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Bronchial angiogenesis in asthmatic horses

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Résumé

L'asthme équin est une maladie inflammatoire chronique des voies respiratoires inférieures caractérisée principalement par des changements structuraux menant à un épaississement de la paroi des bronches et à l'obstruction du débit d'air. Le traitement de l'asthme équin inverse partiellement ce remodelage. Dans l'asthme, chez l'humain, la démonstration que l'angiogenèse contribue à l'épaississement de la paroi bronchique en augmentant la vascularisation de la muqueuse respiratoire ouvre une nouvelle fenêtre pour un traitement plus ciblé. Cependant, peu d'information est disponible sur le rôle potentiel exercé par l'angiogenèse dans l'asthme équin. L'objectif de cette étude est de documenter la présence d'angiogenèse dans les voies respiratoires inférieures des chevaux asthmatiques. Des échantillons bronchiques récoltés chez sept chevaux asthmatiques éprouvant une exacerbation de la maladie, sept chevaux asthmatiques en rémission clinique et chez sept chevaux sains du même âge ont été étudiés. L'analyse immunohistochimique a été réalisée en utilisant le collagène de type IV comme biomarqueur pour les membranes basales des vaisseaux sanguins. Le nombre de vaisseaux, la densité vasculaire, l'aire vasculaire et les valeurs moyennes de taille des vaisseaux ont été mesurés par histomorphométrie à l'aide d'un logiciel d'analyse d'images (Image J) et les valeurs provenant des trois groupes comparés à l'aide d'une ANOVA à une voie ($p < 0,05$). Un test post hoc Benjamini-Hochberg par paire a été effectué pour corriger le niveau alpha pour les mesures répétées. Une augmentation significative du nombre de vaisseaux chez les chevaux asthmatiques en exacerbation ($p = 0,007$) et chez les chevaux en rémission ($p = 0,02$) a été observée par comparaison aux chevaux sains. De plus, l'aire vasculaire était augmentée chez les chevaux souffrant d'asthme en exacerbation comparativement aux chevaux sains ($p = 0,02$) et ceux en rémission ($p = 0,04$). Aucune autre différence significative n'a été observée. En conclusion, les voies respiratoires centrales des chevaux asthmatiques présentent des indices d'angiogenèse, ce qui suggère qu'elle puisse contribuer à l'épaississement de la paroi des bronches. D'autres études sont justifiées afin d'évaluer la réponse à un traitement ciblé.

Mots-clés : Asthme, angiogenèse, chevaux, collagène IV

Abstract

Equine asthma is a chronic inflammatory disease of the lower airways characterized by structural changes that lead to bronchial wall thickening and airflow obstruction. Treatment for equine asthma partially reverse these remodeling changes. Angiogenesis has been shown to increase vascularization of the bronchial mucosa, which contributes to the thickening of the bronchial wall in humans with asthma, opening a new window for a targeted treatment. However, little information is available related to the occurrence of angiogenesis in asthmatic horses. The objective of this study is to document the presence of angiogenesis in the bronchi of asthmatic horses. Bronchial samples from seven asthmatic horses collected during an episode of exacerbation of the disease, seven asthmatic horses in clinical remission, and seven age-matched healthy horses were studied. Immunohistochemistry analysis was performed with type IV collagen as a biomarker for basement membranes. The number of vessels, vascular density, vascular area and mean vessel size values were measured by histomorphometry using an image analysis software (Image J) and values from all three groups were compared using a one-way ANOVA ($p < 0.05$). A Benjamini-Hochberg pairwise post hoc-test was performed to correct the alpha level for repeated measurements. A significant increase in the number of vessels in asthmatic horses in exacerbation ($p = 0.007$) and in horses in remission ($p = 0.02$) was observed in comparison to controls. Similarly, the vascular area was increased in horses with asthma in exacerbation when compared to controls ($p = 0.02$) and to horses in remission ($p = 0.04$). No other significant differences were observed. In conclusion, angiogenesis is present in the central airways of asthmatic horses, suggesting that it may contribute to the thickening of the airway wall. Further studies are warranted in order to assess the response to a targeted treatment.

Keywords: Asthma, angiogenesis, horses, collagen IV

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List of Abbreviations

Adj.: Adjective

BAL: Bronchoalveolar lavage

TTW: Transtracheal wash

CCSP: Clara cell secretory protein

Etc.: Etcetera

NANC: Non-adrenergic-non-cholinergic

ECM: Extracellular matrix

MMP: Matrix metalloproteases

PG: Proteoglycans

GAGs: Glycosaminoglycans

SM: Smooth muscle

EP: Epithelium

NBI: Narrow band imaging

COPD: Chronic obstructive pulmonary disease

VEGF: Vascular endothelial growth factor

HIF: Hypoxia-inducible factor

ANG-1: Angiopoietin-1

ANG-2: Angiopoietin-2

VVG: Verhoeff's Van Gieson

IHC: Immunohistochemistry

PECAM-1: Platelet endothelial cell adhesion molecule 1

CD-31: Cluster of differentiation 31

CD-34: Cluster of differentiation 34

vWF: von Willebrand Factor

"No one who achieves success does so without acknowledging the help of others. The wise and confident acknowledge this help with gratitude"

Alfred North Whitehead

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To Dr. Jean-Pierre Lavoie, I would like to express my immense gratitude for welcoming me into the research world. Thank you so much for your guidance and support during this time, your great experience and immeasurable knowledge made me more analytical and thorough, a quality needed for conducting research. I could not imagine having a better mentor for my Master study.

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INTRODUCTION

Disturbances of the respiratory system may lead to poor performance in horses and secondary worsening of their condition overtime. For this reason, respiratory abnormalities have been considered of great importance for several years. In 2016, facing the variety of processes inducing lower airway inflammation in horses, the consensus statement on Inflammatory airway disease of horses – Revised Consensus Statement from the American College of Veterinary Internal Medicine pooled together horses with mild (previously known as inflammatory airway disease, IAD) and severe (formerly recognized as recurrent airway obstruction, RAO) clinical signs of asthma under the term of "Equine asthma".

Equine asthma syndrome is defined as a chronic inflammation of the lower airways characterized by structural changes that lead to remodeling of the airway wall contributing to impairment of the lung function [1]. Airway changes are only partially reversible with current conventional treatments, predisposing to the establishment of permanent baseline deficits in lung function of asthmatic horses.

Previous studies in asthmatic horses have demonstrated that structural changes, such as an increase in the bronchial smooth muscle mass, collagen deposition and epithelial hyperplasia contribute to the impairment of normal lung function in asthmatic horses. The vascular component associated with asthma in humans has been studied, suggesting that an increased angiogenesis is present in asthmatic patients when compared to non-asthmatic patients (controls). Several studies have proposed that angiogenesis in asthmatic humans is likely due to an increased number of vessels as well as an increase in vascular area. However, no association between airway wall vascularization and bronchial wall remodeling in equine asthma patients has been investigated to date. Based on similarities between equine and human asthma, an analogous phenomenon with increased angiogenesis may be expected. The objective of this study was to document the presence of angiogenesis in the bronchi of asthmatic and healthy control horses and determine whether asthmatic horses demonstrate greater indices of angiogenesis than healthy horses.

The results of this study may contribute to a better understanding of the pathophysiology of equine asthma, which may open a new window for treatment of this syndrome.

LITERATURE REVIEW

1. Respiratory system overview

The respiratory system of the horse is a unique and highly specialized system that can move large volumes of air in and out of the lungs each minute. It is composed by the upper (nares, nasal passages, paranasal sinuses, guttural pouches, nasopharynx and extrathoracic part of the trachea) and lower respiratory tract (intrathoracic trachea divides into bronchi, bronchioles and alveoli) [2]. The respiratory system can also be divided into a conducting and respiratory portion, according to the role played by its components [3]. The conducting portion, with the upper respiratory tract, allows transit of air to the pulmonary portion. It does not intervene in gas exchange. The respiratory portion is composed of respiratory bronchioles, alveolar ducts, alveolar sacs and pulmonary alveoli, main regions where gas exchange occurs. Lastly, the diaphragm serves as a pumping mechanism altering the negative pressure of the pleural cavities and promoting air distribution with each movement [3].

1.1. Gross anatomy of the trachea and equine lung.

The trachea is a tubular structure that connects the upper and lower airways and can be classified as extrathoracic or intrathoracic. The intrathoracic portion bifurcates at the level of the carina into the mainstem bronchi (left and right) and enters the hilum of the lung [4] (Figure 1). The equine lung along with cattle and pigs have a different morphology in comparison with other species such as companion animals [5; 6]. The separation of the right and left lungs into lobes is not visible externally as it is in dogs and cats, but their lungs are septate by elastic and collagenous tissue fibers into two lobes in both the right and left hemithorax. Furthermore, the right lung does have an accessory lobe [7; 8]. The absence of respiratory bronchioles and this characteristic morphology of the equine lung contributes to limit the collateral ventilation to a minimum (i.e. ventilation of alveolar structures through accessory pathways) [9], increasing the horse susceptibility to gas exchange problems, more pronounced in horses with airway obstruction [10]. With an incomplete septa, and a minimum collateral ventilation, the high resistance of the accessory pathways to airflow (e.g. pores of Kohn, the canals of Lambert, etc.) allows only an air maximum of 16% of its required volume [11]. The equine lung morphology has been classified as a type III,

characterized by incompletely developed secondary pulmonary lobules, thick vascular visceral pleura and well defined interlobular septae [12]. In addition, the lungs are covered by a thick serous membrane named pleura, divided into visceral and parietal pleurae. The visceral pleura covers the lung whilst the parietal pleura covers the rest of the thoracic cavity [3].

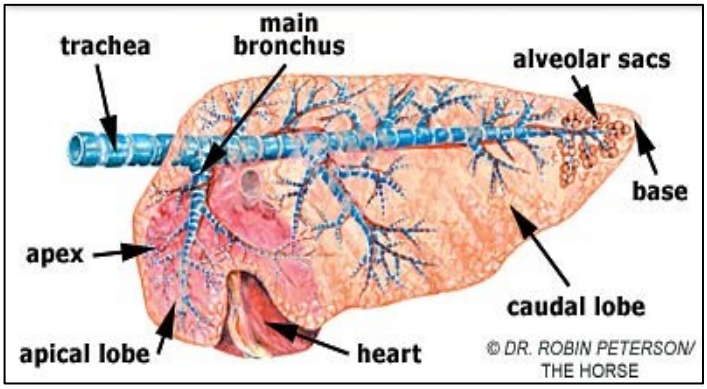

Figure 1. Representation of gross lung morphology 1. The equine lung is divided into left and right lobes in the corresponding hemithorax. The most cranial aspect is defined as the apex, and the most caudal part as the base.

Starting from the tracheal bifurcation, the mainstem bronchi will undergo multiple divisions, each daughter having a lumen of smaller diameter than the branching parent, giving rise to a multitude of branches. The smallest airways, the bronchioles are further differentiated into terminal bronchioles and poorly develop respiratory bronchioles, if present, which lead to the alveoli, where gas exchange occurs. The airway branching in the horse is pseudo-dichotomous or monopodial, meaning that it's an asymmetrical branching (one branch is larger than the other) and all branches (daughter bronchi) arise from a main axis (parent bronchus) [13]. Branching begins at the level of the carina (tracheal bifurcation) and continues until the distal airways. The distance between the carina and the distal airways depends on the size of the horse, with small horses having the shortest distances [13]. The principal bronchi divide into lobar bronchi, which supply air to each lobe of the lung. Lobar bronchi are further divided in segmental and subsegmental bronchi.

[1] Taken from and authorized by *Dr. Robin Peterson at www.fernwoodstudio.com*.

The right mainstem bronchus yields air to the cranial, accessory and caudal lobar bronchi, which are subsequently divided into smaller segmental bronchi. Divisions of the left mainstem bronchus follows the same pattern, with the exception of the left accessory lobar bronchi that is inexistent [3; 14] (Figure 2). The diameter of the lung branches according to their location has previously been studied [13]. In brief, airways closer to the carina are larger than distal airways, located in the periphery of the lung. Due to the large inter-animal and intra-animal variations, there is no information on the exact number of branches [15], but some studies report that horses may undergo 38-43 divisions from the carina to the alveolar ducts [8].

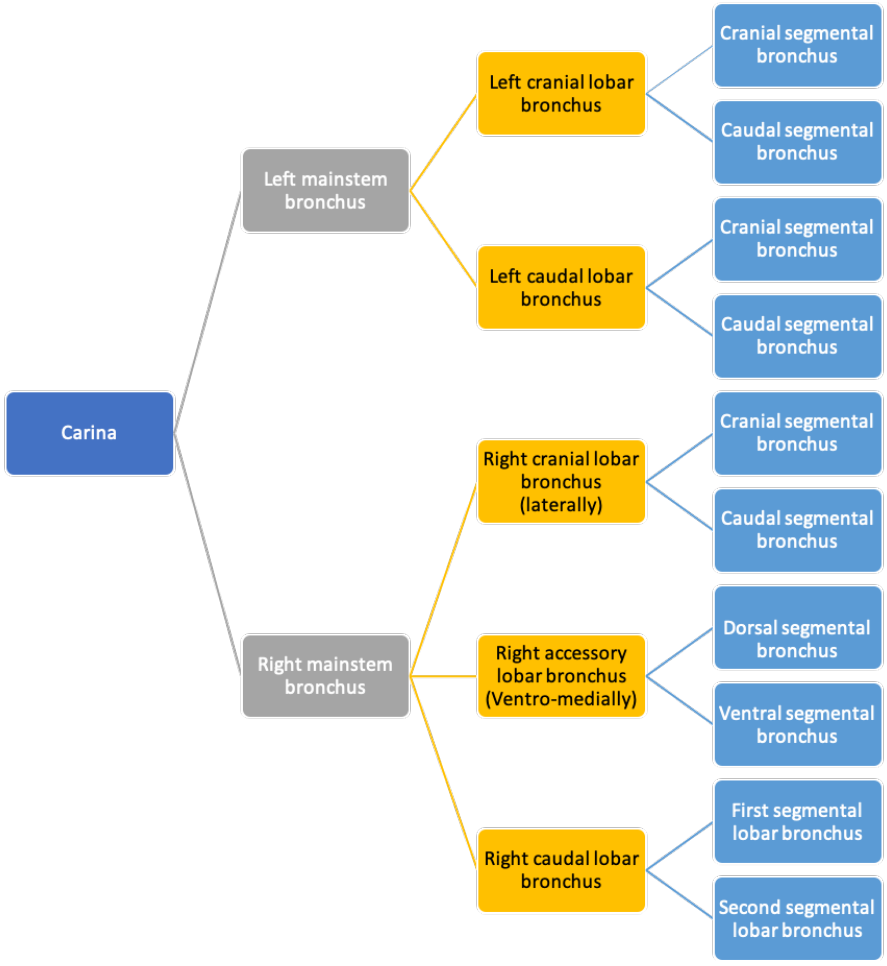

Figure 2. Schematic representation of the bronchial tree of the horse. Left and right mainstem bronchi begin their division at the carina, followed by lobar and segmental bronchi.

Bronchi can be differentiated from other structures in most species, by the presence of cartilage in their wall and their caliber, which is usually larger than 2mm [15]. The bronchus further divides into small airways, called bronchioles, which are characterized by the absence of cartilage in their wall and by their small diameter (approximately <2mm) [16]. Furthermore, bronchi and bronchioles are characterized by the presence of a layer of smooth muscle in their wall, which plays a fundamental role in bronchoconstriction-bronchodilation. The bronchioles connect the small bronchi to the alveoli. In contrast to other species having well developed respiratory bronchioles, the horse has non-respiratory or poorly developed respiratory terminal bronchioles (most distal bronchiole) that connect directly to either the alveolar duct or the alveoli [12; 15]. Located distal to the bronchioles, the alveolar ducts and alveoli constitute the basic unit of ventilation, where the gas exchange occurs. The equine lung is formed at least by 10^7 alveoli, and 1000 times more capillary segments available for gas exchange. This surface is considerably larger than in other species [11; 17].

1.2. Subgross anatomy of the lower airways

Tracheal and bronchial histology consists of ciliated and non-ciliated cells organized in a tall pseudostratified columnar epithelium overlying the basement membrane. The non-ciliated goblet cells, mainly produce mucus (mucin), a portion of the mucus layer of the airways, which is transported cranially by the ciliated cells [15]. The epithelium gradually changes from a pseudostratified to a simple columnar epithelium as bronchi become smaller with each division. Underneath, a connective tissue layer termed the lamina propria is observed. Different elements constitute this lamina, collagen being the main component, followed by elastic fibers, blood vessels and nerves among other structural components. Spiral bands of smooth muscle are also displayed beneath the lamina propria. The set of all the layers previously described are defined as the airway mucosa [15; 18]. The submucosa is the layer observed underneath and is formed by a mix of connective tissue, bronchial glands (only in large bronchi) and blood vessels. Hyaline cartilage surrounds the submucosa, however this structure disappears in small bronchi, allowing differentiation between bronchi and bronchioles [16]. Finally, located at the most external region, is an adventitial layer formed by connective tissue that fuses with the lung parenchyma (Figure 3).

