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EVALUATION OF PULMONARY ARTERY STIFFNESS IN ASTHMA AFFECTED HORSES

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RESEARCH PROJECT

LIST OF ABBREVIATION

ΔRPA: right pulmonary artery fractional dimensional change

ANOVA: analysis of variance

AT: acceleration time

AT_{HR}: acceleration time adjusted for heart rate

AT/ET: ratio of acceleration time to ejection time

AT/ET_{HR}: ratio of acceleration time to ejection time adjusted for heart rate

BAL: bronchoalveolar lavage

BMI: body mass index

BSA: body surface area

CFU: colony-forming unit

CMRI: cardiac magnetic resonance imaging

COPD: chronic obstructive pulmonary disease

CT: computed tomography

cTnI: cardiac troponin I

cTnT: cardiac troponin T

dPAP: diastolic pulmonary artery pressure

ET: ejection time

ET_{HR}: ejection time adjusted for heart rate

EIPH: exercise-induced pulmonary haemorrhage

HR: heart rate

IAD: inflammatory airways disease

IQR: interquartile range

LA: left atrium

LV: left ventricle

MEA: mild equine asthma

MFS: maximal frequency shift

mPAP: mean pulmonary artery pressure

MRI: magnetic resonance imaging

OSAS: obstructive sleep apnoea syndrome

$PaCO_2$: arterial partial pressure of carbon dioxide

PAD: pulmonary artery diameter

PAD/AOD: ratio of pulmonary artery diameter to aorta diameter

PAH: pulmonary arterial hypertension

PaO_2: arterial partial pressure of oxygen

PAS: pulmonary artery stiffness

PAS_{HR}: pulmonary artery stiffness adjusted for heart rate

pCO_2: partial pressure of carbon dioxide

PHT: pulmonary hypertension

PLH: pharyngeal lymphoid hyperplasia

pO_2: partial pressure of oxygen

PRV: pulmonary regurgitation velocity

PVOD: pulmonary veno-occlusive disease

PVR: pulmonary vascular resistance

PW: pulsed-wave

RA: right atrium

RAO: recurrent airways obstruction

RAP: right atrial pressure

ROC: receiving operator characteristics

RPA: right pulmonary artery

RV: right ventricle

RVSP: right ventricular systolic pressure

RVSTIs: right ventricular systolic time intervals

SEA: severe equine asthma

sPAP: systolic pulmonary artery pressure

STE: 2D speckle tracking echocardiography

s_w: within-subject standard deviation

TDI: tissue Doppler imaging

TRV: tricuspid regurgitation velocity

TW: tracheal wash

V/Q ratio: ratio of ventilation to perfusion

ABSTRACT

The present research evaluated Pulmonary Artery Stiffness (PAS) and right ventricular systolic time intervals (RVSTIs) in horses with mild/moderate (MEA) and severe (SEA) equine asthma and in healthy horses.

In human medicine, PAS is a pulsed-wave (PW) Doppler echocardiographic parameter useful in assessing an increase in pulmonary artery stiffness due to remodeling of the vessel wall caused by chronic diseases. Moreover, PAS in humans is used as an early indicator of pulmonary hypertension. RVSTIs, such as acceleration time (AT), ejection time (ET) and acceleration time index (AT/ET), are other PW Doppler parameters useful for the evaluation of changes in the pulmonary vascular bed.

Like human asthma, equine asthma is able to induce remodeling of the pulmonary artery wall even in horses, leading to a decreased pulmonary artery elasticity and consequently pulmonary hypertension. Therefore, it is conceivable that PAS could be a useful parameter also in horses. However, there are no studies on PAS in veterinary medicine.

The aims of this research were: to assess feasibility of PAS in horses, to evaluate possible influence of age, bodyweight, sex and heart rate on PAS and RVSTIs, to investigate possible differences between healthy, MEA and SEA horses regarding those parameters, to evaluate possible correlation between PAS and RVSTIs and ratio of pulmonary artery diameter to aorta diameter (PAD/AOD) and to determine PAS and AT cut-off values for diagnosis of SEA.

Echocardiographic examination and PW Doppler of the pulmonary flow were performed in 23 MEA affected horses, 15 SEA affected horses and 15 healthy horses.

Results demonstrated that PAS can been measured consistently in horses and that, as well as RVSTIs, it is not influenced by age, bodyweight, sex and heart rate. Moreover, a significant higher PAS and lower RVSTIs were detected in SEA affected horses compared to healthy subjects and MEA affected ones. In addition, considering the whole sample, a positive correlation between PAS and PAD/AOD and a negative correlation between AT or AT/ET and PAD/AOD were found. These findings, in association with several similarities between equine asthma and human asthma, suggest that these parameters could be correlated to pulmonary pressure even in horses. Finally, this study determined that a PAS value of 8.18 kHz/sec and a AT value of 0.202 sec are the best cut-off values, with a very high sensitivity (PAS: 93.33%; AT: 86.67%) and specificity (PAS: 86.84%; AT: 89.47%), for the diagnosis of SEA.

