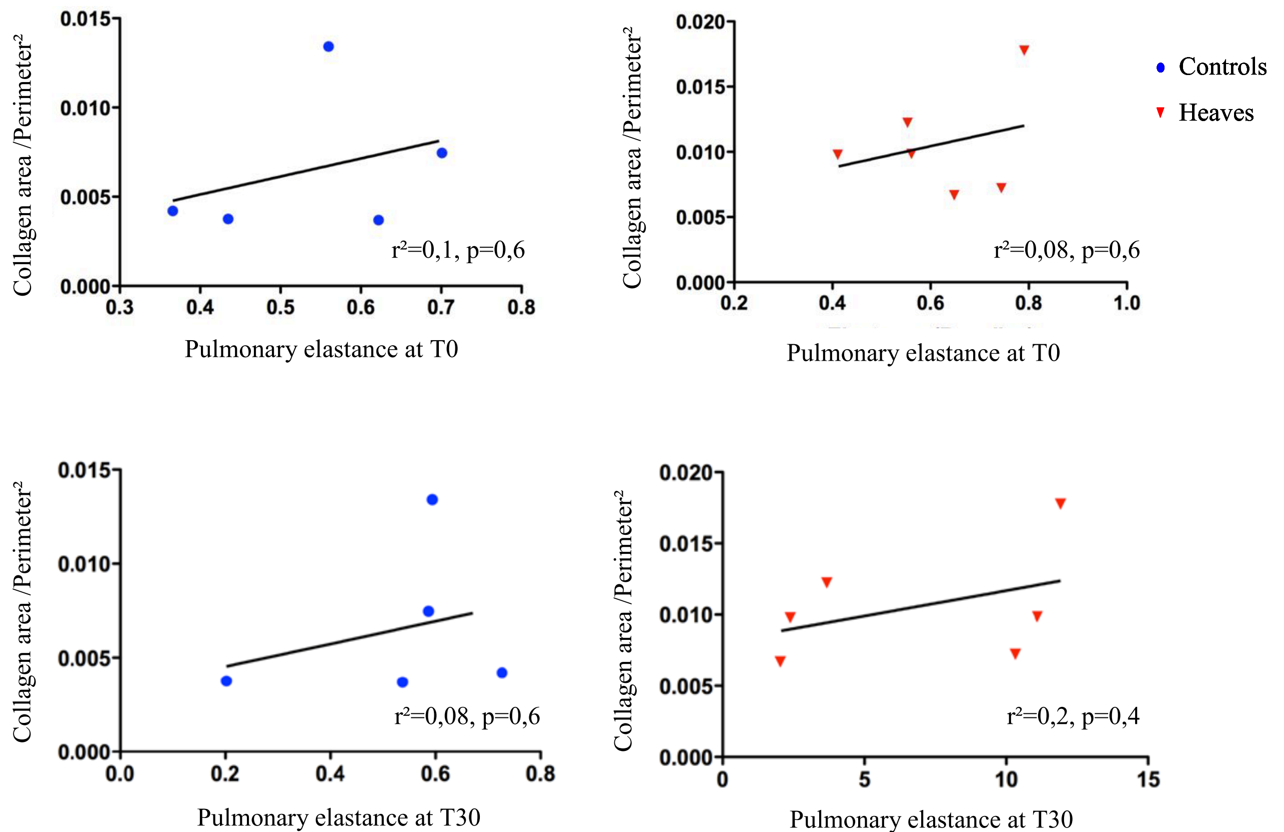


Figure 24 *Pulmonary elastance as a function of peripheral airway subepithelial area collagen content of horses with heaves and controls after 3 months of antigen withdrawal (T0) and after 30 days of antigen exposure (T30).*

8. Peripheral airway subepithelial area collagen content as a function of BAL total cell counts (%) and neutrophil cell count (%)

No association was observed between peripheral airway subepithelial area collagen content and BAL total cell counts (%) or neutrophil cell count (%) in either group, at both T0 and T30.

9. Descriptive observations of picrosirius-red stained subepithelial area collagen under polarized light

No qualitative changes in polarized colors (bright red, yellow, orange, green) could be visually appreciated in the subepithelial area of the airway. Furthermore, this region was difficult to delimitate. No quantitative assessment could be performed due to difficulty in separating polarized colors using the Image J® program threshold color tool.

10. Airway smooth muscle (ASM) qualitative assessment of collagen fiber presence under polarized light

Airways were divided into two categories: 1) presence of collagen fibers in the ASM and 2) absence or very little presence of collagen fibers in the ASM. Of the fifty airways which had similar polarization color results and were sorted by a blinded observer; twenty-three heaves-affected airways and twelve control airways were sorted as having presence of collagen fibers in the airway smooth muscle; fifteen controls and five heaves-affected horses were sorted as having no or very little collagen in the airway smooth muscle layer. The two-sided Fisher exact test revealed that heaves-affected horses had statistically more airways with presence of collagen fibers in the ASM, than control horses ($p=0.0052$). Most of the collagen fibers observed in the ASM were of the green polarization color (c.f figure 25).