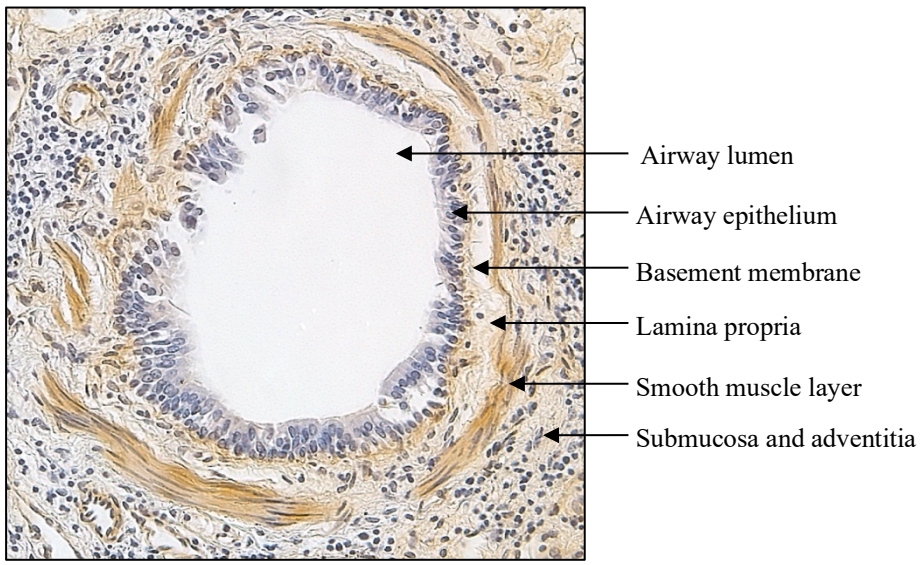

Figure 3. Schematic representation of the equine bronchial structural components. Lung tissue sample stained with type IV collagen.

The bronchioles have a different epithelium when compared to the bronchi. The epithelium consists of a layer of short ciliated cuboidal cells superficially, and non-ciliated secretory club cells (previously known as Clara cells) underneath, all surrounded by a layer of smooth muscle. The goblet cells present in healthy bronchi are absent in bronchioles, unless airway inflammation is present [15; 19]. The secretory club cells represent approximately 60% of the epithelium, each cell containing approximately 6 to 28 granules in the bronchi of a normal horse. These cells secrete Clara cell secretory protein (CCSP) and in a lesser degree, mucin. When airway inflammation is present, an increase in mucus production by the club cells occurs, leading to the "Clara-goblet cells" [20].

The most distal structure of the lung is the alveolus, which is formed by type I and II pneumocytes (Figure 4). Type I pneumocytes surround each alveolus and are characterized by a thin cytoplasmic extension. Type II pneumocytes are more numerous and metabolically active than type I pneumocytes [15; 21]. Type II cells are characterized by cytoplasmic inclusions that contain surfactant, contributing to maintain alveolar stability [22; 23]. Type II pneumocytes have an important role in repairing lung injuries, absorbing alveolar edema and differentiating into type I pneumocytes when needed during the repair process.

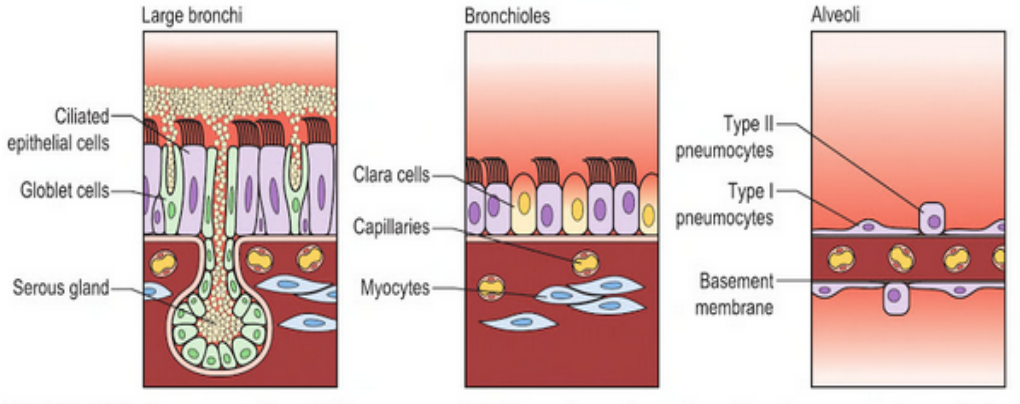

Figure 4. Epithelial composition in different parts of the lower airway: large bronchi, bronchioles and alveoli [2].

1.3. Cytological features of the lower airways

To evaluate cytological features of non-septic diseases of the lower airways, different diagnostic modalities are available. Bronchoalveolar lavage (BAL) is superior to trans-tracheal wash (TTW) due to the possible degeneration of the cells in TTW samples [24; 25]. BAL may serve to sample a specific portion of the lung, however it does not necessarily constitute a true representation of the cytologic features of the entire lung any more than an exact representation of the bacteria present in the entire lung [26]. The cellularity observed in BAL samples (Figure 5) is normally low and predominantly includes macrophages (50-70%), small lymphocytes (30-50%), and in a lesser extent, neutrophils (<5%), mast cells (<2%) and eosinophils (<1%). Epithelial cells may also be present [27; 28]. The BAL composition becomes important when identification of airway inflammation is warranted. Bronchoalveolar lavage features of airway inflammation include an increase in total cell count, increased percentage of neutrophils, some of which can show degenerative changes,

[2] Taken from and authorized by Art T. and Bayly W (2014). 27-Lower airway function: response to exercise and training. Equine Sports Medicine and Surgery (Second Edition). K. W. Hinchcliff, A. J. Kaneps and R. J. Geor, W.B. Saunders: 587-603.

increased percentage of mast cells and eosinophils [27; 29]. Caution should be taken when performing a BAL since the cytological features, such as the percentage of neutrophils can be altered according to the volume of saline instilled [30].

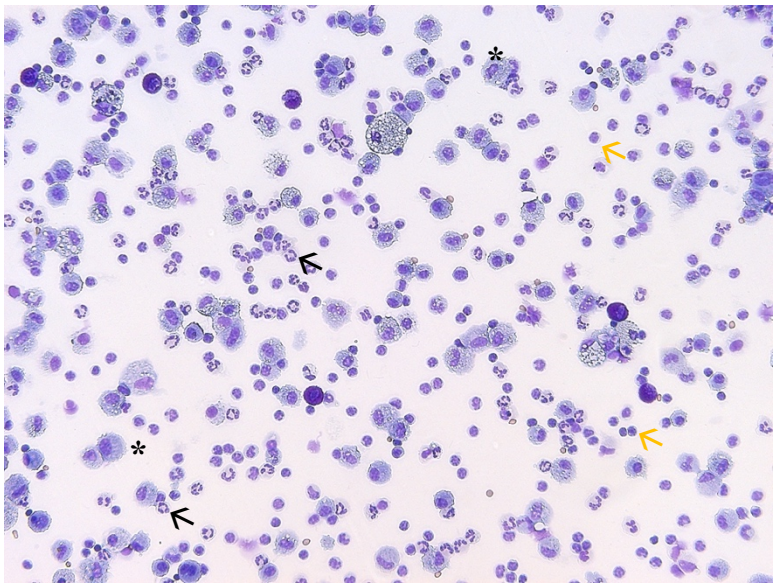

Figure 5. Cytology of bronchoalveolar lavage fluid in an asthmatic horse. The predominant cell types are pulmonary alveolar macrophages (asterisk), neutrophils (black arrow) and lymphocytes (yellow arrow).

1.4. Blood supply of the lower airways

The equine lung is perfused from two different pathways, the pulmonary and the bronchial circulations. The bronchial circulation is a low flow, high pressure system [31] that originates from two different sources. The first source, the broncho-esophageal artery, supplies most of the airways, the interlobular septa and the sub-pleural connective tissue [12]. The second source, the bronchial artery (branch of the aorta), supplies the right lung and receives approximately between 1% and 2% of the cardiac output from the left ventricle in a horse at rest [22; 32]. This circulation is considered as a branch of the systemic circulation and supplies the intrapulmonary airways components with arterial blood. The more peripheral structures are supplied by the pulmonary artery [8]. The terminal

bronchioles are vascularized by an anastomosis between the capillaries and the vein, arising from both types of circulation (pulmonary and bronchial). This phenomenon will contribute to maintain blood flow in situations where the pulmonary perfusion is impaired [22; 33]. The pulmonary arteries alongside the bronchi are elastic, whereas the arteries surrounding the bronchioles and alveolar ducts are muscular [31]. The hypoxia receptors of the smooth muscle are responsible of the response of vessels to hypoxia [11]. A difference in size between the two types of arteries is also observed, the pulmonary artery being larger than the bronchial artery. The venous drainage from the extra-pulmonary airways is carried by the azygos vein, which will drain into the cranial vena cava. The venous drainage from the intrapulmonary airways circulates into the pulmonary veins at the pulmonary capillary level. The outflow pressure of this venous drainage varies depending on the who is performing the drainage, either the azygos vein or the pulmonary circulation [31].

The pulmonary circulation is a high flow, low pressure system that holds the total cardiac output arising from the right side of the heart [32]. The deoxygenated blood is carried to the lungs via the pulmonary artery and its branches. Blood is then oxygenated (arterialized) at the level of the alveolar capillaries and returned by the pulmonary veins to the left atrium of the heart [11; 22]. Oxygenation of blood constitutes the principal function of the pulmonary circulation. However, it can also accomplish filtration and removal of chemical substances. Blood flow distribution in the equine lung is believed to be similar to that of the human lung [22], with the ventral region receiving more perfusion due to the influence of gravity and the generated vertical gradient. However, recent studies have shown the opposite, where blood flow was greater in the dorsal regions in comparison to the ventral regions, suggesting that vessel length and resistance may represent the major factors, and excluding gravity as previously thought [22; 33; 34]. Characteristics of both bronchial and pulmonary circulations are detailed in Table 1.

	Pulmonary	Bronchial
Aims	• Gas exchange • Venous blood filtration • Blood reservoir	• Nutrition of airways, vessels, and visceral pleura. • Thermoregulation
Structure	Right ventricle ↓ Pulmonary artery ↓ Pulmonary arterioles ↓ Pulmonary capillaries ↓ Pulmonary veins	Left ventricle ↓ Bronchial and bronchoesophageal arteries ↓ Peribronchial plexus ↓ Subepithelial plexus ↓ Pleural, vascular, and ganglia plexi ↓ Azygos vein
Blood flow (liters/min)	± 99 percent of the right ventricle: 30 (280)	± 2 percent of left ventricle: 0.6 (6)
Pressure (mmHg)	Arterial: 30 (100) Capillary: 20 (80) Venous: 10 (60)	Arterial: 100 (200) Capillary: 20 (>60) Venous: 15 (60)
Capillary flow	Pulsatile	Constant
Vascular resistance (mmHg/liter/min)	0.7 (0.25)	140 (20)
Effect of hypoxia	Vasoconstriction	Vasodilation
Effect of hyperthermia	-	Vasodilation
Effect of pleural pressure changes	+++	+

Table 1. Comparison between Pulmonary and Bronchial Circulations.[3]

A continuous production of lymph occurs in the lung, due to fluid transport between the pulmonary microvasculature and the interstitial tissue. The lymphatic vasculature is organized in a superficial and a deep layer. The superficial layer creates a network of vessels along the pleura (sub-pleural); while the deep layer follows the bronchi and pulmonary vessels [11]. These lymphatics, drain into the thoracic duct, and connect the hilum and mediastinal lymph-nodes [15]. All lymphatic vessels enclose the airways and non-capillary

[3] Taken from and authorized by Lekeux, P., et al. (2014). Chapter 9 - The respiratory system: Anatomy, physiology, and adaptations to exercise and training. The Athletic Horse (Second Edition). D. R. Hodgson, K. H. McKeever and C. M. McGowan, W.B. Saunders: 125-154

blood vessels, without penetrating the alveolar septa. During respiration, fluid transport is increased in the lymphatic vessels, since they can accommodate large quantities of fluid. Additionally, they have the capacity of removing fluid and other substances such as antigens and leukocytes from the different blood vessels (arterioles, capillaries, and veins). The lymphatic system is not only important for the movement of fluid, but also for alveolar hydration. The lack of permeability of the alveolar epithelium in comparison to the capillary endothelium, allows the alveoli to be exempt of interstitial fluid under normal conditions [31]. When the epithelium is present in the interstitium, fluid accumulates in the alveoli, a condition known as pulmonary edema [32]. Clearance of alveolar fluid accumulation by the lymphatic system is fundamental. However, an increase in the interstitial hydrostatic pressure associated with pulmonary edema, may lead to the compression of the lymphatic vessels, impeding their normal function [35; 36].

1.5. Innervation of the lung

The lung is innervated via different nervous systems: sympathetic, parasympathetic and non-adrenergic ± non-cholinergic (NANC) inhibitory and excitatory nervous systems [4; 22]. The sympathetic and parasympathetic systems form a pulmonary plexus from which they innervate the lung. The sympathetic motor fibers through the middle cervical and cervicothoracic ganglia, control bronchodilation of the airways; whereas the parasympathetic fibers through the vagus nerve control bronchoconstriction [37; 38]. The parasympathetic cholinergic nerves secrete acetylcholine to produce bronchoconstriction of the airway smooth muscle via muscarinic receptors, and to the submucosal glands via nicotinic receptors. The sympathetic system is smaller than the parasympathetic, and is believed to induce relaxation of the airway smooth muscle by activation of β-adrenoreceptors [39]. A dysregulation between both systems seems to play an important role on the pathophysiology of diseases like asthma [40], where inflammatory mediators can lead to an increased cholinergic neurotransmission contributing to the bronchospasm observed [41-43]. NANC excitatory system contribute to smooth muscle contraction, inflammatory cell activation and mucus secretion among others, by the release of neuropeptides such as tachykinins. In addition, NANC inhibitory system modulates the excitatory response of the

airways by the secretion of nitric oxide. This is important in the control of airway hyperresponsiveness in asthmatic horses [39].

1.6. Function of the respiratory system

The main function of the respiratory system is to oxygenate blood, and to remove carbon dioxide from the blood, a process defined as gas exchange [22]. The gas exchange occurs in the alveoli and the alveolar duct coated with a capillary structure, and then diffuses into the bloodstream. The oxygenated blood then returns to the heart who pumps it through the whole body. During exercise, the demand for oxygenated blood and disposal of carbon dioxide increases considerably [44]. There are multiple factors participating in the gas exchange, such as ventilation, perfusion, diffusion, and gas transport. Other non-pulmonary functions accomplished by the respiratory system include defense mechanisms, surfactant production, thermoregulation, filtration and humidification of the inhaled air, among others [11; 22].

2. Extracellular matrix and components

The extracellular matrix (ECM) is a dynamic three-dimensional fibrous network that provides physical scaffolding for the cellular components and is also fundamental for biomechanical and biochemical properties of the airways [45-47]. The ECM components can be primarily divided into two sections, the pericellular matrix or basement membrane, adjacent to epithelial cells, and the interstitial matrix, which surrounds the cells. The ECM is mainly composed of fibrous connective tissue proteins such as collagen, elastic fibers, laminin, structural glycoproteins and proteoglycans [48; 49]. The continuous remodeling of the ECM allows growth and regeneration; a regulation of the degradation is crucial. The ECM degradation is carried out by proteolytic enzymes such as proteases, especially the matrix metalloproteases (MMPs) [50; 51]. A dysregulation on the ECM has been associated with the development of pathologic conditions such as asthma [49].

2.1. Components

2.1.1. Basal membrane

The basal membrane is a type of pericellular matrix that serves as an anchor between the alveolar epithelium and the adjacent connective tissue, and contributes to the regulation of the epithelial cells' differentiation [52; 53]. This membrane is composed of a multilayer structure that comprises the lamina densa, lamina lucida and lamina fibroreticularis. The lamina lucida being the closest to the epithelium, followed by the lamina densa. The last two layers (lucida and densa) are often grouped under the term of basal lamina [54]. Basal membranes are mainly composed of laminin, collagen, proteoglycans and entactin. Integrins connect the basal lamina to the epithelium, whilst collagen type IV connects it to the ECM [48; 49; 53].

2.1.2. Collagen

Collagen is the main protein component of the ECM in all animals. It is constituted by three α polypeptide chains that are intertwined with each other and form a right-handed triple helix that grants tensile strength to the ECM [48]. Collagen represents 60% of the lung connective tissue and comprises 30% of the total proteins in humans [49; 55]. The different collagen subtypes can be grouped into fibrillar or non-

fibrillar. The types more commonly found in the lung are collagen type I, II, III, IV, V and VI [48]. The distribution of collagen in the lung varies regionally in the ECM. Previous studies have shown type I, III and VI in large bronchi and blood vessels; collagen II in bronchial and tracheal cartilage, and type IV and V in alveolar and capillary basement membranes in humans [47; 56]. However, little is known about the distribution of collagen subtypes in the equine lung. Studies showed the presence of types I and III in the lamina propria and adventitia of small equine airways [46; 57](Figure 6).