LITERATURE REVIEW

Chapter 1

PULMONARY VASCULAR BED

1.1 Anatomy

The circulation of the lung is unique; in fact, the lung is the only organ that receive blood from two sources: the pulmonary circulation and the bronchial circulation. The main function of pulmonary circulation is gas exchange; instead, the primary role of bronchial circulation is to provide nutrition to airways, vessels, parenchyma, and visceral pleura. From the anatomical point of view, these two circulations present different and peculiar characteristics (Marlin and Vincent, 2007; Suresh and Shimoda, 2016).

1.1.1 Pulmonary circulation

The subdivision of both the arterial and venous components of the pulmonary circulation presents slight interspecific differences, depending on the bronchial branches of the species considered. In veterinary anatomy, it is universally accepted the division of the equine lung into five lobes: left cranial, right cranial, left caudal, right caudal and right accessory (Figure 1.1). Nevertheless, a study proposed a more precise subdivision of the lung parenchyma into cranial, middle, caudal and accessory lobes bilaterally. This different subdivision is based on the study of bronchial and pulmonary artery ramifications, rather than on the external aspect of the organ (Nakakuki, 1993).

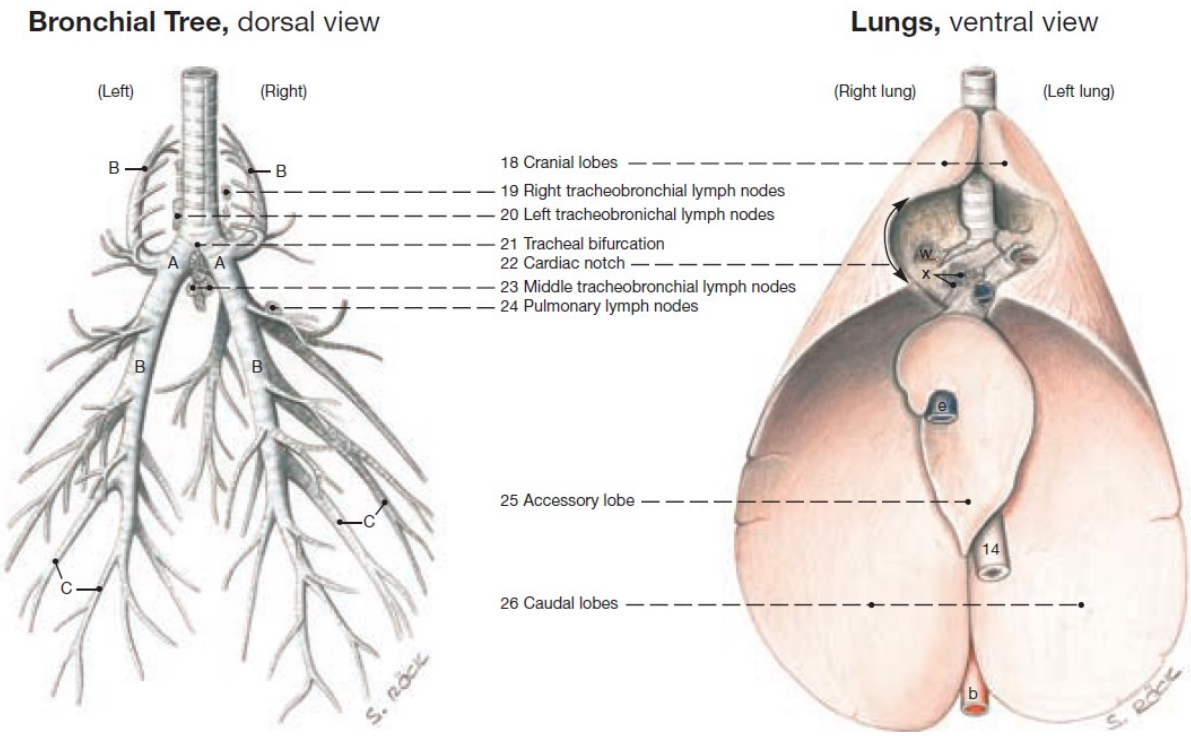

Fig. 1.1 – Schematic representation of the dorsal view of bronchial tree and the ventral view of equine lungs. A: principal bronchus; B: lobar bronchus; C: segmental bronchus; b: aorta; e: caudal vena cava; w: pulmonary artery; x: pulmonary veins; 14: esophagus (Budras *et al.*, 2009).