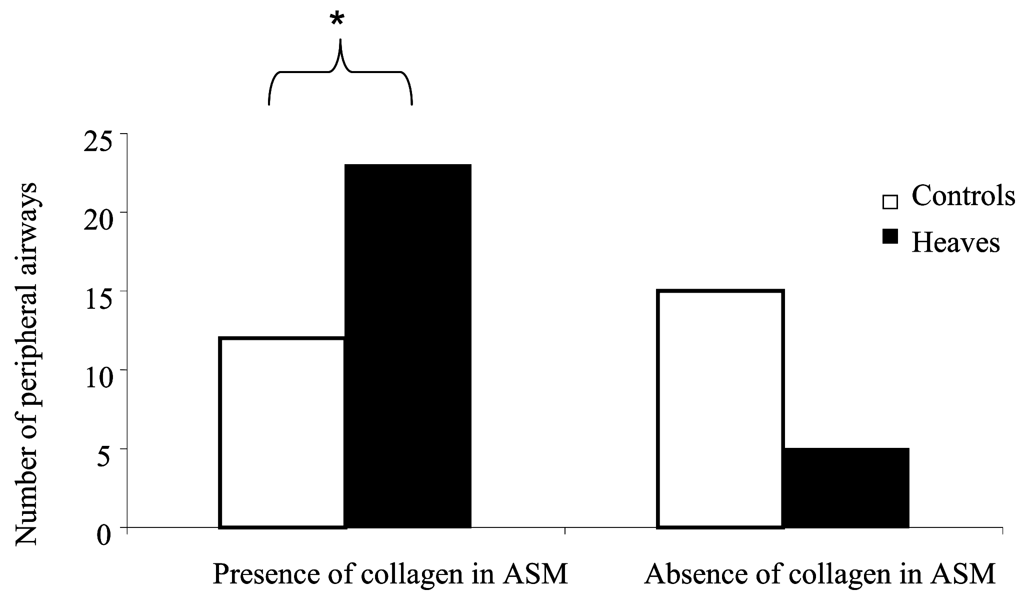


Figure 25 *Presence or absence of collagen fibers in the airway smooth muscle of horses with heaves and controls * p<0.01: Different for Presence of collagen in ASM between groups.*

DISCUSSION

1. Subepithelial area collagen content in the airways of heaves-affected horses and controls

In light of these results, fibrosis of the subepithelial area appears to be another feature of airway remodelling occurring in heaves, likely contributing to disease pathophysiology. Our results are difficult to correlate to prior studies on lung fibrosis in heaves (Kaup, et al., 1990; Thurlbeck & Lowell, 1964; Winder & von Fellenberg, 1988), since the qualitative changes described in the latter pathology reports refer mostly to the interalveolar and peribronchial region of the lung. However, our results corroborate and extend these reports by showing that collagen content also increases in the subepithelial area of small peripheral airways in diseased animals in comparison to controls.

While the difference in subepithelial collagen content between the two groups of subject horses reached significance (p=0.0586), there are several possible explanations that may account for the lack of greater significance. The relatively low number of sampled diseased individuals (n=6) combined with the observed high interindividual variability in collagen content in the subepithelial region, are probable contributing factors. The arithmetic mean ($\bar{x}$) of all the airways' collagen content in the subepithelial area was used to compare remodelling changes between groups. However, the extensive dispersion of data points and presence of outliers observed within the airways of a same horse could skew the mean value, and thus inaccurately represent airway collagen deposition for a given animal. Additionally, digitized picrosirius-red stained airways for which the smooth muscle layer didn't completely encircle the airway required subepithelial area collagen to be arbitrarily delimited from surrounding connective tissue. Such estimations may account for some of the variability observed.

A recent study used an immunolabeling method to identify collagen type I and type III fibers in the lamina propria and adventia of bronchioles in heaves-affected and control

horses, and to assess if fibrosis was present in diseased animals (Furness, et al., 2010). A semi-quantitative score was used to evaluate differences in collagen content between the two groups of horses; however no significant difference was appreciated. While at first sight these results appear to contradict our findings, limitations of the latter study could account for our diverging conclusions.

First, the number of diseased horses sampled (n=4) and tissue sections obtained per horse (n=8) were small. Our study attests that interindividual variation is high in sampled individuals, making a type II statistical error plausible. Remodelling changes may have occurred at different stages in a given horse at the time lung samples were obtained. Since heaves is a naturally occurring chronic respiratory condition, the degree of ECM turnover may manifest itself only at certain periods of time in disease progression, and thus could require a greater number of individuals to properly assess remodelling changes. Yet, heaves-affected horses were aged between sixteen and twenty eight years at the time of death and three out of four individuals had a long term history of heaves, suggesting that the disease was advanced enough that a change in collagen deposition would have been present, making this hypothesis less likely.

In addition, airway size was not taken into consideration when assessing collagen content in the airways of subjects in the Furness et al. (2010) study. Airways of subject horses were not infused at a constant rate following lung removal from the cadavers, making the application of a correction factor for airway size pivotal. Our study showed that fibrosis of the subepithelial area was more markedly increased in smaller airways (>175µm), further justifying the use of a correction factor. The epithelial membrane internal perimeter (Pi) applied as a correction factor for airway size has been shown to be independent of lung volume or the degree of airway dilation or contraction due to bronchoconstriciton (James, et al., 1988), and should have been used in the Furness et al. (2010) study. Pi is believed to be a consistent parameter in the airway because of its attachment to the underlying inextensible basement membrane.

Furthermore, while the collagens evaluated with the immunolabeling method (Col I and III), are the most abundant in the lungs of mammalian species (Last & Reiser, 1984), other collagen types are known to be present in the lung and were not evaluated. In human asthmatics, collagen types I and III are known to be increased in the subepithelial region of

the airway. However, collagen type V is also present in increased proportions in this region of the airway as well as in the submucosa (Bergeron, Al-Ramli, & Hamid, 2009; Elias, 2000; Hoshino, et al., 1998; Wilson & Li, 1997). A method to characterize its presence in normal or heaves-affected individuals has never been investigated, and its increased presence in heaves-affected horses could partly account for the studies' diverging results.

Additionally, collagen types were compared individually between the two groups of horses. It is possible that their combined assessment would yield an appreciable difference in the airways of diseased versus controls airways.