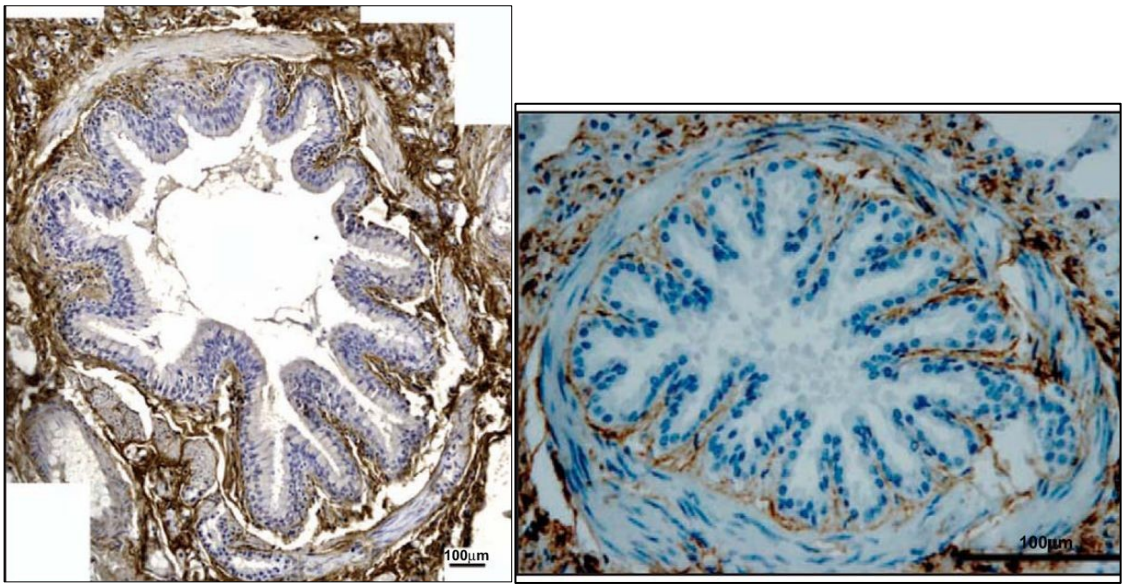

Figure 6. Identification of collagen type 1 (left) and type 3 (right) in the equine airways [4]. Collagen types 1 and 3 were present in the lamina propria and the adventitia of the airways, but not in the basement membrane.

2.1.3. Elastin

The intrinsic recoil properties of the lung depend on the presence of elastic fibers, which have two major components: elastin and microfibrils. Elastin is an insoluble matrix protein composed mainly of hydrophobic amino acids and glycine that can endure

[4] Taken, modified and authorized from Setlakwe, E. L., et al. (2014). "Airway collagen and elastic fiber content correlates with lung function in equine heaves." American Journal of Physiology: Lung Cellular and Molecular Physiology 307(3): L252-260. Doi: 10.1152/ajplung.00019.2014

high linear stress-strain, with a half-life similar to the lifespan of the organism [47; 53]. The distribution of elastin in the lung differs from the one observed with collagen, with the parenchyma having the highest elastin content. In 1955, elastin was considered fundamental during normal breathing, whereas collagen importance was thought to rely on total lung capacity. However, recent data propose an equal importance even at lower lung volumes [58; 59], which suggest that both component may modulate both bronchoconstriction and bronchodilation. In addition, elastin content in the airways has been positively correlated with pulmonary elastance (E_L) in healthy horses [46].

2.1.4. Glycosaminoglycans and Proteoglycans

Proteoglycans (PGs) are a family of major core proteins synthetized by most cells. They have diverse functions such as permeability barriers and binder component to different molecules like growth factors, cytokines and proteases [53]. According to their location, PGs can be classified as intracellular, cell surface, pericellular-basement membrane, and extracellular [49]. These proteins are constituted of glycosaminoglycans (GAGs) covalently combined with a core protein, and together they control all cellular processes from signaling and proliferation, to apoptosis and adhesion [47; 49; 58].

2.2. Functions and Importance

The ECM functions in the lung are fundamental for tissue homeostasis and normal organ function. This includes tissue compliance, control of cell behavior and tissue repair and remodeling [48]. Disturbances of any of these components may lead to modifications of the environment and can lead to different pathologies. For example, the dysregulation of proteoglycans may jeopardize the protective duty of the ECM and lead to interstitial edema [48; 49].

3. What is Equine Asthma?

3.1. Equine Asthma

Equine asthma is the term used to describe a chronic inflammatory syndrome of the lower airways, which groups together horses with mild to moderate (previously known as inflammatory airway disease, IAD) and severe asthma (formerly recognized as recurrent airway obstruction, RAO). Rather than a single condition with different manifestations, equine asthma is a variety of conditions leading to similar clinical manifestation [1]. Clinical signs associated with this syndrome can vary in severity, but it is mainly characterized by inflammation of the lower airways resulting in airway obstruction, airway remodeling, excessive mucus accumulation and poor performance.

3.1.1. Airway remodeling

Airway remodeling develops as a consequence of the chronic inflammation occurring in asthmatic horses. Remodeling is characterized by histological changes including epithelial hyperplasia and denudation, subepithelial collagen deposition, smooth muscle hypertrophy/hyperplasia, leading to thickening of the airway wall. Furthermore, mucus accumulation results in luminal obstruction [41; 60; 61]. All these histological changes contribute to an impairment of the normal lung function leading to breathing dyspnea. Airway remodeling is reported to be only partially reversible with standard treatment [62; 63].

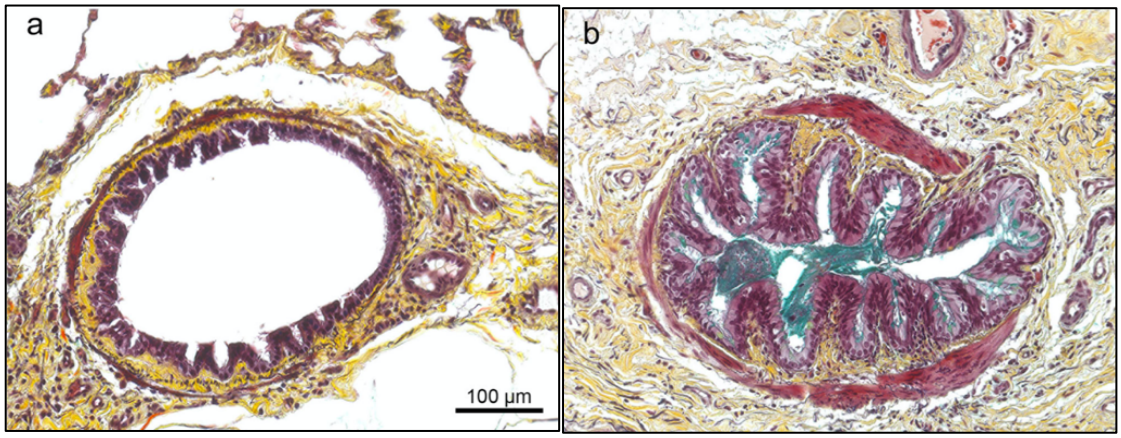

Figure 7. Non-asthmatic (a) and asthmatic (b) bronchial sections of two horses stained with modified Russel-Movat Pentachrome. Structural changes such as smooth muscle thickening (pink right image) are observed in conjunction with the presence of mucus in the lumen (green right image).[5]

3.1.1.1. Extracellular matrix remodeling

Subepithelial collagen deposition underneath the epithelium (lamina propria) is considered one of the main causes of the thickening of the airway wall, in conjunction with increased bronchial smooth muscle mass [62]. This deposition is most likely secondary to the activation of fibroblasts, key unit in the secretion of ECM components. Furthermore, this increased deposition has only a partial response to treatment [64]. A 2014 study showed an increase in elastic fibers and collagen deposition (collagen I and III) in horses with severe asthma when compared with non-asthmatic animals, and reported a positive correlation between collagen and pulmonary resistance (R_L) in asthmatic horses in

[5] Taken from and authorized by Bullone, M. and J.-P. Lavoie (2019). "The equine asthma model of airway remodeling: from a veterinary to a human perspective." Cell and Tissue Research. Doi: 10.1007/S00441-019-03117-4.

remission, suggesting that collagen deposition may contribute to airway obstruction [46].

3.1.1.2. Smooth muscle hypertrophy/hyperplasia

Different techniques have been used to assess the characteristics of the bronchial smooth muscle in asthmatic horses. Studies showed a smooth muscle thickening in airways of different sizes, being more pronounced in peripheric than in the central airways [65; 66] (Figure 8). This increased mass contributes to the narrowing of the lumen of the airway. Furthermore, the bronchoconstriction observed in asthmatic horses contributes to an impairment of the normal lung function [62].

Smooth muscle hypertrophy and hyperplasia have been suggested to represent an early feature of asthma [67; 68]. However, more recent studies have pinpointed a dynamic process described as an increase in myocyte apoptosis occurring in conjunction with *in situ* proliferation [66; 69]. This hypertrophy is only 30% reversible with current conventional treatments (inhaled corticosteroids and bronchodilators) [62; 70].

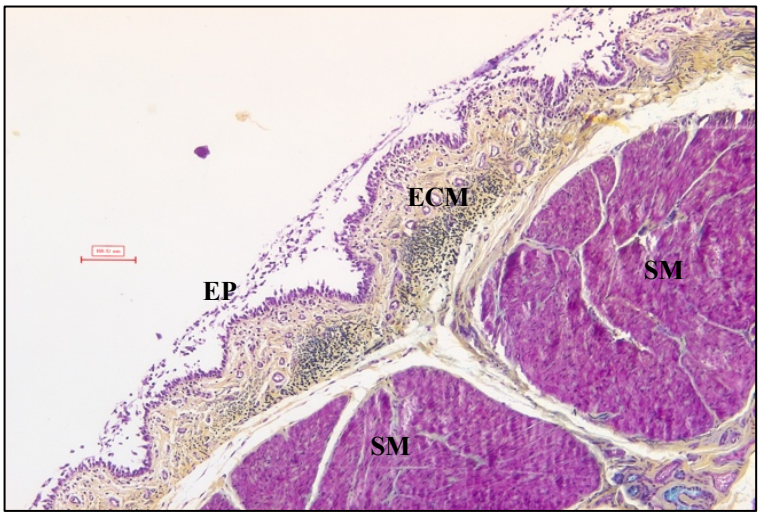

Figure 8. Smooth muscle hypertrophy in an asthmatic horse stained with Russel-Movat Pentachrome. EP, Epithelium; SM, smooth muscle; ECM, extracellular matrix. Scale 100µm.

3.1.1.3. Mucus accumulation

Mucus is a complex mixture of mucins (high weight molecular glycoproteins), water, enzymes, electrolytes, mixed with epithelial cells and leukocytes. The exact mechanism for mucus accumulation in airways of asthmatic horses, is not completely understood. Multiple theories exist such as, an increase in mucin due to the presence of hyperplasic secretory cells (goblet cells) [71]. Other theories include an excessive production of mucin being produced by normal cells, and without a change in their number, or by decreased clearance from a damaged ciliary apparatus [72].

3.1.1.4. Epithelial hyperplasia and denudation

Epithelial cells are fundamental for homeostasis of the equine lung. Multiple studies using electron and light microscopy have been conducted in order to describe the epithelial cell hyperplasia observed in asthmatic horses [73; 74]. These studies have shown a loss of ciliated cells in large diameter airways and goblet cell hyperplasia in airways of small diameter. Additionally, studies showed that asthmatic horses have epithelial damage secondary to inflammation which may lead to an impartment of the epithelial barrier function [20; 75].

4. Angiogenesis

Angiogenesis is defined as the formation of new vessels from pre-existing ones, an important difference with vasculogenesis, which is the formation of entirely new vessels (de novo). Vascular changes such as an increase in the number of vessels and vascular density, are among the structural modifications observed in humans with asthma [76]. In asthmatic horses, information regarding angiogenesis in airways is lacking.

4.1. Pathophysiology of angiogenesis

Angiogenesis is a fundamental process during embryonic development and physiological functions of the organism, including tissue repair and healing, chronic inflammation, etc. [77; 78]. In addition, angiogenesis is implicated in multiple pathologies, namely neoplasia, ocular disorders (e.g. neovascular glaucoma), and vascular malformations (e.g. cutaneous angiectasias and benign processes such as angiofibroma, hemangiomatosis) among others [77]. The formation of new vessels can be described according to their characteristics as sprouting or non-sprouting angiogenesis (i.e. intravascular subdivision).

The first step starts with an angiogenic stimulus that contributes to the activation of endothelial cells. This stimulus play a fundamental role on physiological processes of the endothelial cells such as growth, proliferation and migration [78]. This activation leads to an increase in vascular permeability of the pre-existing vessels, and activation of growth factors of the endothelial cells. These latter will then release enzymes (proteases) triggering the proteolytic degradation of the basement membrane. This degradation allows endothelial cells to migrate from the vessel wall towards the angiogenic stimulus, where they proliferate in the surrounding matrix. Following their proliferation, endothelial cells undergo changes in shape through a process called morphogenesis leading to the development of a lumen. The newly formed solid sprouts connect adjacent vessels [77-79](Figure 9).

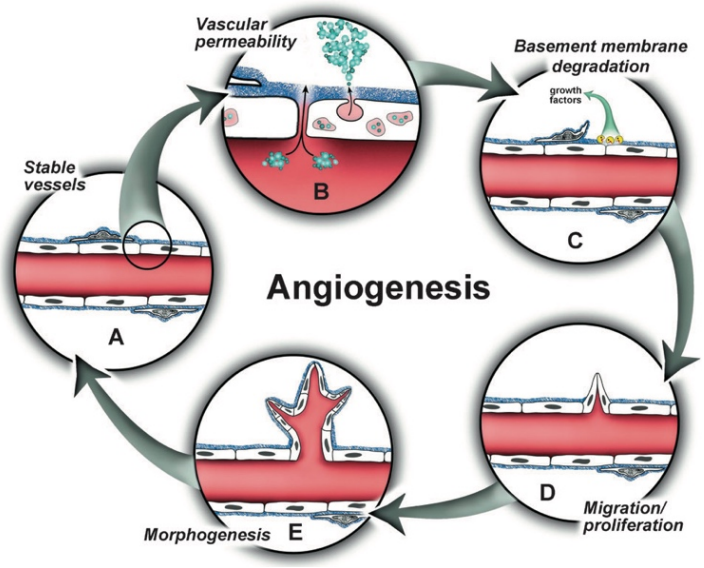

Figure 9. Schematic representation of the angiogenic cascade. Once angiogenesis is initiated, stable vessels (A) undergo an increase in vascular permeability, allowing extravasation of plasma proteins (B). Degradation of the ECM by MMPs relieves pericyte-EC contacts and ECM-sequestered growth factors are then released (C). ECs then proliferate and migrate to their final destination (D), where they assemble as lumen-bearing cords (E). ECM, extracellular matrix; MMPs, matrix-metalloproteases; EC, endothelial cell. [6]

4.2. Angiogenesis in lung diseases

The exact mechanism for angiogenesis in diseases with complex pathophysiology such as asthma, has been the object of interest of multiple studies. [80; 81]. Chronic inflammation has been shown to have a codependent relation with angiogenesis [82]. The proliferation and migration of inflammatory cells observed will contribute to the release of pro-angiogenic factors. Additionally, hypoxic tissues damaged by chronic inflammation can also participate to the release of angiogenic factors [83]. An example is the hypoxia-inducible factor (Hif), a transcription factor

[6] Taken from and authorized by Bryan, B. A. and P. A. D'Amore (2007). "What tangled webs they weave: Rho-GTPase control of angiogenesis." Cellular and Molecular Life Sciences 64(16): 2053-2065. Doi: 10.1007/s00018-007-7008-z

stimulated when low oxygen levels are detected in the tissues. The Hif regulates pro-inflammatory genes and has been shown to increase expression of vascular endothelial growth factor (VEGF) in horses with asthma [84]. This growth factor, VEGF, participate in the migration of endothelial cells from pre-existing vessels to the surrounding extracellular matrix, where they elaborate solid sprouts. Furthermore, angiogenesis contributes to the maintenance of the chronic inflammation observed in pathologies such as asthma, by transporting cells and nutrients to the inflammation site [82]. Chronic inflammation and hypoxia may lead to the concentric thickening of the airway wall, and thus, leading to narrowing of the airway lumen [80].

Multiple studies have been conducted evaluating the presence of angiogenesis in asthma and other chronic lung diseases in humans [85-87]. A 2010 study [88] demonstrated an increased number of vessels in the small airways of patients with asthma and chronic obstructive pulmonary disease (COPD), when compared with healthy individuals. In addition, other studies have shown an increased vascular area and number of subepithelial vessels in bronchial biopsies from both adults and children with mild to moderate asthma when compared to controls [89-91] (Figure 10). *In vivo* studies have also shown an increased number of vessels in the trachea of asthmatic patients versus control groups [92]. Treatment in humans aims to decrease airway inflammation and hyperresponsiveness. A 2002 study [93], showed that inflammatory cell counts in BAL and basement membrane thickness were reduced after 3 and 12 months of inhaled fluticasone treatment, respectively. No difference was observed in collagen deposition with the same treatment. Even though a partial reversibility is seen, with a decrease in basement membrane and smooth muscle thickness, remodeling in asthma is considered to be poorly reversible [94].