The authors further submitted the hypothesis that albeit the control horses (n=10) didn't have a history of exercise induced pulmonary hemorrhage (EIPH), the condition is known to cause collagen remodelling in the interlobular veins and in the pleura and interlobular septa of the lung (Williams, et al., 2008). It is conceivable that if the control horses had suffered a prior episode of this condition, it could confound the difference in collagen content between the two groups of horses. Moreover, seven of the ten controls in the study were either Thoroughbreds or Standardbreds, and both these breeds are at risk for EIPH (Williams, et al., 2008).

Research in progress in our laboratory is attempting to quantify the presence of collagens type I and III in the lungs of heaves-affected horses and healthy controls, using immunohistochemical techniques. The information provided by these results will likely elucidate the present unanswered questions.

2. Etiology of airway fibrosis in heaves

Airway inflammation and hyperresponsiveness are believed to be involved in the mechanisms leading to fibrosis in human asthma, although the exact pathways have yet to be explained. As previously discussed, we suspect that similar mechanisms occur in heaves, because of shared aspects of airway inflammation and hyperresponsiveness with asthma.

Inflammation of allergic origin in asthma is one of the proposed etiologies of airway remodelling. The Th2 and Th17 type cytokine profiles present in asthmatics results in the activation of many mediators known to have fibrogenic properties. TGF-ß1, a fibrogenic mediator discussed earlier, has been associated with the presence of subepithelial fibrosis in human asthmatics and in murine models. A study in human asthmatics correlated the severity of subepithelial fibrosis with the expression of TGF-ß1 (Minshall, et al., 1997). Similarly, investigators showed that the presence of subepithelial fibrosis in a mouse model of asthma could be correlated with increased levels of TGF-ß1 in the bronchoalveolar lavage (BAL) fluid of the subjects (Tanaka, et al., 2001). However, another report in human asthmatics failed to positively correlate type III collagen deposition with TGF-ß1 expression, suggesting other factors are involved in remodelling changes (Tomkowicz, et al., 2008).

A study by Jenkins et al. (2003) reported various remodelling changes in airway biopsies obtained from children with severe asthma. In five out of six subjects, little or no evidence of airway inflammation was present, again suggesting other mechanisms are responsible for the remodelling observed. However, the authors could not exclude the possibility that airway inflammation was present in the individuals, albeit more distally than the sampled area.

A problem involving the epithelial repair process is another proposed etiology for the remodelling changes observed in asthmatics. Close interactions between the epithelium and subepithelial layer, defined as the epithelial-mesenchymal trophic unit (EMTU), are important in lung development, and believed to be impaired in asthma (Holgate, et al., 2004). Chronic airway inflammation causes repeated insults to the epithelium, and the latter's inability to properly recover could lead to ongoing activation of the EMTU and consequent remodelling of the airway. It is suspected that the repair process of the damaged epithelium is abnormal in asthmatics, leading to activation of secondary repair mechanisms and subsequent deposition of collagen in the airway. Activated epithelial cells have the ability to secrete pro-fibrotic mediators, such as TGF-ß2 and fibroblast growth factor-2, which promote fibroblastic cells to produce ECM proteins. Minshall et al. (2000) showed that an increase in IL-11 expression, a cytokine secreted by subepithelial cells, could be correlated to the deposition of type I and III collagen in a localized airway region.

Swartz et al. (2001) used an *in vitro* model of cultured human airway epithelial and fibroblast cells to study the effects of mechanical stress on airway remodelling. Epithelial cells submitted to mechanical stress resulted in an increase of collagen type III and V production by the unstressed fibroblast cells. The degree of remodelling was correlated to the magnitude of stress placed on the epithelial cells (Swartz, et al., 2001). These results suggest that independent of airway inflammation, mechanical forces acting on the airway during episodes of airway hyperresponsiveness may modulate some of the airway wall remodelling occurring in heaves.

All of the proposed etiologies could be contributing to the development of fibrosis in heaves and need further exploration.

3. Airway fibrosis and lung function

The role of airway fibrosis in asthma or heaves in relation to lung function is not completely understood. While some have suggested that fibrosis serves as a protective response to airway disease, others have proposed mechanisms in which airway fibrosis is detrimental to lung function and contributes to airway narrowing.

A mechanism by which fibrosis of the subepithelial area in heaves could protect against airway hyperresponsiveness is through stiffening of the airway wall. A thickened airway would theoretically result in decreased distensibility, also known as compliance, which in areas of limited air flow (bronchoconstriction) could assist in preserving peak expiratory flow (PEF), even if the airway lumen was reduced in size (McParland, Macklem, & Pare, 2003). Such a hypothesis was tested in a study using a rat model of asthma induced with aerosolized ovalbumin (OVA). Increased collagen and fibronectin deposition was present in the airways of asthmatic rats in comparison to controls after the twelve week challenge. Interestingly, airway response to carbachol, a cholinergic agonist, was similar in both groups of rats at the end of the trial, suggesting airway remodelling was protecting against hyperresponsiveness (Palmans, Kips, & Pauwels, 2000).

Increased collagen deposition around smooth muscle cells has also been proposed to limit airway smooth muscle shortening and airway narrowing by altering the "elastic

afterload". "Elastic afterload" develops as narrowing compresses tissue structures, and increase in collagen content could potentially intensify this phenomenon (McParland, et al., 2003).

Mechanisms by which airway fibrosis leads to a detrimental impact on lung function have also been advanced. The principle by which an increase in airway wall thickness exerts a magnifying effect on smooth muscle shortening was initially proposed by *in vitro* mathematical models (Moreno, Hogg, & Pare, 1986). A post-mortem study on the airways of asthmatic and non-asthmatic individuals further corroborated the model, determining that asthmatic airways, in comparison to controls, had a greater airway wall thickness, and thus required a lesser degree of smooth muscle shortening to completely occlude the airway (James, Pare, & Hogg, 1989).

Another potential negative impact of increased remodelling in the airway wall is through the loss of parallel elastance of connective tissue to the airway smooth muscle (McParland, et al., 2003). Products of airway inflammation could hypothetically result in proteolysis and subsequent architectural alterations of collagen fibers and other ECM proteins. An earlier *in vitro* study on human bronchial tissue by Bramley et al. (1995) supports this theory. The group demonstrated that collagenases, enzymes of collagen degradation, could alter bronchial tissue function and lead to loss in tissue elastance and intensify smooth muscle shortening (Bramley, Roberts, & Schellenberg, 1995).

The relationship between subepithelial fibrosis and lung function is further complicated by the presence of conflicting information on the subject. A study by Shiba et al. (2002) correlated subepithelial thickening in mild asthmatics to duration of disease, airflow limitation and airway hyperresponsiveness. Others have found similar parallels between subepithelial fibrosis and lung function deficits (Chetta, et al., 1997) or airway hyperresponsiveness (Hoshino, et al., 1998), supporting the hypothesis that remodelling can impair lung function. Nevertheless, such a correlation is not a universal finding (Brewster, et al., 1990; Jenkins, et al., 2003).

Our results indicate the presence of a positive correlation between subepithelial area collagen content in the peripheral airways of heaves-affected horses and the maximal changes in transpulmonary pressure (PL) measurements, indicating that airway fibrosis has

a detrimental effect on airway function. A similar tendency is present with pulmonary resistance (RL). This may explain the higher RL and PL measures in diseased horses while in remission (free of clinical symptoms of airway obstruction). These findings suggest that fibrosis of the subepithelial area plays a role in passive airway obstruction.

The positive association between fibrosis of the subepithelial area and RL did not reach significance. However, RL is a calculated value derived from a linear regression equation, which may possibly not adequately represent the mechanics of the diseased equine lung. A greater number of sampled individuals may have allowed significant changes to become evident, as observed with PL.

The lack of association between fibrosis of the subepithelial area and PL or RL in the control group suggests that in healthy individuals with normal respiratory patterns, airway subepithelial area collagen content does not influence lung function parameters.

The lack of association between PL and RL with fibrosis of the subepithelial area after a thirty day antigenic exposure (T30) could be explained by the predominating influence of bronchospasm on lung function in diseased animals. Therefore, the association with fibrosis of the subepithelial area is no longer observed. While these results were expected, a negative correlation between fibrosis and lung function may have indicated a protective effect as discussed above.

No correlation between fibrosis of the subepithelial area and EL was established. These results were surprising since EL is a measure believed to be representative of the mechanical elastic properties of peripheral airways (Robinson, et al., 2000), and remodelling changes in heaves and asthma are suspected to be mostly contained to the smaller peripheral airways. RL is a better indicator of airflow obstruction occurring in the large and small central airways (Robinson, et al., 2000). It is thus possible that our population of heaves-affected horses is part of a sub-phenotype of disease, as is seen in human asthmatics (Kaczka, Ingenito, Israel, & Lutchen, 1999). Type A asthmatics are known to have increased RL values but EL values similar to that of healthy individuals. High RL and EL values are more typical of Type B asthmatics, associated with a more severe form of disease. These different pulmonary mechanic patterns exhibited by asthmatics are likely due to differences in the viscoelastic properties of lung tissue, shunting of the airway wall and severity of airway obstruction (Kaczka, et al., 1999;

Kaczka, Ingenito, Suki, & Lutchen, 1997). No sub-phenotypes of disease have yet been described in heaves.

Also, and as suggested previously, it is possible that the method we used to calculate E_L may not accurately reflect the elastic properties of the diseased lung.

4. Airway remodelling over time

Our present findings indicate that fibrosis of the subepithelial area fails to increase after a thirty day antigenic challenge with mouldy hay and straw in heaves-affected horses. These results were expected, since the development of fibrosis is known to be a chronic process evolving over a long period of time, possibly years. It is also generally accepted to be poorly reversible. The population of heaves-affected horses used in our study consisted of mature animals, having been repeatedly exposed to environmental antigens over several years. A thirty day challenge would have been surprisingly rapid for fibrotic changes to occur.

No previous equine study has addressed airway remodelling progression in the same subjects, in clinical remission or disease exacerbation. In human asthmatics, techniques are not available in the clinical setting to track airway remodelling progression in the peripheral airways, making the horse a useful model for such an evaluation. Rodent models are not naturally-occurring, and poorly represent the course of disease progression in humans (Shin, Takeda, & Gelfand, 2009). Other than cats, horses are the only other mammal known to develop spontaneous respiratory disease characterized by airway obstruction, when exposed to environmental allergens (Lowell, 1964).

Small rodent models have led to breakthroughs in the understanding of asthma. The importance of a Th2-cytokine profile in the pathophysiology of disease evolved from studies in mice, for instance (Kumar & Foster, 2002). Advances in genetic manipulation have led the development of numerous transgenic mouse models, adapted for the study of more specific aspects of disease. Furthermore, small rodent models are cost-effective and

relatively easy to breed and manage, when compared to large animal models (Shin, et al., 2009).

Nonetheless, there are limitations to the use of small animal models for the study of asthma, especially in evaluating chronic changes to airway architecture. Chronic murine models of human asthma have been created to mimic certain features of airway remodelling, and to address the problem posed by primary or secondary challenge models. The latter models develop little airway remodelling and only transitory airway inflammation or hyperresponsiveness (Shin, et al., 2009). A research group recently compared acute, subacute and chronic murine models of asthma sensitized with OVA (Locke, et al., 2007). The chronic challenge model was used to reproduce the long-term characteristics of airway remodelling in asthma, such as subepithelial fibrosis and goblet cell hyperplasia. However, an increase in airway smooth muscle mass, a dominant feature of asthmatic airway wall remodelling, was not observed (Locke, et al., 2007), suggesting the chronic model remains incomplete in representing all features of airway remodelling. It has also been shown that some strains of the chronic murine models develop tolerance to antigenic exposure and a gradual decrease in airway inflammation and hyperresponsiveness can be seen despite continued antigen challenge (Koya, et al., 2006).