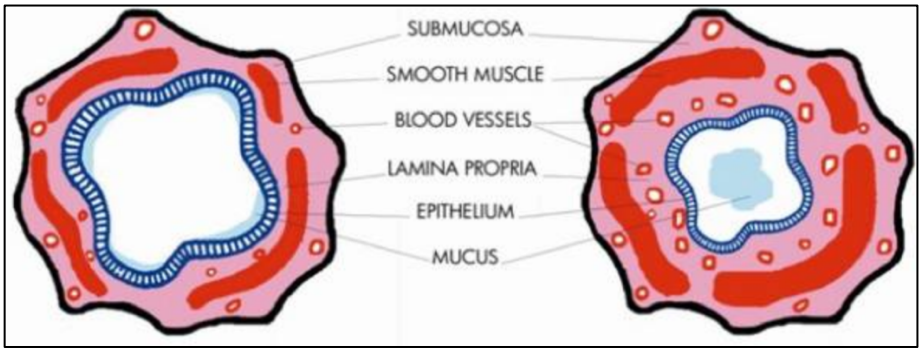

Figure 10. Schematic picture of a normal (left) and asthmatic airway (right) [7]. The different layers of the bronchi are outlined on the image. A larger number of vessels is seen on the asthmatic airway (right).

Due to the similarities in the immunopathology and clinical presentation between equine and human asthma, the angiogenic changes described in humans would be expected to occur in horses. However, the limited information currently available suggests otherwise. A 2012 study [95], evaluated the presence of angiogenesis in the large airways of horses with asthma using narrow band imaging (NBI). Superficial and deep vessels of three different sites along the airways were evaluated (trachea, carina and intermediate bronchi). The results obtained were not those expected since only a significant increase in vascular density of the superficial vessels of the trachea was observed. In face of these results, the authors concluded that the lack of significance at the level of the carina and bronchi, may suggest that the technique lacks sensitivity, or that angiogenesis only occurs in the trachea, contrary to the results obtained in human studies.

4.3. Angiogenic factors

Increased vascular permeability in humans with asthma has been related to the release of inflammatory mediators and growth factors, among others [88](Table 2).

[7] Taken from open access article Zanini, A et al. (2010) The role of the bronchial microvasculature in the airway remodeling in asthma and COPD. Respiratory Research 11, 132. Doi: 10.1186/1465-9921-11-132

Although *in vitro* studies have associated multiple factors with angiogenesis, vascular endothelial growth factor (VEGF) is considered to be the central protein leading to bronchial vascular remodeling [83; 96]. Furthermore, increased VEGF has been demonstrated *in vitro* in myeloid cells and *in vivo* in the BAL of asthmatic horses after exposure to hay dust [84]. Additionally, angiopoietins have been shown to play an important role in asthma due to their synergetic effect with VEGF on angiogenesis [97-100].

Angiogenesis	
VEGF	VIP
FGF	IL-8, IL-13
TGFβ	TNFα
HGF	NKA
HIF	Angiogenin
Ang-1	MMPs
Histamine	IGF-1
PGD_2	Chymase
PGI_2	VCAM-1
LTC2	E-selectin
PAF	$\alpha_v\beta_3$
SP	

Table 2. Factors involved in bronchial angiogenesis in humans with asthma and COPD[8]

4.3.1. Vascular endothelial growth factor (VEGF)

VEGF, previously known as vascular permeability factor, is a potent proangiogenic factor that prompts endothelial cell migration and proliferation [101]. Studies have shown an increase of VEGF expression in tracheal epithelial

[8] Modified from an open access article Zanini, A et al. (2010) The role of the bronchial microvasculature in the airway remodeling in asthma and COPD. Respiratory Research 11, 132. Doi: 10.1186/1465-9921-11-132

cell culture of asthmatic specimens when compared with controls [96]. This element has also been directly related to an increased vascular permeability, contributing to the thickening of the airway in human asthmatic patients [102]. An increased expression of VEGF by the airway epithelium after exposure to allergens may trigger vascular remodeling [96]. In addition, a correlation between this factor and subepithelial fibrosis has been reported, suggesting an effect not only on the number of vessels, but also on basement membrane thickness [103]. Furthermore, a 2012 study [84] showed that Hif has a strong positive correlation with the expression of VEGF in horses with asthma.

4.3.2. Angiopoetin-1 (Ang-1) and angiopoietin-2 (Ang-2)

The angiopoietin family constitutes another important family of factors directly related to the angiogenic process. Ang-1 is considered a ligand for endothelial cell surface receptor. It stimulates sprouting and helps stabilize the new formed vessels [88; 101]. In contrast, Ang-2 acts as an antagonist of Ang-1, destabilizing and disrupting the formation of new vessels [88; 99].

5. Angiogenesis detection and quantification

5.1. Laboratory techniques

Multiple laboratory techniques are available for the detection and quantification of blood vessels in tissues including immunohistochemistry, immunofluorescence and standard histological stains [89; 104; 105].

Standard histological stains such as Hematoxylin & Eosin (H&E), Movat Pentachrome and Verhoeff's Van Gieson (VVG) have been used to outline blood vessels. Movat Pentachrome stains red blood cells in yellow and elastic fibers in dark purple. Elastic lamina of large blood vessels adopts a dark brown/black color with VVG. However even though their sensitivity is high for large blood vessels, the specificity of these colorations for small vessels such as capillaries is low. Furthermore, regular stains such as H&E do not differentiate between blood and lymphatic vessels, which may lead to an increase in false positives [106-109].

Immunohistochemistry (IHC), is a laboratory technique used to identify a particular tissue component via specific antigen-antibody reactions in normal and pathological conditions [110; 111]. This technique uses an antibody (immunoglobulin) to react with a small area of the antigen of interest called epitope. These reactions are detected with fluorescent or enzymatic cell immunolabeling allowing detection of the precise location of the protein of interest [112]. Immunofluorescence (IF) is a specific type of IHC that uses fluorescent antibody labeling and can be further classified into direct or indirect according to their labeling technique [113]. Direct methods bind the primary antibody, which is immunolabeled, to the antigen, whereas the indirect process involves an additional step in which the secondary antibody binds to the primary, amplifying the signal (Figure 11). Indirect methods of immunostaining are more commonly used, due to their higher sensitivity [114; 115]. Although both techniques share a similar goal and both can outline blood vessels, IHC is considered the gold standard technique to quantify bronchial microvessels [88].

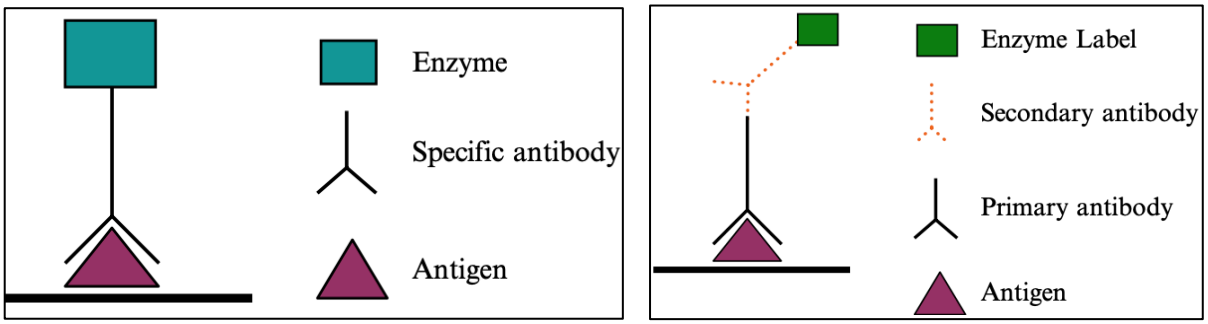

Figure 11. Representation of the direct (left) and indirect (right) immunochemical staining methods [9].

5.1.1. Antibody selection

Antibody selection is one of the key elements for successful IHC. These immunoglobulins are produced by B lymphocytes. According to their characteristics, antibodies can be classified into monoclonal and polyclonal. Monoclonal antibodies correspond to a homogenous group of molecules produced by the same clone of plasma B cells. Polyclonal antibodies refer to a variety of immunoglobulins which act against a specific antigen and are produced by different clones of plasma B cells [116](Table 3).

MONOCLONAL ANTIBODIES	POLYCLONAL ANTIBODIES
Produced by the same clone of plasma B cells	Produced by different clones of plasma B cells
A homogeneous antibody population	A heterogeneous antibody population
Interact with a particular epitope on the antigen	Interact with different epitopes on the same antigen
Production is expensive	Production is inexpensive
Possess low cross reactivity	Possess comparatively high cross reactivity

[9] Taken from and authorized by Katikireddy, K. R. and F. O'Sullivan (2011). "Immunohistochemical and immunofluorescence procedures for protein analysis." Methods Mol Biol 784: 155-167. Doi: 10.1007/978-1-61779-289-2_11

Advantages include immortal supply, high specificity, and high reproducibility	Advantages include high affinity, tolerance of minor changes, and more robust detection

Table 3. Comparison between polyclonal and monoclonal antibodies [10].

5.1.2. Biomarkers

Numerous studies have evaluated the ability of certain antibodies such as collagen IV, PECAM-1 (CD31), von Willebrand factor (vWF) and cluster of differentiation 34 (CD-34) to outline blood vessels [117-120]. PECAM-1 has been recognized as a superior endothelial marker due to its strong and homogeneous expression when compared to others such as vWF and CD-34 [119]. Similarly, collagen IV allows a strong outlining of the basement membrane, which facilitates the recognition of blood vessels outlining the vascular basement membrane.

5.1.2.1. Platelet endothelial cell adhesion molecule (PECAM-1)

PECAM-1 or cluster of differentiation 31 (CD-31) is a transmembrane glycoprotein, found mainly at the surface of endothelial cells where they join each other (cell junctions). This glycoprotein is also present at the surface of platelets and macrophages among others. Its role in biological functions such as cell migration, adhesion and angiogenesis, is fundamental [121-123]. Multiple studies have been conducted with PECAM-1 as a biomarker for blood vessels in normal structures like human alveolar capillaries [117], and pathological processes such as wound repair in horses [124], canine tumors [118] among others. PECAM-1 is one of the most commonly used biomarkers for blood vessels [104; 118] (Figure 12).

[10] Taken and modified from Panawala, L. (2017). "Difference Between Monoclonal and Polyclonal Antibodies." http://pediaa.com/difference-between-monoclonal-and-polyclonal-antibodies/

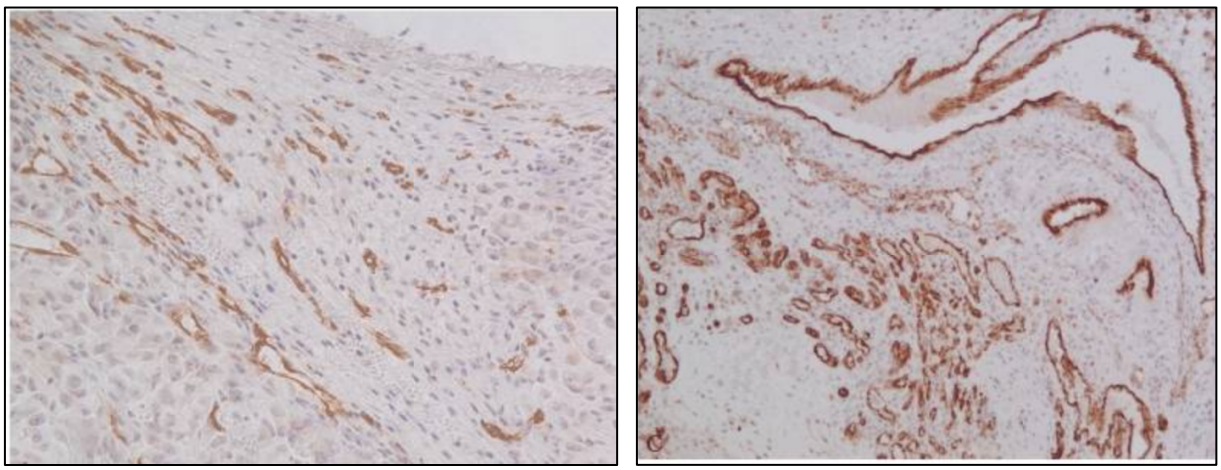

Figure 12. IHC to outline blood vessels of a breast cancer xenograft with CD31[11]. Dark brown staining of endothelial cells highlights the localization of blood vessels on both images.

5.1.2.2. Collagen IV

Collagen IV is a major basement membrane protein found in the extracellular matrix. It is essential for preservation of the basement membrane integrity and is composed of six different α-chains (α1–α6) [119; 125; 126]. In addition, collagen IV is fundamental in biological processes such as cell adhesion and migration, among others [127]. Multiple studies have been conducted using collagen IV as a biomarker to determine the presence of blood vessels in different tissues [128; 129]. Furthermore, collagen IV has been used to outline the microvasculature of patients with asthma and COPD [88](Figure 13).

[11] Taken and modified from Wang D. et al (2008) Immunohistochemistry in the evaluation of neovascularization in tumor xenografts. Biotechnic & Histochemistry, 83:3-4, 179-189. Doi: 10.1080/10520290802451085

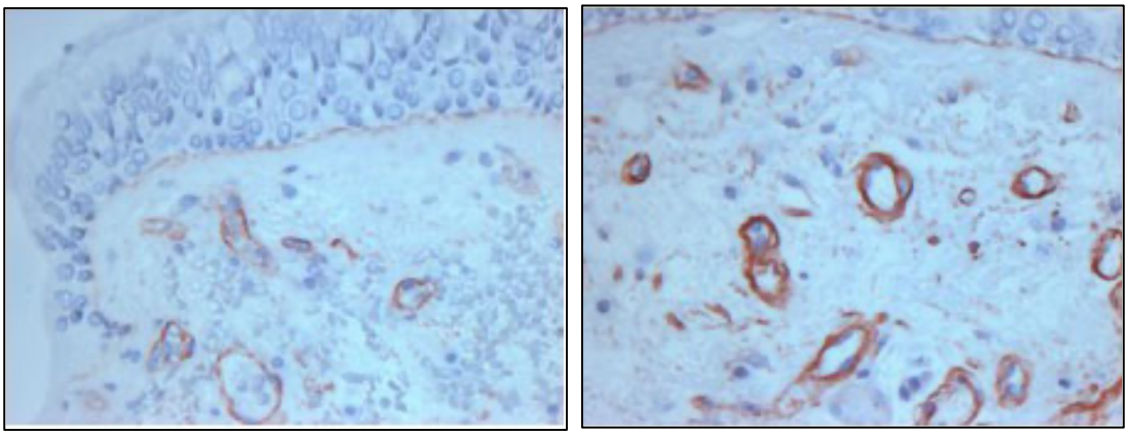

Figure 13. Microphotographs showing bronchial mucosal staining with antibody directed against Collagen IV to outline blood vessels from a normal subject (left image) and asthmatic patient (right image)[12].

5.2. Angiogenesis quantification

Different morphometric methods are available to quantify vascularity in lung tissues including manual quantification, stereology or imaging techniques such as NBI or micro-computed tomography [130; 131].

Standard histomorphometric analysis consist in the evaluation of a digitalized image of a sample of interest using a dedicated computer software with manual counting or with a semiquantitative analysis [90; 132-134]. Different quantification methods are available such as the Weidner's method for the measurement of microvascular density. This method consists in the evaluation of "hot spot" areas with a high magnification using one of the following: (1) manually on the digitalized image, (2) with computerized methods or (3) with the Chalkley method directly with the microscope [91; 104; 135; 136]. The Chalkley technique involves the quantification of vessels intersecting with the points of an array grid mounted into the microscopes' eyepieces [91; 137].

Recently, other quantification techniques have gained popularity such as stereology and imaging evaluation based on an alleged unbiased quantification analysis [136; 138;

[12] Taken and modified from open access article Zanini, A et al. (2010) The role of the bronchial microvasculature in the airway remodeling in asthma and COPD. Respiratory Research 11, 132. Doi: 10.1186/1465-9921-11-132

139]. Stereology is a statistical technique that evaluates the properties of a 3D tissue with geometric tests based on quantification of a two dimensional histological image [130; 138]. Even though, stereology is considered to reduce the bias associated with this analysis, it is difficult to perform, especially in tissues stained by IHC, and therefore manual counting continues to be an accepted procedure [130].

The parameters used to quantify angiogenesis include the number of vessels per square millimeter of area, the percentage of vascular area in relation to the total ECM area, and the mean vessel size calculated when dividing the vascular area by the number of vessels [88; 120; 132]. Numerous studies suggested to analyze vessels located in the lamina propria at 100-160 μm from the epithelial basement membrane [90; 140; 141]. All the areas internal to the vascular endothelial basement membrane on each vessel were manually drawn (free hand tool) and the computer software was able to determine the area of these traces to estimate the vascular area. The total surface area of the airway and vessel wall can be calculated following the same process than for the vascular area, by drawing either manually or automatically the area to be measured, and automatically with the image software analysis estimate this parameter [142].

6. Purpose of study

Little information is known about angiogenesis in the lower airways of asthmatic horses. The inaccessibility of the bronchi in a clinical setting and the low sensitivity of the current diagnostic techniques available contribute to the lack of scientific studies evaluating angiogenesis in equine patients [95]. In humans with asthma, angiogenesis is observed has demonstrated by increases in vascular density and vessel size in lower airways? [83]. In this thesis, an equine asthma model has been used to investigate the presence of angiogenesis in the bronchi.

6.1. Objectives

The main objective of this project is to evaluate the presence of bronchial angiogenesis in bronchi of horses with asthma compared to control horses using immunohistochemistry.

6.2. Hypothesis

We hypothesized that an increase in bronchial angiogenesis occurs in the bronchi of severe asthmatic horses which is partially decreased by conventional treatment due to the decrease of inflammation.

ARTICLE 1.