Another drawback of the mouse model for studying remodelling occurring in asthma is the marked difference in pulmonary anatomy and physiology compared with human subjects. Mice have a different airway branching system and a limited musculature of the airway in comparison to people (Shin, et al., 2009). Humans have a thicker blood-gas barrier than mice. These features may contribute to different pulmonary mechanics and gas exchange parameters between species (Irvin & Bates, 2003).

Accurate lung function tests are also more difficult to perform in small subjects. The use of non-invasive methods in rodent models such as plethysmography is the center of much debate. With this technique, airway function is deduced from a calculated parameter known as Penh (enhanced pause), which is derived from alterations in breathing pattern (Bates & Irvin, 2003). While plethysmography has the advantage of permitting repeated measures in conscious, unrestrained animals, it has been shown to be easily influenced by the subject's metabolism (Irvin & Bates, 2003). While some have demonstrated plethysmography as a useful tool in evaluating airway hyperresponsiveness in allergen

sensitized and challenged mice (Hamelmann, et al., 1997), it is now generally accepted that Penh is a poor representation of lung function (Adler, Cieslewicz, & Irvin, 2004; Petak, Habre, Donati, Hantos, & Barazzone-Argiroffo, 2001).

Invasive methods are more precise, as they offer a direct means of obtaining the "Gold standard" pulmonary function parameters; airway resistance and dynamic compliance. The use of tracheostomy with endotracheal intubation has the advantage of eliminating the bias presented by interfering upper airway resistance. The main disadvantage with the latter technique, however, is the impossibility of obtaining repeated measurements (Hoymann, 2006). Other variations of invasive and non-invasive techniques exist and can be used to assess pulmonary function in small rodent models, each with various advantages and limitations.

In equines, the technique described earlier permits lung function measurements repeatedly, over time, and in a non-invasive manner.

5. Thoracoscopic guided lung biopsy procedures for histomorphometric analysis

Ethical considerations preclude the use of thoracoscopic guided lung biopsies for the study of airway remodelling in human asthma. Therefore, evaluation of peripheral airway tissue in live subjects is rarely feasible (Balzar, Wenzel, & Chu, 2002), making the horse a useful model for evaluating distal airway remodelling in a longitudinal manner. Thoracoscopic guided lung biopsy procedures in equines have the advantage of being minimally invasive, providing adequate biopsies for histological evaluation and allowing repeated sampling over time of peripheral airway tissue in the same subjects (Lugo, et al., 2002). The two thoracoscopic biopsy procedures performed at a thirty day interval were generally well tolerated in the study horses, and recovery was uneventful. Biopsy samples were of suitable size for histomorphometric analysis (Relave, et al., 2008).

Endobronchial biopsies are less appropriate for evaluating remodelling changes in the distal airways, as restrictions in equipment length limit sampling to the proximal portion of the tracheobronchial tree. Tissue crushing caused by the biopsy forceps can cause artifacts which are detrimental to morphometric analysis. Such biopsy specimens tend to be smaller in size and only sample a superficial section of the airway wall.

Researchers evaluating airway smooth muscle mass in airways of heaves-affected horses and controls observed that endobronchial sampling in airways undergoing bronchospasm tended to yield more superficial biopsies due to difficulty in properly positioning forceps at a thickened carina. It was speculated that this introduced a bias in relation to the percentage of smooth muscle present in the airway (Leclere, et al., 2010). Endobronchial biopsies used to quantify fibrosis of the subepithelial area could potentially yield this same bias. Furthermore, as noted above, fibrosis of the subepithelial area is more prevalent in distal airways, making endobronchial biopsies less appropriate for evaluating these changes.

Percutaneous lung biopsies, acquired using a Tru-Cut punch in horses can yield appropriate samples for histological analysis in heaves; however the technique requires repeated sampling and many complications have been reported (Savage, Traub-Dargatz, & Mumford, 1998).

In human asthmatics, transbronchial biopsies, a half-way method between other lung sampling procedures, has proved successful in obtaining good quality samples for morphometric analysis and histological differentiation of smaller vs. larger airways (Balzar, et al., 2002). Nonetheless, the method is technically challenging and presents risks of severe complications such as hemorrhage and pneumothorax (Anders, Johnson, Bush, & Matthews, 1988; Bergeron, Tulic, & Hamid, 2007). There are other drawbacks; the majority of tissue sampled contains primarily the alveolar wall as opposed to distal airways (Kraft, et al., 1996) and obtaining full airway cross-sections is not possible (Bergeron, et al., 2007). Its usage has never been investigated in equines.

CONCLUSION

Results from our study indicate that fibrosis of the subepithelial area is present in the airways of heaves-affected horses as diseased horses have an increased collagen content in the subepithelial layer in comparison to controls after three months of low antigenic exposure (T0). Fibrosis may also extend to other components of the airway wall as the proportion of green birefringent polarized collagen fibers in the airway smooth muscle layer of heaves-affected horses was increased when compared to controls. Fibrosis of the subepithelial area may contribute to the persistent airway obstruction observed in heaves-affected horses given that it was positively associated to pulmonary resistance (R_L) and to the maximal changes in transpulmonary pressure (P_L) in these animals. Airway collagen remodelling appears to be a gradually evolving process as no significant change was observed in collagen content after thirty days of antigenic challenge in either group. Morphometric quantification on lung biopsies obtained by a thoracoscopic guided procedure was shown as a suitable method to evaluate changes in collagen content in the airway of picrosirius-red stained histological sections.

While our research results contribute valuable information on airway remodelling and its relationship to lung function in heaves-affected horses, there are nonetheless certain limitations to the study which must be considered. Owing to the experimental design, a blinded observer microscopically examined all the histological sections, resulting in an unpredictable and unevenly distributed number of airways available for analysis for each subject horse at both time periods. As well, due to the high interindividual variation in subepithelial area collagen content observed between airways, a greater sample number may have revealed greater changes than those obtained.

Histological and morphometric analyses allow a general appreciation of different connective tissue remodeling events in the lung; they do not, however, give information on the biological or secretory activity of ECM cells at the time the sample is acquired. Additionally, the picrosirius-red staining procedure does not permit the differentiation of collagen type or fiber thickness. Immunohistochemical techniques specific for collagen types or cellular constituents of the ECM could offer complementary information as to the

type of fibers present in the subepithelial area of the airway as well as the biological activity of cells responsible for their synthesis, respectively.

Future investigations into airway remodelling of other matrix constituents in heaves-affected horses; elastic fibers and glycosaminoglycans, for example, will be necessary as these components are known to be important in asthmatics and small animal models of human asthma.

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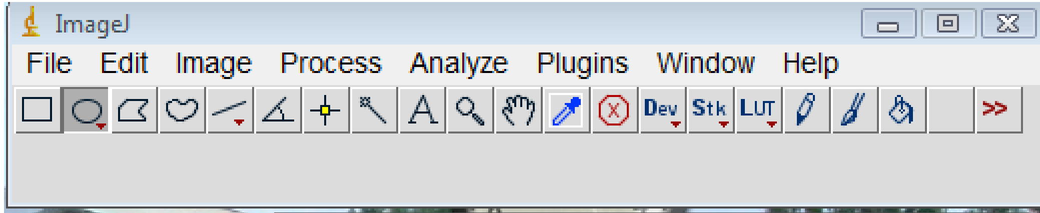
ANNEXE A

IMAGE J® PROTOCOL FOR MORPHOMETRIC ANALYSIS OF EQUINE PERIPHERAL AIRWAYS

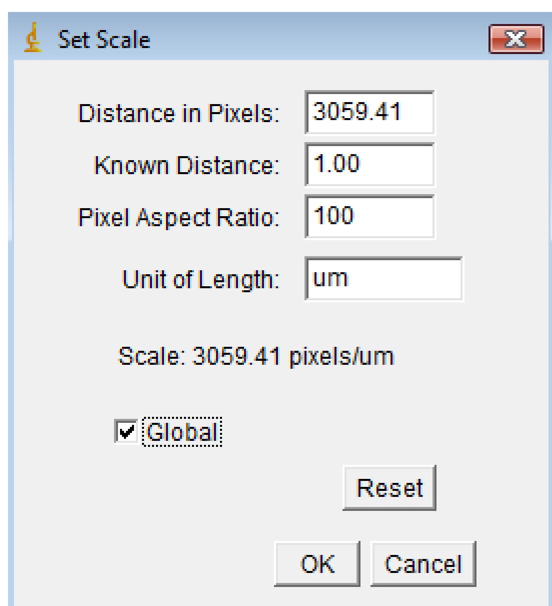


Calibration (measurement comparison)

1. Using the tool **STRAIGHT LINE SELECTIONS**, trace a known distance on the image of the stage micrometer at a given magnification (10X or 20X).

2. Go to **ANALYZE**, click on **SET SCALE** and enter the known distance (Ex. in µm, in mm, or in cm).

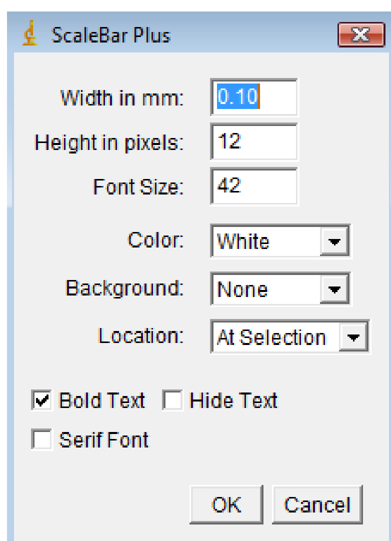
3. Select the **GLOBAL** option in the designated box.



4. Go to **ANALYZE**, and then click on **TOOLS**, and then click on **SCALE BAR**.

5. Choose the **LOCATION** and **COLOR** of the scale bar if desired.

6. Transpose the **SCALE** on the image in its designated location by re-clicking on **SCALE BAR**

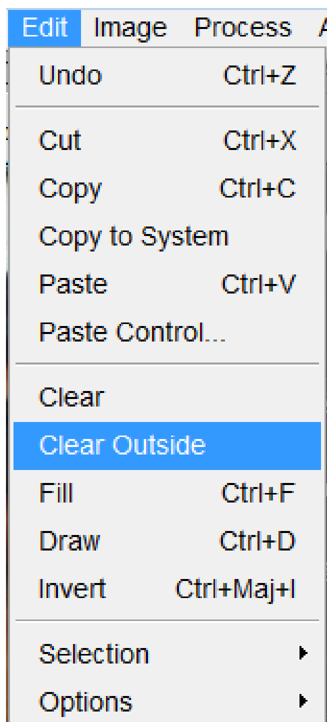


Cutting or extracting a part of the image

1. Example: Removing the airway smooth muscle layer and advential connective tissue from the rest of the airway image. Using the image in its original color format, select **FREEHAND SELECTIONS**, and using the **TRACING TOOL**, delimitate the region to extract.

2. Choose **EDIT** and click on **CLEAR OUTSIDE.**

3. Choosing **EDIT** and clicking on **CLEAR** performs the opposite, the part of the image interior to the region delimitated will be removed.