In preparation for submission to Journal of Veterinary Internal Medicine (JVIM- Standard Article)

Bronchial angiogenesis in severely asthmatic horses

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Keywords: Asthma, angiogenesis, horses, collagen IV, immunohistochemistry, bronchi.

Abbreviations:

ECM: Extracellular matrix

R_L: Pulmonary resistance

E_L: Pulmonary elastance

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Funding & Conflict of Interest

This work was made possible with support from the Canadian Institutes of Health Research (CIHR). Authors declare no conflict of interest.

Off-label antimicrobial declaration

Authors declare no off-label use of antimicrobials.

Institutional Animal Care and Use Committee (IACUC) or other approval declaration

All experimental procedures were performed in accordance with the Canadian Council for Animal Care and the research protocol was approved by the University of Montreal Animal Care Committee (Rech-1578).

Human ethics approval

Authors declare human ethics approval was not needed for this study.

1. Abstract

Background: Equine asthma is a chronic inflammatory disease of the lower airways characterized by structural changes that contribute to airway thickening and airflow obstruction. Treatment for equine asthma partially reverses these remodeling changes. Angiogenesis has been shown to increase vascularization of the bronchial mucosa which contributes to the thickening of the bronchial wall in humans with asthma, opening a new window for a more targeted treatment. However, little information is available on angiogenesis in equine asthma.

Objectives: To document the presence of indices of angiogenesis in the lower airways of severely asthmatic horses.

Animals: Bronchial samples from 7 asthmatic horses in exacerbation of the disease, 7 asthmatic horses in clinical remission, and 7 age matched healthy horses were studied.

Methods: Prospective, blinded, randomized controlled study. Immunohistochemistry analysis was performed with collagen IV as a biomarker for basement membranes. The number of blood vessels, vascular density, vascular area and mean vessel size values were measured by histomorphometry using an image software (Image J) and compared using a one-way ANOVA ($p<0.05$).

Results: A significant increase in the number of vessels was observed in asthmatic horses in exacerbation ($p=0.007$) and in horses in remission ($p=0.02$) in comparison to controls. Furthermore, the vascular area was increased in horses with asthma in exacerbation when comparted to controls ($p=0.02$) and to remission ($p=0.04$). No significant differences were observed in mean vessel size between the groups.

Conclusions and clinical importance: Angiogenesis is present in the bronchi of asthmatic horses, suggesting that it may contribute to the thickening of the airway wall. Furthermore, results of

the current study may offer a new avenue for targeted treatment. Further studies are warranted in order to assess response to those treatments.

2. Introduction

Equine asthma syndrome is an umbrella term encompassing various chronic inflammatory processes occurring in the lower airways [1-4]. This syndrome is considered one of the most common causes of lower airway inflammation [5,6], with a prevalence of severe asthma in the northern hemisphere of 14% [7,8]. The clinical signs associated with this syndrome varies according to the severity of the disease. Equine asthma syndrome is characterized by an inflammation of the lower airways associated with airflow obstruction, airway remodeling and hyperresponsiveness, excessive mucus accumulation and poor performance [9]. The airway remodeling occurring in response to the chronic inflammation include epithelial hyperplasia, collagen deposition and smooth muscle hypertrophy/hyperplasia [10-12]. These structural modifications contribute to the impairment of lung function in asthmatic horses [13-16]. Interestingly, an association between chronic inflammation of the airways and the development of angiogenesis has been demonstrated in asthmatic humans [17,18]. Moreover, a study showed increased airway mucosal blood flow in human asthmatic airways when compared to normal subjects [19]. Similarities between equine and human asthma, indicate that this association between chronic inflammation and angiogenesis may also be present [9,20]. Airway remodeling is only partially reversible with current therapy (i.e. inhaled and systemic corticosteroids, and bronchodilators) [7,21,22]. If angiogenesis is demonstrated to be a feature of equine asthma, a new opportunity for targeted treatment may open.

Angiogenesis is defined as the formation of new vessels from pre-existing ones. The stimulus for angiogenesis in humans with asthma is unclear [23]. However, angiogenesis and chronic inflammation have been shown to be codependent [24]. Furthermore, hypoxic pulmonary tissues release angiogenic growth factors such as vascular endothelial growth factor (VEGF) that

contribute to the migration of endothelial cells from pre-existing vessels to the surrounding extracellular matrix to form solid sprouts. Inflammatory cells such as macrophages, mast cells and fibroblasts can also lead to an increase in vessel growth [25], which may contribute to thickening of the bronchial wall. Even though multiple studies have shown the presence of angiogenesis in humans with asthma [26-30], literature on lung angiogenesis in asthmatic horses is scarce. A 2012 study [31] showed an increased vascular density of superficial tracheal vessels from asthmatic horses, when evaluated with narrow band imaging. However, no differences were observed at the level of the carina or bronchi, contradicting the results obtained in humans. The authors attributed those findings to the lack of sensitivity of the imaging technique.

The main objective of this study was to indirectly evaluate the presence of bronchial angiogenesis using immunohistochemistry by quantitatively documenting the presence of mature bronchial vessels in the airways of both asthmatic and healthy horses. We hypothesized that an increase number of vessels would be observed in the airways of asthmatic horses compared to controls.

3. Material and Methods

Animals

Prospective, blinded, randomized controlled study. Frozen bronchi from 21 horses belonging to the Equine Respiratory Tissue Biobank (ERTB, www.ertb.ca, Faculty of Veterinary Medicine, University of Montreal) were studied. Horses were divided into three groups based on clinical history (respiratory signs) and antemortem pulmonary resistance R_L and elastance E_L values, namely exacerbation (n=7, R_L>1cmH_2O/L/s, E_L>1cmH_2O/L), remission (n=7, $R_L \leq$1cmH_2O/L/s, $E_L \leq$1cmH_2O/L) and control (n=7, no respiratory signs, $R_L \leq$1cmH_2O/L/s, $E_L \leq$1cmH_2O/L). Sixteen mares and five geldings were included in the study, with a weight (mean ± SD) of 504

± 74.5 kg and age (mean ± SD) of 16.8 ± 5.1 years. Most horses were housed individually in a barn, each stall bedded with wood shavings, except for 4 horses in remission that were kept on pasture. Horses kept inside the barn were fed hay twice daily whereas those outside on pasture also received pellets. Turnout was allowed daily for all the stabled horses. Horses stayed in a remission status for a minimum of 3 months either by reduced exposure to dust by turning them out into pasture (n=4), or by the administration of corticosteroids (n=3). Experimental procedures for euthanasia and necropsy were performed in accordance with the Canadian Council for Animal Care and the research protocol was approved by the University of Montreal Animal Care Committee (Rech-1578).

Tissue sampling

Equine bronchi specimens from the ERTB dissected immediately after euthanasia, frozen in liquid nitrogen and maintain at -80°C as previously described [66; 143] were thawed in phosphate buffered saline (PBS1x) at room temperature and cut into 0.5 cm sample size. Tissue samples were fixed for 24 h in 10% neutral-buffered formaldehyde and embedded in paraffin blocks until analyzed. All blocks were stored at room temperature prior to use.

Immunohistochemistry

Collagen IV, a major basement membrane protein, was used to outline the endothelial basement membrane of the bronchial extracellular matrix vasculature [17]. Two complete airways were studied for each horse. Histologic sections of 5 µm thickness were used for immunohistochemistry evaluation. Enzymatic antigen retrieval with pepsin was performed (Ready-to-use, ImmunoBioScience #AR-6543-0). Collagen IV (Mouse Anti-Human, IgG1 monoclonal, Dako #M0785) was used as the primary antibody with an overnight incubation at

4°C. Donkey anti-mouse biotinylated (Jackson immunoresearch laboratory #715-065-150) was used as a secondary antibody, and DAB peroxidase (Substrate kit, Vector Laboratories, Inc. #SK4100) was applied producing a brown coloration of the bronchial extracellular matrix capillaries' basement membranes (Figure 1). Histologic slides were counterstained with Harry's hematoxylin and mounted with Leica Micromount (Surgipath Micromount Mounting Medium, Leica #3801731).

Histomorphometric analysis

Histologic sections were evaluated with a 20x magnification and digitalized with an automated upright microscope system with a FLIR camera (Life Science Research, Leica DM4000-B) (FLIR Integrated Imaging solutions, Inc) and a specialized software for image acquisition (Panoptiq 5.0, ViewsIQ Inc). Six regions of interest were randomly selected from each airway for histomorphometric evaluation. A region of interest was defined as the area between the epithelial basement membrane and a distance of 150μm in the extracellular matrix (ECM). A software analysis system (Image J, Version 1.52p, National Institutes of Health, Bethesda, MD) was used to evaluate the number of vessels, vascular density, vascular area and mean vessel size in each area of interest (Figure 2). Additionally, the epithelial basement membrane length of each section analyzed was measured manually by tracing with a free hand line tool to ensure that the images analyzed were of approximately similar size. The number of vessels was determined by adding the stained vessels in each of the regions of interest, and values obtained were corrected for the area occupied by the ECM on each section analyzed. The vascular area was computed directly by the software following manual tracing of all the area internal to the vascular endothelial basement membrane of each vessel using the freehand selection tool. The vascular density was calculated by dividing the vascular area by the total ECM area and

multiplying the value obtained by 100 generating a percentage. The last parameter calculated was an estimation of the mean vessel size, obtained by dividing the vascular area by the number of vessels. The values obtained for the six regions of interest of each airway were averaged, resulting in a single value for each parameter and each horse. A single operator (EMR), blinded to group allocation, performed the analysis.

Statistical analysis

Age, sex and weight were analyzed to verify an equal distribution between groups with a one-way ANOVA (age, weight) or Chi-Square test (sex). Data were analyzed using a one-way ANOVA and Benjamini-Hochberg procedure (GraphPad Prism 7.0, GraphPad Software, Inc. La Jolla, California, USA). Statistical significance was set at $P \leq 0.05$.

4. Results

Animals

No association was observed between sex and group when performing an exact Chi-Square test ($p=0.3$). Likewise, a one-way ANOVA showed no association between group and weight or age ($p>0.05$).

Lung function

As expected, a significant increase in R_L was observed in horses in exacerbation when compared with control ($p<0.0001$) and remission ($p<0.0001$). Likewise, a significant increase in E_L was observed in horses in exacerbation when compared with control ($p=0.0008$) and remission ($p=0.0012$) (Figure 3A and 3B).

Histomorphometric analysis

The number of vessels in each region of interest were corrected per micrometer of ECM. A statistically significant increase of 37% in the number of vessels was observed in horses in exacerbation ($p=0.007$) and remission ($p=0.02$) when compared to control horses (Figure 4). Furthermore, the vascular area measured by tracing the area internal to the vascular endothelial membrane of each vessel, was significantly increased in horses in exacerbation when compared to control horses ($p=0.02$) and to horses in remission ($p=0.04$) (Figure 5). The percentage of vascular density (percentage of the vascular area divided by the total ECM area) was similar between the three groups of horses ($p=0.04$, not significant after correction for multiple comparisons) (Figure 6). No statistically significant difference was observed between groups in regard to epithelial basement membrane length ($p=0.86$; Figure 7). Furthermore, no difference on the estimation of mean vessel size (vascular area divided by number of vessels) was observed ($p=0.4$; Figure 8).

5. Discussion

Angiogenesis is defined as the development of new vessels formed from preexisting vessels. The codependence between angiogenesis and chronic inflammation plays a fundamental role in chronic lung diseases [24], in addition to its involvement in other pathologies such as neoplasia and vascular malformations [17,28,29]. The contribution of angiogenesis in asthma has not been completely elucidated; however, it is considered a component of the chronic inflammation observed in patients with asthma[30]. Regardless of the underlying cause, a chronic inflammatory state will stimulate the proliferation and migration of inflammatory cells to the inflamed site, leading to organ damage over time, and potentially compromising oxygenation of affected tissues. The hypoxia that can result from chronic inflammation will trigger the release of pro-angiogenic factors by inflammatory mediators [26]. Furthermore, angiogenesis is believed to

participate to the maintenance of chronic inflammation observed in pathologies such as asthma by transporting cells (eg. inflammatory cells), oxygen and nutrients to the inflammation site [24,31]. The current study provides the first indirect evidence of angiogenesis occurring in bronchial tissues of the central airways in severely asthmatic horses. Horses in exacerbation or in a remission status of the disease were chosen. Both groups included severe asthmatic horses, and therefore, were assumed to have chronic airway inflammation. Horses in exacerbation were mainly used to demonstrate the presence of angiogenesis when the disease is uncontrolled, whereas the remission group was used to assess the potential reversibility of angiogenic changes, since horses in remission presumably have the disease under control. The increased number of vessels in asthmatic horses, either in exacerbation or remission, when compared to control horses and increased vascular area in horses in exacerbation are in agreement with similar observations in children and adults with mild to moderate asthma [18,32,34]. In both groups of asthmatic horses an increased number of vessels was observed, but only an increased vascular area was observed in horses in exacerbation when compared to controls. The difference observed in the remission group could be due to these vessels being smaller in size even though mean vessel size was not statistically different between the three groups. In addition, mean vessel size is considered to be an estimation, since correction for vessel dilation was not performed, which could be the reason for the differences observed. Furthermore, increased airway mucosal blood flow has been observed in humans with asthma, however, it has not been demonstrated whether this was due to vasodilation, angiogenesis or a combination of both [35]. Conventional treatment for equine and human asthma includes the administration of systemic or inhaled corticosteroids and bronchodilators [7,21,36]. However, these treatments only partially reverse the airway remodeling observed in asthmatic horses [37,38]. Confirmation of the presence

of angiogenesis in the airways of asthmatic horses may offer a new opportunity for treatment by targeting angiogenesis and possibly reducing the edema observed on the extracellular matrix of asthmatic horses.

Immunohistochemistry allows the identification and localization of a molecule of interest with the aid of immunolabeling of specific antigen-antibody reactions [39,40]. Albeit manipulations and analyzing results can be challenging with this technique [41,42], it is considered the gold standard method when quantifying the bronchial vasculature [17]. In the current study, the basement membrane protein, collagen IV, was used as a biomarker for evaluating the presence of angiogenesis[17,18]. This marker is superior to endothelial biomarkers such as PECAM-1 [43], since it allows differentiation between blood and lymphatic vessels, because of the absence of basement membrane in lymphatic vessels. Furthermore, inter and intra-observer variation was reduced by the development of a systematic protocol.

The study was performed on a small number of animals. The strict experimentally controlled environmental conditions under which severe asthmatic horses or control horses were maintained during the experimentation allow reducing the number of animals required to reach statistical differences. The lack of differences in vascular density after correction for multiple comparisons is likely due to the underpower of study, since a post hoc power calculation (G*Power 3.1.9.4) showed that eight horses would have been needed to achieve power of 0.8 and an effect size of 1.6. An additional limitation of the study was the absence of mild clinical cases of equine asthma and therefore results may not be directly applicable to these horses.

In conclusion, this study demonstrates that in addition to the pulmonary structural changes, there is an increased in vascularity in severe asthmatic horses, suggesting an increased angiogenesis in severely asthmatic horses. This finding could help to better understand the pathophysiology

of the disease. Further studies are needed to assess the angiogenic response to conventional asthma treatments in horses.

6. References

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7. Figure legends

Figure 1. Representative image of a bronchus of an asthmatic horse stained with Collagen IV highlighting in brown the basement membrane of blood vessels. EP, Epithelium; BM, Basement membrabe; SM, smooth muscle; ECM, extracellular matrix. Scale bar 100μm.

Figure 2. Example of the tracings for the calculation of vascular area and the number of vessels. Luminal circumference of each vessel included in a region of interest is outlined in red (vascular area). Each vessel was manually assigned a number for subsequent vessel count. Scale bar 100μm.

Figure 3. (A) Pulmonary resistance (R_L) and (B) pulmonary elastance (E_L) measured in control (n=7) and asthmatic horses in exacerbation (n=7) and remission (n=7). The error bars indicate the SD of the mean. A significant increase in R_L and in E_L was observed in horses in exacerbation when compared with control ($p<0.0001$) ($p=0.0008$) and remission ($p<0.0001$) ($p=0.0012$), respectively.

Figure 4. Total number of bronchial vessels per μm of ECM in control and asthmatic horses in exacerbation and remission. An increase in the number of vessels in horses in exacerbation

(p=0.007) and remission (p=0.02) when compared to control horses was observed. The error bars indicate the SD of the mean.

Figure 5. Vascular area of control and asthmatic horses in exacerbation and remission. An increased was observed in horses in exacerbation when compared the control (p=0.02) and remission horses (p=0.04). The error bars indicate the SD of the mean.

Figure 6. Vascular density of control and asthmatic horses in exacerbation and remission. No differences were observed between the three groups of horses after statistical corrections. The error bars indicate the SD of the mean.

Figure 7. Total epithelial basement membrane length values of control and asthmatic horses in exacerbation and remission. No differences were observed between the three groups. The error bars indicate the SD of the mean.

Figure 8. Mean bronchial vessel size of control and asthmatic horses in exacerbation and remission. No differences were observed between the three groups. The error bars indicate the SD of the mean.

Figure 14 (Figure 1 article). Representative image of a bronchus of an asthmatic horse stained with Collagen IV highlighting in brown the basement membrane of blood vessels. EP, Epithelium; BM, Basement membrabe; SM, smooth muscle; ECM, extracellular matrix. Scale bar 100µm.