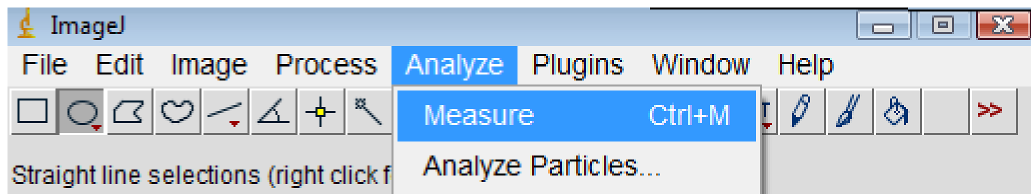


Measuring a perimeter

1. Example: Measuring the internal perimeter of the epithelium layer of the airway. Choose **STRAIGHT LINE SELECTIONS**, perform a right click on the mouse and select **FREEHAND LINES**.

2. Trace the internal border of the epithelium by holding the left click on the mouse.

3. Go to **ANALYZE**, followed by a click on **MEASURE**.



4. The **MEASURE** option gives various information including the length of line selection, according to calibrated units.

5. To change or increase the number of measurement parameters, go to **ANALYZE**, and choose **SET MEASURMENTS**.
6. Once the **SET MEASUREMENTS** window unfolds on the screen, click on the desired parameters such as area, density etc.

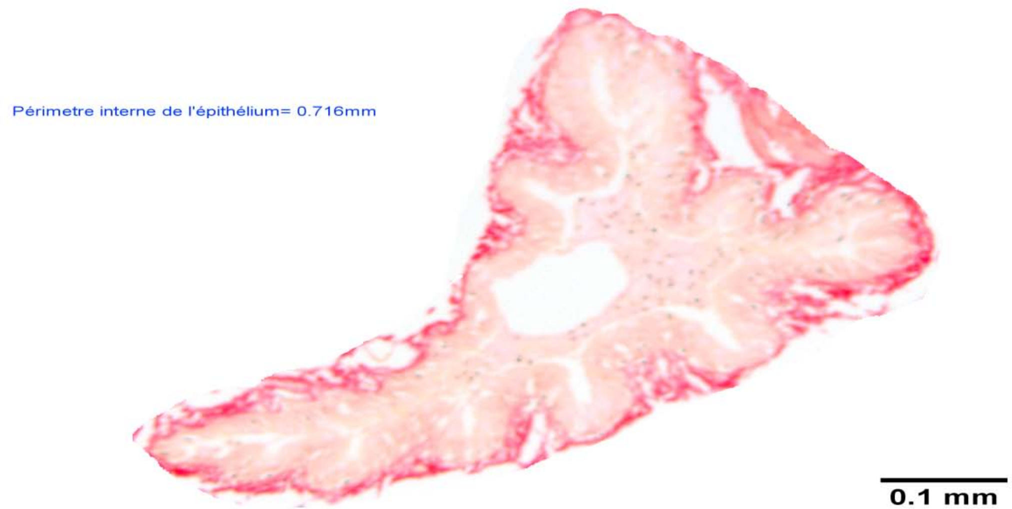
7. Verify that the **SCALE BAR** is well calibrated depending on the magnification strength of the image. Otherwise, perimeter measurements will be erroneous.

To add written text in an image

1. Example: To write that the internal airway perimeter is 2, 70 mm in length beside the image.

2. Click on **TEXT TOOL** and a text rectangle will appear on the image, using the mouse this rectangle can be moved to a designated location.

3. Once at the designated location, the desired information can be entered in the rectangle (ex. Internal perimeter = 2, 70 mm) and ctrl + D function can be used to insert the information.



Using the wand tool for automatic trace selection of an image area

(Will work better in **8 BIT** mode or in **THRESHOLD COLOR** mode)

1. Select the **WAND TOOL** in the tool bar.

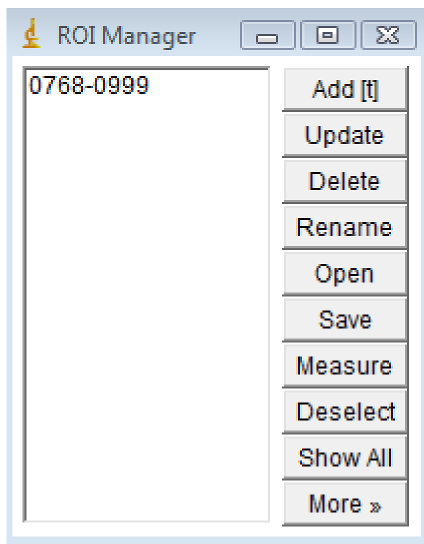
2. Place the **WAND TOOL** at the edge of a border of the region of interest in the image (Example: collagen of the subepithelial layer). The wand tool will automatically trace and highlight the region of interest.

3. By clicking on the left cursor of the mouse and pressing the **SHIFT** key on the keyboard at the same time, other image areas can be traced and added to the initial wand selection.

Data storage using the ROI MANAGER tool

1. Go to **ANALYZE**, then choose **TOOLS**, and then choose **ROI MANAGER**.

2. **ROI MANAGER** allows add-ins of additional traced regions in the image, which for example, were not picked up with the **WAND TOOL** on first try.



3. A measurement can also be made directly in the **ROI MANAGER** by clicking on **MEASURE**. This will give desired parameters such as an area, perimeter etc.

4. In order to verify that the traced selections are correct (and that no subepithelial area has been forgotten for example), by pressing the **DELETE** key on the keyboard, the entire traced selection will disappear from the image and allow such as assessment.