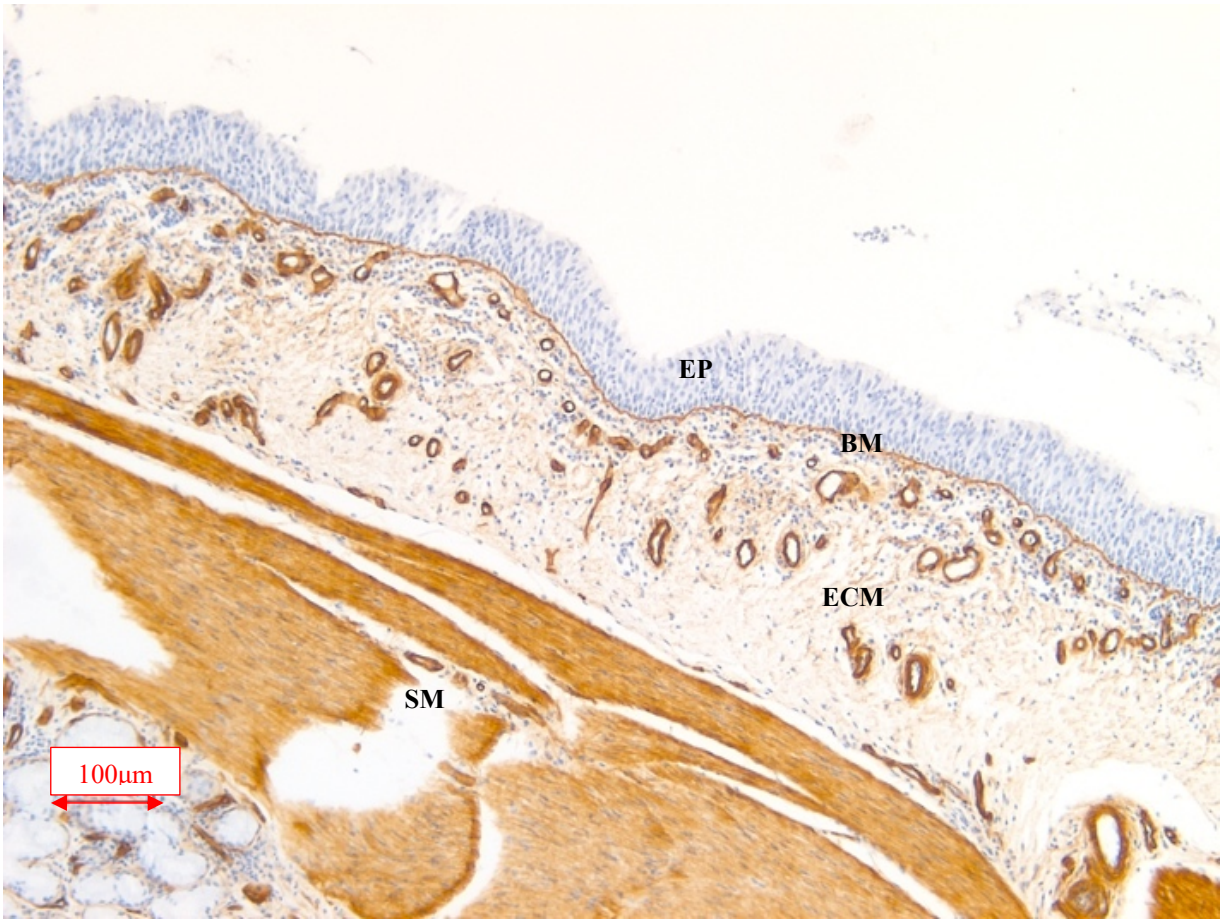


Figure 15 (Figure 2 article). Example of tracings used for the calculation of vascular area and the number of vessels. In the inset, a specific number was assigned to each vessel permitting their subsequent count.

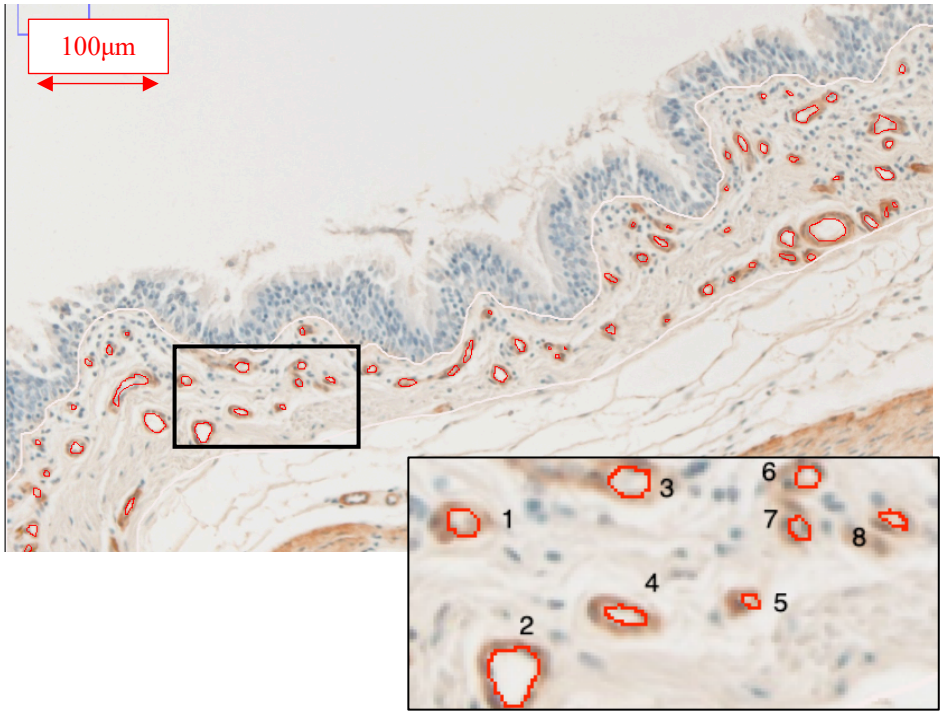


Figure 16. (Figure 3 article) Lung function parameters. (A) Pulmonary resistance (R_L) and (B) pulmonary elastance (E_L) measured in control (n=7) and asthmatic horses in exacerbation (n=7) and remission (n=7). A significant increase in R_L and in E_L was observed in horses in exacerbation when compared with control (p<0.0001) (p=0.0008) and remission (p<0.0001) (p=0.0012), respectively.

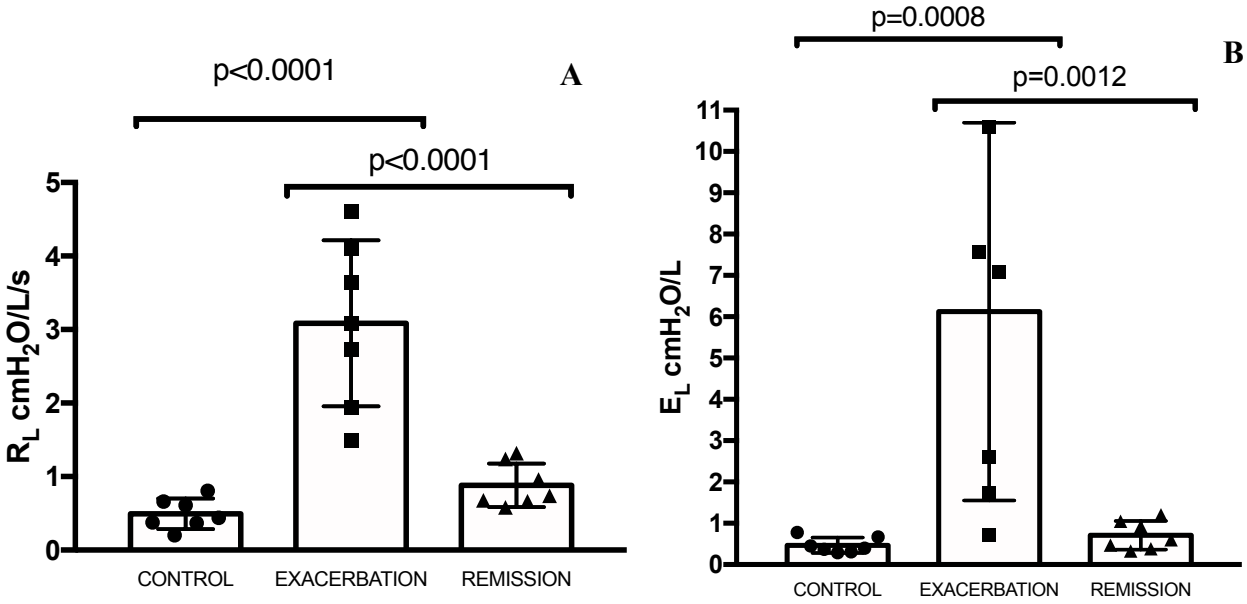


Figure 17 (Figure 4 article). Total number of bronchial vessels per μm of ECM in control and asthmatic horses in exacerbation and remission. An increase in the number of vessels in horses in exacerbation (p=0.007) and remission (p=0.02) when compared to control horses was observed.

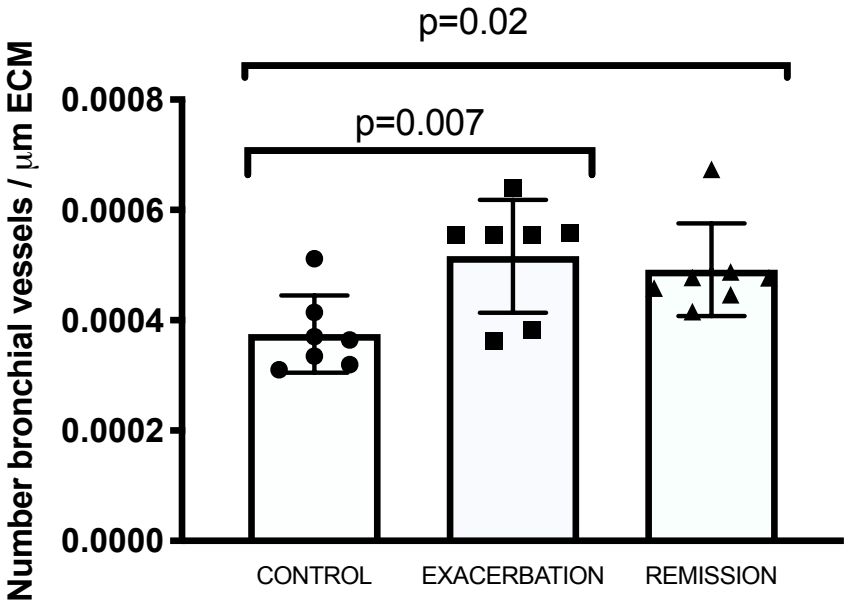


Figure 18. (Figure 5 article). Vascular area of control and asthmatic horses in exacerbation and remission. An increased was observed in horses in exacerbation when compared the control (p=0.02) and remission horses (p=0.04).

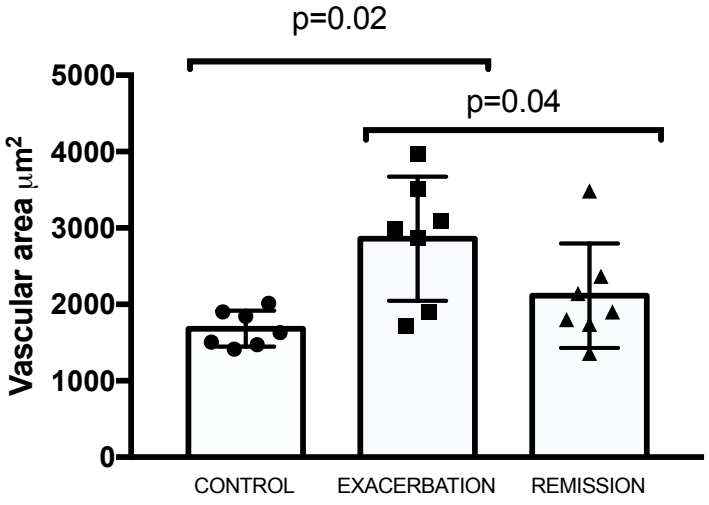


Figure 19. (Figure 6). Vascular density of control and asthmatic horses in exacerbation and remission. No differences were observed between the three groups of horses after statistical corrections.

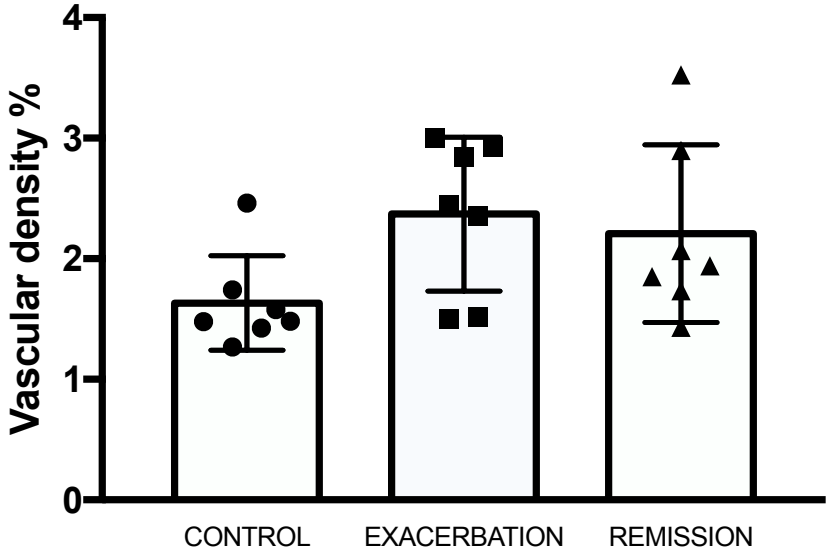


Figure 20. (Figure 7 article). Total epithelial basement membrane length values of of control and asthmatic horses in exacerbation and remission. No differences were observed between the three groups.

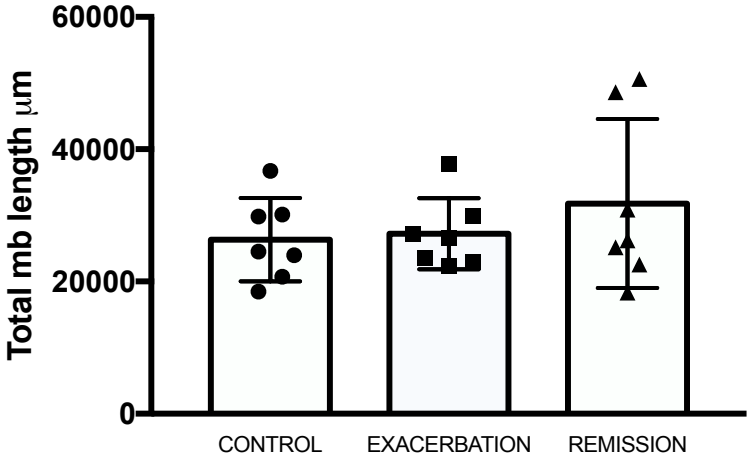
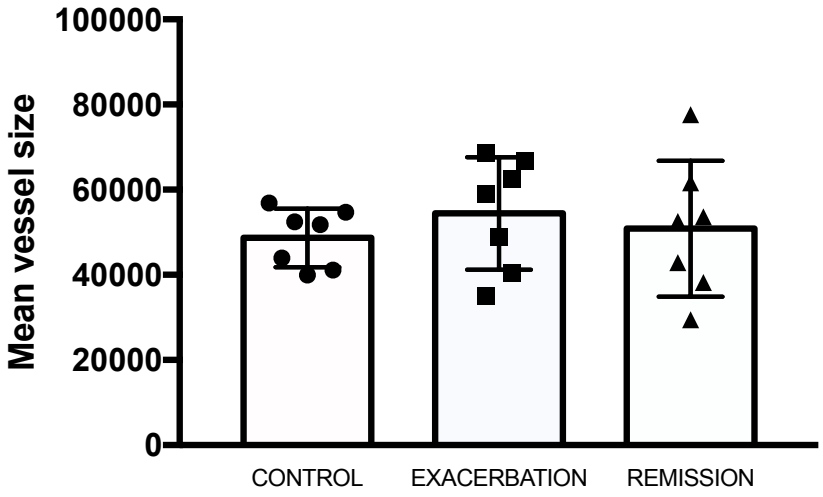


Figure 21 (Figure 8 article). Mean bronchial vessel size of control and asthmatic horses in exacerbation and remission**.** No differences were observed between the three groups.



GENERAL DISCUSSION

The aim of this thesis was to evaluate the presence of bronchial angiogenesis in severely asthmatic horses compared to healthy horses (control group) using immunohistochemistry for quantification of bronchial microvasculature. We hypothesized that an increase in vascularity would be observed as a consequence of the chronic inflammation of the bronchi of severe asthmatic horses when compared to healthy animals, suggesting an increase in bronchial angiogenesis. The objective of the thesis was accomplished, and our hypothesis was validated. An increased number of vessels corrected for micrometers of extracellular matrix was found in the airways of asthmatic horses, both in the exacerbation and the remission phases, when compared to healthy control horses. Additionally, an increased vascular area was noted in asthmatic horses in the exacerbation phase of the disease in comparison to healthy horses and to asthmatic horses in the remission phase. The number of vessels and vascular area were deemed important in establishing whether or not chronic inflammation of bronchial airways resulted in structural alterations of the existing vessels, for example by an increase in size, and/or whether new vessels had developed leading to an increase in their number. The differences observed between both groups of asthmatic horses, either exacerbation or remission, in regard to vascular area were not investigated. However, an increased airway mucosal blood flow has been shown in humans with asthma, yet the origin has not been elucidated. Therefore it is unknown whether this was due to vasodilation, angiogenesis or a combination of both [144]. Mean vessel size was obtained by dividing the vascular area by the number of vessels. This parameter serves as an estimation of the true value, since no correction was performed to take into consideration vessel dilation. Furthermore, the length of the epithelial basement membrane was calculated to verify that the areas selected in each horse were similar in size.