5. By pressing **UNDO** or (crtl + z) on the keyboard; the traced selection can be made to re-appear.

6. To fill the selection with another color on the image, select **FILL**.

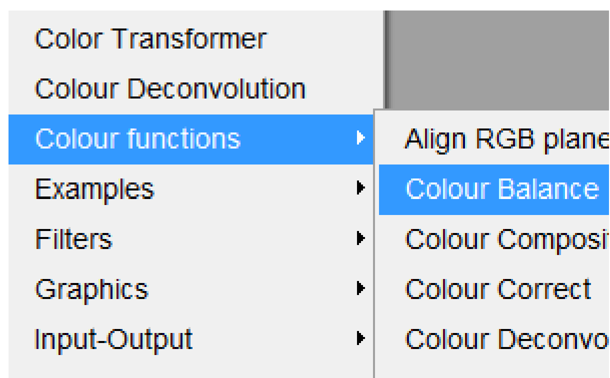
7. The **ROI MANAGER** cannot add stored measures together, this must be done manually.

8. Another way to measure the traced selections is by choosing **ANALYZE** in the tool menu, and then choosing **MEASURE**, as explained previously.

Background correction

1. Choose **PLUGINS** from the tool menu, then choose **COLOUR FUNCTIONS**, and then choose **COLOUR BALANCE**.

2. Choose a sample from the image background to be corrected; it will be converted to a whiter shade. All images can then be standardized with the same background.



Threshold colour (Method 1)

1. Go to **IMAGE**, select **TYPE** and choose the **8 BIT** option. This will convert the image in black and white.

2. Go to **IMAGE** again, choose **ADJUST**, and then choose **THRESHOLD**. The image can then be thresholded in various ways with the **BLACK AND WHITE**, **RED**, or **OVER AND UNDER** options.
3.
4. These threshold options allow differentiation of colors in an image. This can be achieved by scrolling down the intensity bar with the mouse cursor.

5. A **THRESHOLD COLOUR** plug-in exists which can give more specific colour differentiation and is detailed below.

Thresholding colour (Method 2)

1. Go to **PLUGINS**, then choose **THRESHOLD COLOUR** and then choose again **THRESHOLD COLOUR**.

2. Choose a sample of the colour that needs to be thresholded. (Example: to sample the subepithelial collagen stained red).

3. Click on **SAMPLE**, and only that thresholded colour will remain on the image.

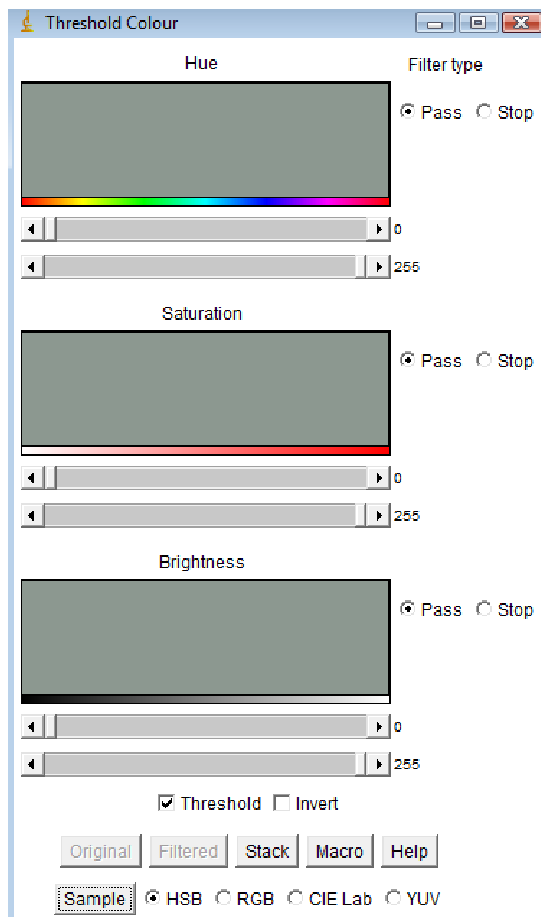
4. Click on **THRESHOLD**, and the selection will appear in black.

5. Click on **ORIGINAL** for the image the resume its initial appearance.

6. Once the selected colour has been thresholded, the **ROI MANAGER** can be opened as previously described.

7. Using the **WAND TOOL** and **SHIFT** key, desired image areas can be selected, and using **ADD INS**, can be grouped together in the **ROI MANAGER**. Thereafter, measurements can be obtained.

8. It is possible to highlight the area selections in the **ROI MANAGER**, and superimpose the latter on the original image, and thus verify the success of the colour threshold for a given area.



ANNEXE B

PICROSIRIUS-RED STAINING PROTOCOL FOR COLLAGEN FIBERS

Reagents Required:

0.05 M phosphate buffer[12]

0.5% papain solution[13]

0.1% picro-sirius red[14]

Staining Procedure:

1. Dewax section in toluene for 5 minutes

2. Dehydrate for 2 minutes in 100% ethanol solution

3. Dehydrate for 2 minutes in 95% ethanol solution

4. Dehydrate for 2 minutes in 80% ethanol solution

5. Wash in running tap water for 25 minutes.

6. Wash in a few changes of distilled water.

7. Wash in phosphate buffer (pH 4.7 and 0.05 M).

8. Incubate at 37°C in 0.5% papain (pre-warmed) solution pH 4.4 for 90 minutes.

9. Wash in a few changes of distilled water.

10. Place slides in 0.1% picro-sirius red for 60 minutes.

11. Wash in 0.01 N HCl for 2 minutes.

12. Dehydrate for 2 minutes in 95% ethanol solution

13. Dehydrate for 3 minutes in 100% ethanol

14. Wash in toluene for 5 minutes

Slides were then covered with a coverslip and left to dry overnight.

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[12] Fisher S-244 Lot 870519 Na2S2O5

[13] Aerus organics company Code 416765000, CAS: 9001-73-4, EC:232-627-2, lot: AO218172001

[14] Direct Red: Alfa Aesar Company, Direct Red 80 code 2610-10-8 FW 1373-09EINECS B211693, lot 10114582