Chronic inflammation and angiogenesis have reportedly a codependent relationship [82]. The release of inflammatory mediators and other cells such as growth factors in chronic inflammation, may contribute to tissue damage leading to hypoxic conditions. In turn, low oxygen levels in damaged tissues stimulate the secretion of pro-angiogenic factors by inflammatory mediators. Over time, the maintenance of chronic inflammation and the

continuous release of angiogenic stimuli promotes the perpetuity of the cycle, supported by the transportation of nutrients and numerous cells to the inflammatory site through angiogenesis [83]. In human asthma, chronic inflammation of the lungs induces increased vascularization of the bronchial mucosa, contributing to the vascular remodeling observed [80; 88]. We believe this thesis provides the first evidence of angiogenesis of bronchial tissues in severely asthmatic horses based on numerous findings. First, horses in exacerbation or in remission of asthma have an increased number of vessels per square micrometer of extracellular matrix in their bronchi, when compared to control horses. Second, asthmatic horses in exacerbation have a greater proportion of their bronchial extracellular matrix occupied by vessels than asthmatic horses in remission or control horses. These results are in agreement with several studies pertaining to humans showing an increased number of vessels, vascular area and vascular density in the bronchi of asthmatic patients when compared to control groups [88-90]. Additionally, similar *in vivo* results were obtained in humans and in horses when evaluating the vascularization of the trachea by endoscopy, showing an increased number of vessels in asthmatics versus control groups [92; 95].

Conventional treatment for equine asthma consists in the administration of corticosteroids (systemic or inhaled) and bronchodilators, in addition to environmental changes aiming to reduce dust exposure [65]. These treatments seek to relieve the airway obstruction observed. However, equine asthma is considered an uncurable disease and a complete reversibility of the structural changes is not expected. A new window for treatment opens with the demonstration that an increased bronchial vascularity occurs in asthmatic horses when compared to healthy horses using IHC with Collagen type IV. Based on our results, it is possible to consider targeting angiogenesis to reduce the number of bronchial vessels present in equine asthma, hoping it would consequently dampen the edema observed in the ECM that contributes to airway obstruction. Specific target to components such as collagen type IV, fundamental for the stability and functionality of the basement membrane, could help inhibit angiogenesis as described in numerous in vitro studies [145-148].

In the current study, we developed an immunohistochemistry protocol for the detection of angiogenesis in the bronchi of severely asthmatic horses (Annex B). Immunohistochemistry (IHC) has been described as the preferential technique for the recognition of a specific target of

interest by the reaction antigen-antibody [149]. Recent studies, have selected IHC as the gold standard technique for the quantification of bronchial microvasculature [88]. However, experimentations and result analyses can be challenging with this technique [150; 151]. Several challenges arose during various stages of development of the study protocol, such as antibody selection, antigen retrieval methods and, tissue quality, among others. The main difficulties encountered are discussed hereafter.

Antibody selection

The most important step in the development of an IHC protocol is the selection of an antibody which is both sensitive and specific for the molecule of interest. Antibodies are frequently available in two forms, either monoclonal or polyclonal antibodies. As previously highlighted in Table 3, polyclonal antibodies have a high affinity and provide a more robust detection than monoclonal antibodies. However, the latter have higher specificity and reproducibility than polyclonal antibodies. In the early stages of our study, we weighed the pros and cons of using monoclonal versus polyclonal antibodies. Limitations include availability of the different types of antibody, polyclonal antibodies often offered in limited quantity and overlooked due to their variability in strength between batches made from various animals at a different time frame. However, advantages of polyclonal antibodies frequently outweigh those of monoclonal antibodies. Numerous studies have evaluated the ability of the different biomarkers to outline vessels such as collagen IV, PECAM-1, vWF, among others [117-120]. We selected Collagen IV and PECAM-1 as our target molecules since they had previously been studied in horses and a few antibodies had been validated in this species [124].

Among them, the previously validated PECAM-1 polyclonal antibody was discontinued by the manufacturer [124]. A substitute was subsequently offered by the company which was expected to work similarly. However, no validation of cross reactivity had been previously performed in horses. Multiple IHC optimization protocols were tested following the manufacturer's recommendations. Despite multiple attempts, no success was achieved. Another manufacturer offered a polyclonal antibody expected to cross-react with equine PECAM-1 due to the similar genomic sequence with humans. Again, no validation in horses had previously been done. Internal validation showed a positive reaction in equine intestine vasculature but not

in bronchial vessels. Further tests using different antigen retrieval methods were performed on equine bronchi. Results showed mild staining of bronchial vasculature with moderate unspecific background. Therefore, no further test was attempted, and all subsequent experimentations were carried out with a monoclonal antibody for the biomarker, collagen IV, which had been previously used in our laboratory (unpublished data). No challenges arose during the various stages of the IHC protocol, and vessels were strongly stained. Therefore, collagen IV was selected as the main antibody for the remainder of the study. Collagen IV, a basement membrane protein, is considered superior to endothelial markers such as PECAM-1 in regard to the differentiation between blood and lymphatic vessels [152]. This differentiation is possible due to the absence of basement membrane on lymphatic vessels. Further differences between PECAM-1 and Collagen IV, suggested that PECAM-1 would be present in early stages of angiogenesis (proliferation phase) which will facilitate recognition of sprouting vessels, whereas collagen IV would be detected in mature vessels, since the basement membrane is developed after the blood vessel is formed [124; 125].

Antigen retrieval

Antigen retrieval is a technique used to expose an antigen site, thus allowing binding of an antibody to it. The most common methods are heat induced epitope retrieval (HIER) and enzymatic retrieval. In the current study, enzymatic retrieval was used for collagen IV whereas PECAM-1, necessitated a combination of both HIER and enzymatic method. HIER was performed by incubating the slides with a citrate buffer in a water bath at 95^{o}C. The prolonged exposure to high temperatures with HIER led to tissue destruction. When attempting to reduce the time of exposure to the temperature of the water bath, samples lacked sufficient staining.

Tissue selection

Lung tissues used in our study came from a local tissue bank, in which tissues are identified by a case number, each corresponding to a specific horse for which demographic and clinical data are available. Immediately after their harvest, lung tissues had been frozen in liquid nitrogen. The day of our experimentation, lung tissues were thawed, fixed in 4% paraformaldehyde and embedded in paraffin blocs until sectioning [112]. Immunohistochemistry on paraffin embedded tissues was selected over frozen sections since

tissue morphology is better preserved with this technique [153]. Quality analysis of the samples was performed since frozen samples may yield poor imaging resolutions. Russell Movat-Pentachrome coloration (Annex A) was thus done prior to IHC to ensure proper tissue quality. Samples demonstrating intact epithelium, extracellular matrix and smooth muscle layer were considered adequate. Tissues samples collected at the level of the intermediate and peripheral airways such as L2, L3, L4 and thoracoscopy samples were evaluated (Figure 22). No evidence of capillaries was observed on peripheral tissues obtained from thoracoscopy. Contrarily, blood vessels were easily observed on sections from intermediate airways. This finding is likely attributed to the small size of the capillaries in peripheral airways or to the inability of collagen IV to detect sprouting vessels. As bronchial vessels were easily observed on intermediate airways, they were selected for the current study.

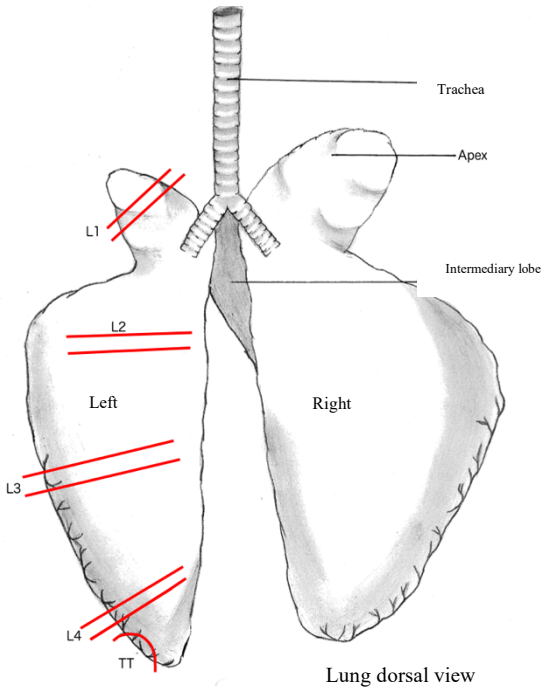


Figure 22. Lung dorsal view sections. Immunohistochemistry was performed on lung tissue sections obtained from intermediate airways (L1-L4) represented on the image by the red lines. Peripheral airways (TT) obtained by thoracoscopy were excluded since they lacked capillaries.

A systematic protocol for histomorphometric analysis was developed (Annex C). First, an area of 1mm^2 was selected as a region of interest according to IHC protocols to analyze blood vessels used in previous quantitative studies [91; 104]. Size was determined with an image acquisition software. Second, we determined the number of images per airway needed for analysis. Therefore, several tests were performed by analyzing all the images that could be created from three different airways (Figure 23) and calculating the mean, standard deviation, and coefficient of variation. Six images per airway were determined to be adequate since the coefficient of variation remained unchanged despite further analysis of additional images. The selection of images used for the analysis was randomized by a computer number randomizer, controlling therefore possible bias. Additionally, the operator was blinded to group allocation. Quantification of vascular structures has been historically considered challenging due to discrepancies between the different methodologies [104]. Therefore, inclusion criteria for vessel selection was established as positively stained vessels with a clear endothelial membrane and a clear vascular area. If a vessel was cut in a longitudinal section, it was excluded from further analysis to reduce potential effects on the vascular area and density values.

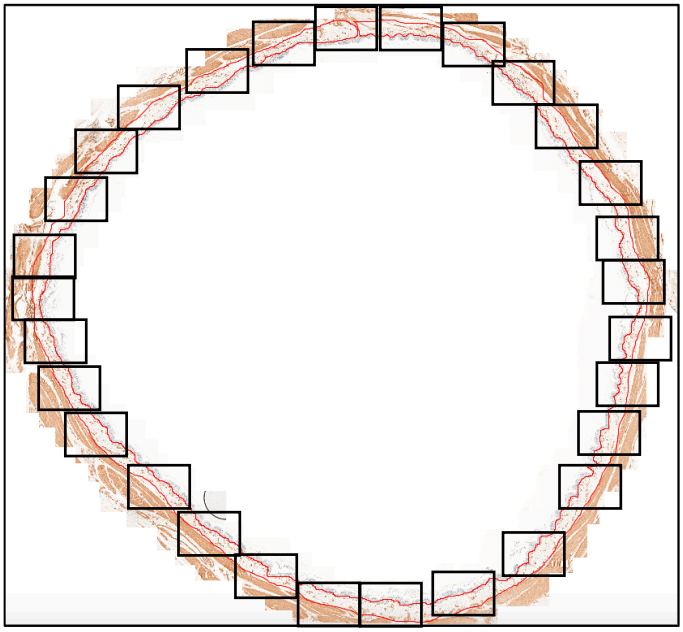


Figure 23. Illustration of the image in an airway. A maximum number of images of 1mm^2 of surface area are represented. A similar pattern of division was repeated for three airways

yielding the number of images required to obtain the mean, standard deviation and coefficient of variation.

CONCLUSION

The increased number of bronchial vessels in the airways of severe asthmatic horses harvested during an episode of exacerbation or in remission compared to airways from healthy horses supported our hypothesis that angiogenesis occurs with asthma in the equine species. Additionally, horses in the exacerbation phase of the disease also have an increased vascular area compared to controls. Based on these results, angiogenesis is likely to contribute to the thickening of the airway wall observed in equine asthma patients. This is not surprising since chronic inflammation, as observed in asthma, has been previously correlated with an increased vascularization of the bronchial mucosa in humans.

Immunohistochemistry was proven to be a useful methodology for the analysis of bronchial vascularization in this study. Our histomorphometric analyses provided a snapshot of the bronchial vascularity in asthmatic and healthy horses at the time of euthanasia. Equine asthma is considered to be a dynamic process; therefore, our research conclusions can only be drawn for the specific time point at which tissues were sampled. Additionally, the number of images selected per airway, the level at which the airway was taken, and the number of airways analyzed for each subject could potentially contribute to interindividual variations.

Additional studies on vascular remodeling in asthmatic horses suffering from mild forms of asthma could be conducted to determine whether an increase in vascularity is also present. The angiogenic response to current standard or pilot treatments for equine asthma could also be the subject of future studies.

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Annex A

Russell-Movat Pentachrome Protocol for tissue quality analysis of respiratory bronchi.

I. Dewaxed:

This step consists in removing paraffin and rehydrating the tissues. It needs to be performed under the hood. Don't forget to annotate how many slides were performed, on the sheet displayed on the hood.

- Xylene: 7 minutes.
- 100% ethanol: 1 x 2 minutes.
- 95% ethanol: 1 x 2 minutes.
- 70% ethanol: 1 x 2 minutes.
- Distilled water: 1 x 5 minutes.

II. Coloration:

1. Dip the slides in 1% Alcian blue solution for 20 seconds in the microwave at maximum power (700 W). Alcian blue is fixed by an electrostatic connection with the acidic mucopolysaccharides and gives a blue color to the basic substances and to the mucin.
2. Wash under running water for 5 min, then rinse with distilled water.
3. Soak the slides in the hematoxylin solution for 8 min. Hematoxylin is very affine to negatively charged elements, especially cell nuclei and elastic fibers, which are colored light red respectively.
4. Rinse in several distilled water baths, until the water comes out clear.
5. Soak the slides in the 2% ferric chloride solution for one minute. The elastic fibers will take on a dark purple color, providing contrast.
6. Rinse with distilled water.

7. Soak the slides in the 5% sodium thiosulfate solution for 1 min. Fixation of hematoxylin staining.
8. Wash under running water for 1 min.
9. Rinse with distilled water.
10. Dip the slides in the scarce acid fuschin crocein solution for 5 min. This solution makes it possible to color the acidophilic elements in red and to differentiate the cellular nuclei which then take on a dark blue to black color.
11. Rinse in several distilled water baths, until the water comes out clear.
12. Soak in 0.5% acetic acid for 30 seconds.
13. Soak the slides in a bath of 5% phosphotungstic acid, 5 min. Phosphotungstic acid helps discolor collagen and reticulin.
14. Rinse in 0.5% acetic acid for 30 seconds.
15. Rinse the alcohol (histological grade or 100% alcohol) for 30 seconds.
16. Soak in the Saffron du Gatinais solution for 4 minutes. Collagen and reticulin get stained in yellow.
17. Rinse the alcohol (histological grade or 100% alcohol) for 30 seconds.
18. Dip into mounting xylene.
19. Dry the xylene slides.
20. Mount the slides with synthetic resin (Leica Micromount).

Annex B

Immunohistochemistry protocol for respiratory bronchi angiogenesis with Collagen IV

I. Dewaxed:

This step consists in removing paraffin and rehydrating the tissues. It needs to be performed under the hood. Don't forget to annotate how many slides were performed on the form hung on the hood.

- Xylene: 7 minutes.
- 100% ethanol: 1 x 2 minutes.
- 95% ethanol: 1 x 2 minutes.
- 70% ethanol: 1 x 2 minutes.
- Distilled water: 1 x 5 minutes.

Preheat the pepsin (ready to use) about 10 min at 37 ° C before its application on the slides

II. Strapping and enzyme unmasking:

This step consists in exposing the antigenic sites, allowing binding of the antibodies to the antigens of interest. This process could be performed with enzymes, with heat or with a combination of both.

- Wipe the slides.
- Surround the tissues with a hydrophobic pencil.
- Apply a drop of PBS on the tissues to prevent them from drying out.
- Apply on the slides and incubate 30 min at 37 ° C in a humid chamber.

IV. Endogenous peroxidase blocking:

Lung tissue is rich in endogenous peroxidase which could lead to high unspecific staining and background. Therefore, it is necessary to block the peroxidase when using chromogenic substances such as DAB.

- Wash 3 times with PBS 1x.
- Remove PBS and apply endogenous peroxidase 3% for 5 minutes at room temperature.

V. Blocking with Donkey serum 10%:

This step will help us reducing unspecific staining by blocking unspecific sites (receptors).

- Wash 3 times with PBS 1x.
- Prepare Blocking Solution at 10% of Normal Donkey Serum in PBS.
- Incubate in the blocking solution for 30 minutes at room temperature.

Meanwhile, prepare the isotype and primary antibody solutions in the blocking solution:

- *Isotype (in blocking solution): Mouse IgG2a Dilution 1: 50 in blocking solution for the isotype control slide (negative control).*
- *Ac. I: Coll.IV, Mouse anti-Human. Dilution 1:50 in blocking solution.*

VI. Application of primary AB:

This step is for the recognition of the desired antigen by the primary antibody.

- Wash 3 times with PBS 1x.
- Apply and incubate 18h at 4 ° C (overnight).

The next day, prepare the secondary AB in PBS: Donkey anti-mouse biotinylated IgG. Dilution of 1: 1000.

VII. Application of secondary antibody:

This step is for the binding of the secondary antibody to the primary antibody. The secondary antibody is biotinylated, which will help with the binding of the chromogenic substance during revelation.

- Wash 3 times with PBS 1X.
- Apply the secondary antibody and incubate for 45 minutes at room temperature.

Meanwhile, prepare the Avidin-Biotin Peroxidase => 5ml PBS 1X + 2 drops of A and vortex then add 2 drops of B and vortex. Leave 45 minutes at room temperature in the dark before use.

VIII. Avidin-Biotin Peroxidase:

- Wash 3 times with PBS 1X.
- Apply AB-Peroxidase solution and incubate for 45 minutes at *room temperature*.

While waiting to prepare the DAB:

1. 2.5ml of distilled water + 1 drop of buffer stock solution => vortex.

2. Add 2 drops of stock DAB solution => vortex.

3. Add 1 drop of hydrogen peroxide => vortex.

IX. DAB:

- Wash 3 times with PBS 1X.
- Apply DAB and incubate for up to 30 min in the dark, monitoring the marking (put white paper under the slides to be able to color).

X. Harry's Hematoxylin Counterstain:

- Rinse the slides 3 times with distilled water and place them on a rack with blades.
- Harris Hematoxylin counterstain baths (Hélène protocol):
  - ✓ Harris haematoxylin: 2 minutes.
  - ✓ Rinse in distilled water.
  - ✓ Hydrochloric alcohol: 2 soaks (about 2 seconds).
  - ✓ Rinse in distilled water.
  - ✓ Lithium carbonate: 20 seconds.
  - ✓ Rinse in distilled water.

XI. Montage:

- 95% alcohol: 2 minutes.
- 100% alcohol: 2 minutes.
- Xylene: 5 minutes.
- Fit the blades to the Micromount (Leica).

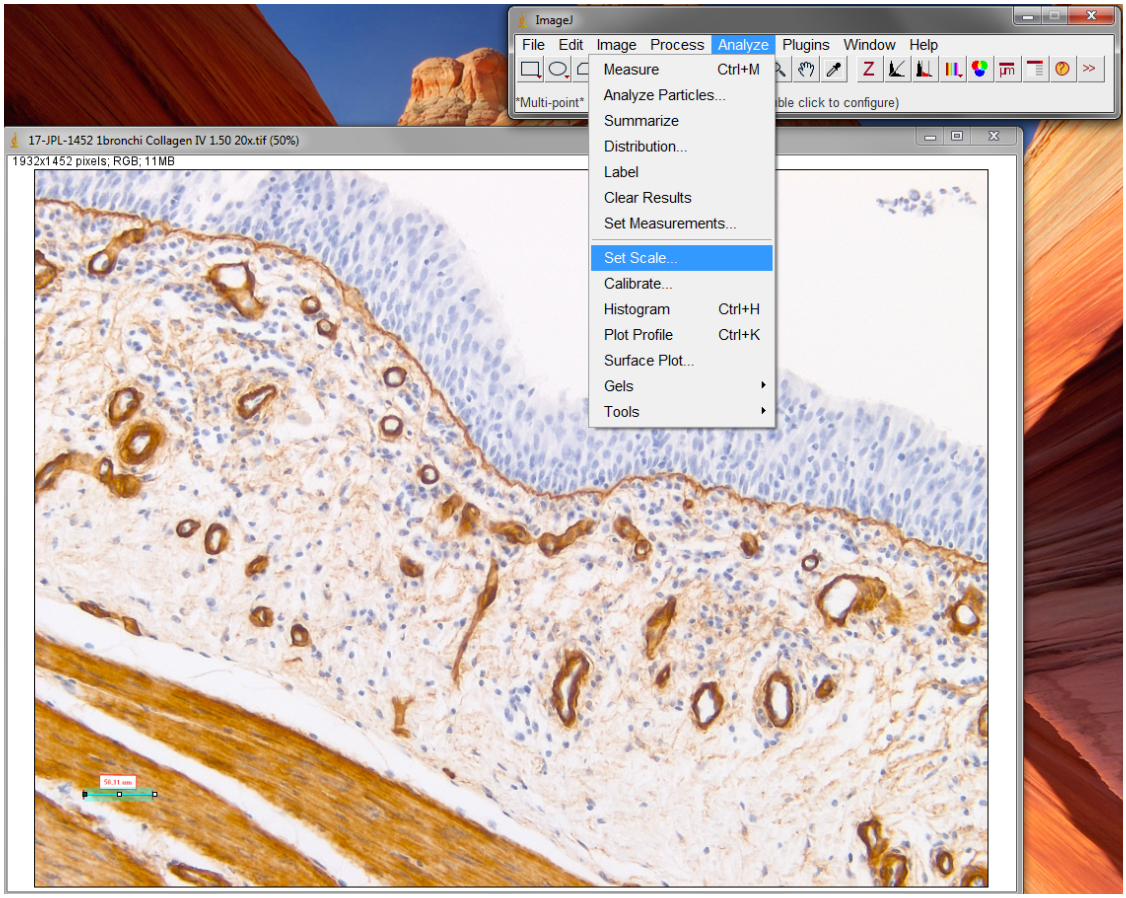
Annex C

Protocol for Histomorphometric Analysis

To do:

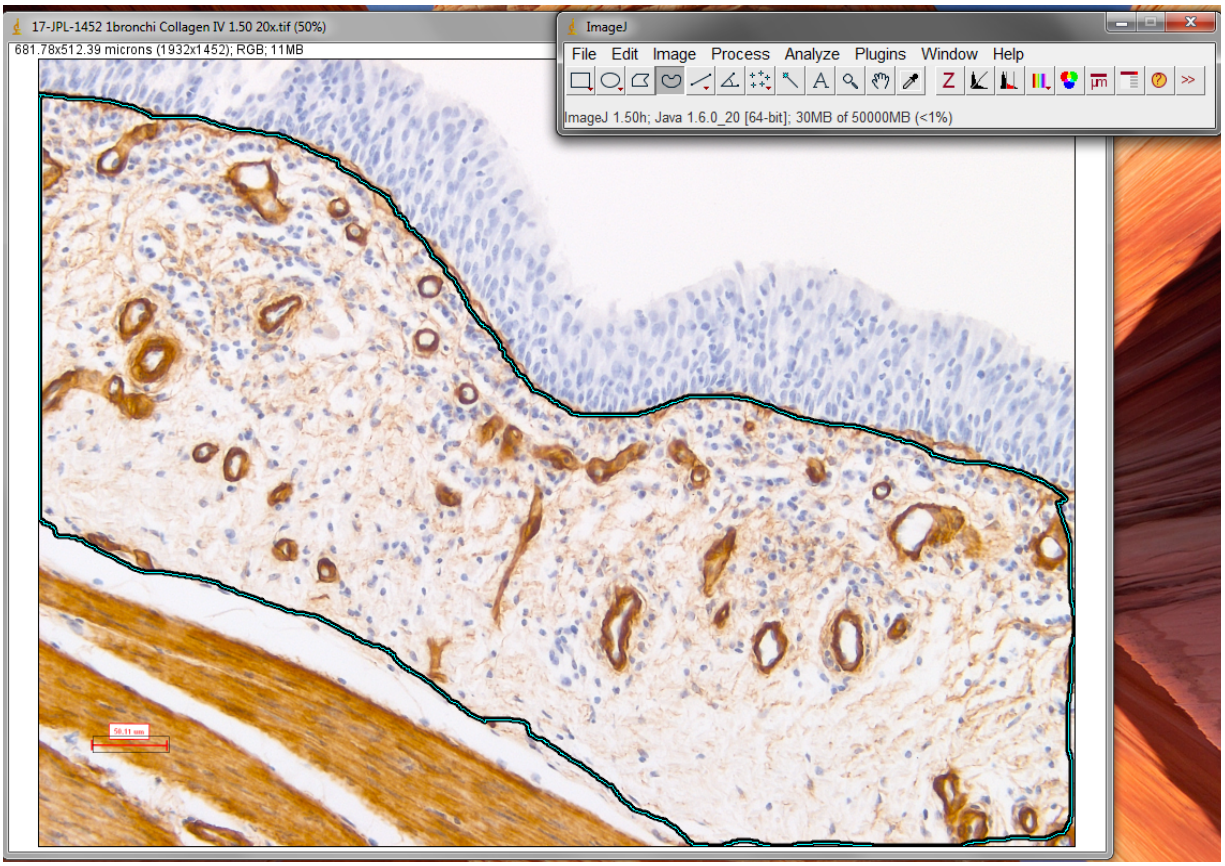
a. Set a scale
b. Draw basement membrane
c. Count vessels- distance 100-160µm from basement membrane. Decided to do 150 µm
d. Vascular Area: Hard to do in really small vessels, but the vascular area refers to the area under the endothelial basement membrane.
e. Vascular density: Number of vessels per square millimeter.
f. Mean vessel size: estimated by dividing the total vascular area by the total number of vessels.

1) Set a scale on the image for adapting later the tools.
2) Draw a line on top of your scale and then go to “analyze-set scale” and add the known size and units.

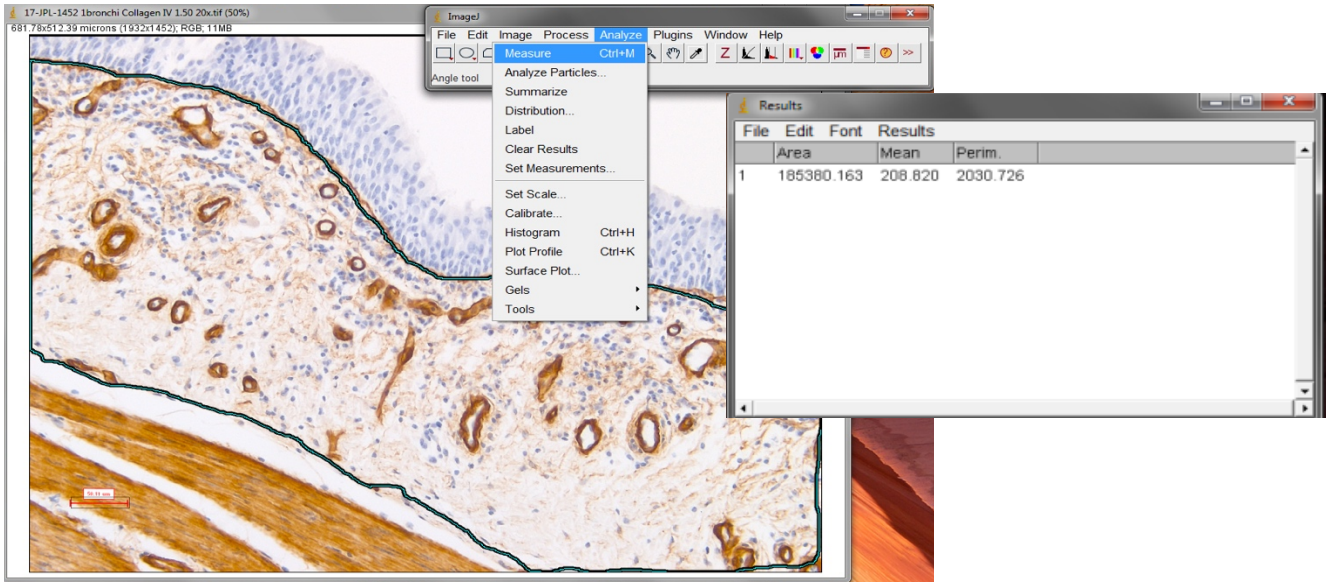


3) Select the free hand tool to outline the desired area needed to be measured. The area should be 150µm from the basement membrane. In some images, the whole extracellular matrix might be selected whereas on others, only a selected area of interest will be outlined.

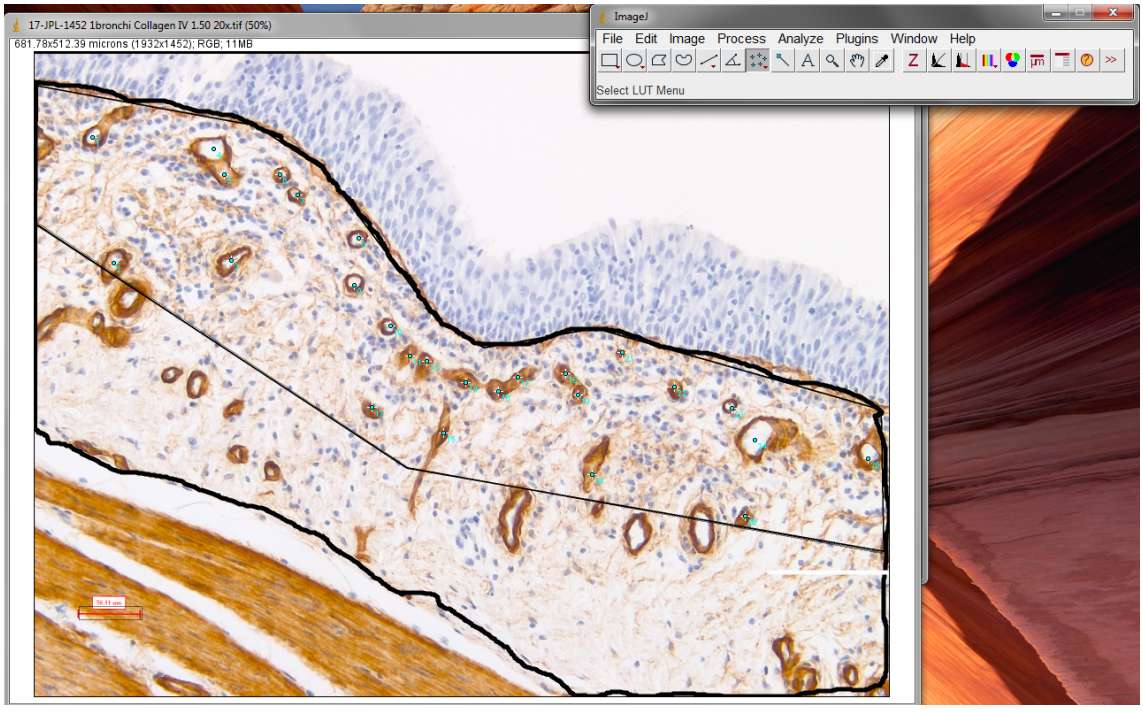
4) Next, don't forget to right click and select draw, to avoid losing your drawing (Command+D in Mac).



5) Measure the outlined area. *Analyze ----measure*



6) Count the vessels on the area selected and calculate the vascular density.

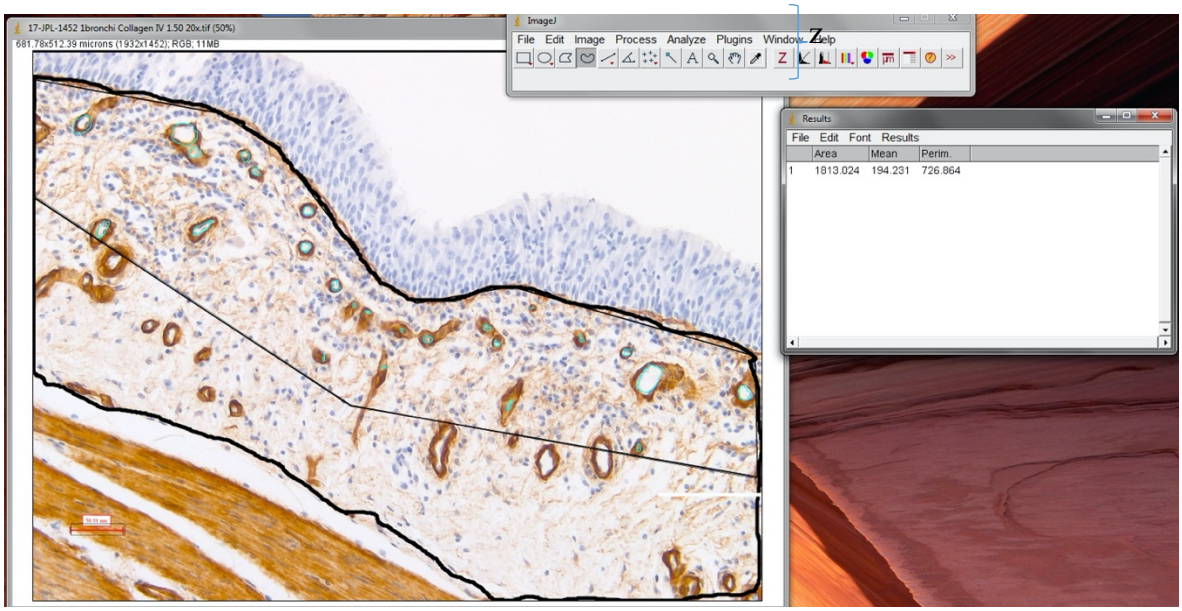


Number of vessels: 26

Area: 95656.685μm² (95.7mm²) } **Vascular density**

7) **Calculate the vascular area**. Select the free hand tool to outline the area under the vascular endothelial basement membrane.

8) Calculate **mean vessel size** by dividing the total vascular area by the total number of vessels.



Total vascular area: 1813.024
Total number of vessels: 26

69.73

9) All measurements obtained need some type of correction, based on what makes more sense for your project. In our case, we divided the number of vessels by the ECM area, this correction allowing comparison between areas of various sizes.