The pulmonary circulation arises from the right ventricle (RV) with the main pulmonary trunk. The main pulmonary trunk extends dorsally to the left and cranial to the aorta, to which is joined by the *ligamentum arteriosum*. As soon as it exits the pericardium the main pulmonary trunk divides into two branches: *arteria pulmonalis dextra* and *arteria pulmonalis sinistra*, which supply the right and left lung, respectively. The two branches have about half the caliber of the main pulmonary trunk and enter the lung at the level of the *hilum*. Then, they divide to form numerous thinner vessels. The transition from pulmonary arteries to arterioles is generally considered to occur from an internal diameter of 100 µm. The arterial component of the pulmonary circulation ends with the subdivision of arterioles into a dense network of capillaries, which surrounds the pulmonary alveoli. The radius of the pulmonary capillaries in horses is about 3 µm, similar to that of dogs and rabbits but with a thicker blood-gas barrier. From the network of capillaries, blood collects into numerous venules, which converge forming veins of ever-increasing caliber, until they form the pulmonary veins, which open in the left atrium (LA). In contrast to veins of systemic circulation, those of pulmonary circulation have no valves. The pulmonary veins do not flow adjacent to the pulmonary arteries but they are located close to the septa, which separate the different segments of the lung (Marlin and Vincent, 2007).

A cadaver study reported an in-depth anatomical description of the venous component of equine lungs (Vandecasteele *et al.*, 2016). Equine lungs are drained from five pulmonary veins, named right cranial pulmonary vein, right caudal pulmonary vein, left cranial pulmonary vein, left caudal pulmonary vein and accessory pulmonary vein. Each pulmonary vein, with its ramifications, drains the respective lung lobe.

The orifice through which the pulmonary veins open into the left atrium is the *ostium*. The coalescence of pulmonary veins associated with each *ostium*, just proximal to this common orifice, determines the formation of a venous space, which takes the name of *antrum*. Four main *ostia* can be identified in the dorsal area of left atrium, between the left auricle and the interatrial septum. The first *ostium* drains the cranial aspect of the left caudal lung lobe and it is positioned caudally and closest to the left auricle. The second *ostium* is the larger orifice and drains the caudal segments of both lungs; it is located to the right of the first *ostium*. The third *ostium* is positioned cranial and to the right of the second *ostium* and drains the cranial and middle segments of the right lung. The fourth *ostium* drains the left cranial lobe and it is located on the left side of the left ventricle, close to the left auricle. The venous component of the accessory lobe is quite variable and can empty directly in the left atrium or through the first or second *ostium* (Vandecasteele *et al.*, 2016).

1.1.2 Bronchial circulation

The bronchial circulation is a branch of the systemic circulation. It originates from the broncho-oesophageal artery, which divides into right, middle and left bronchial arteries. The middle bronchial artery branches at the level of the tracheal bifurcation and supplies the caudal lobes; instead, the cranial lobes are supplied by the right and left bronchial arteries. Bronchial arteries form a circulatory plexus within connective tissue of the airways, from which depart branches that penetrate the bronchial walls to form a subepithelial vascular plexus. The role of this plexus is likely to dissipate heat. Another system of branches of the bronchial circulation supplies the network of alveolar capillaries. In contrast to most species, horses do not have bronchial veins that drain blood in the large bronchi walls. The equine bronchial circulation is drained by the pulmonary veins and the azygos vein. The latter terminates in the superior vena cava (Marlin and Vincent, 2007).

The pulmonary and bronchial circulations anastomose at the level of the terminal bronchioles. These anastomoses locate most at the level of veins and capillaries rather than arteries, and they are frequent in the equine lung. They represent a potential way to shunt blood flow in order to

attenuate the increased capillary pressure that could occur from increases in pulmonary arterial (i.e. during exercise) or venous (i.e. left-side heart failure) pressure (Marlin and Vincent, 2007).

1.2 Histology

From a histological point of view, vessels have three different layers called tunica: *tunica intima*, *tunica media* and *tunica externa*. The *tunica intima*, also called *tunica interna*, represents the innermost component of the vessels and it is formed by layers of epithelial and connective tissue. Endothelium, which is a simple squamous epithelium continuous throughout the vascular system, lined this tunica. Instead, the outer layer of *tunica intima* consists of a thin areolar connective tissue with elastic and collagenous fibers that provide flexibility and additional strength, respectively. Endothelium and connective tissue are bonded together by the basal lamina. The *tunica media* is the middle layer and it consists of layers of smooth muscle supported by connective tissue. The contraction and the relaxation of these smooth muscles permit the vasoconstriction and the vasodilation. This is possible because of the presence of vascular nerves called *nervi vasorum* that are made of sympathetic fibers. The *tunica adventitia*, also called *tunica externa*, is the topmost component of vessels and it is made of a sheath of connective tissue that presents collagenous fibers and a less amount of elastic fibers. The outermost layer of *tunica adventitia* has connective tissue as a support to help holding vessels in the right position. These three tunics have different characteristics according to the type of blood vessel considered (arteries, veins or capillaries), as well as according to the anatomical area supplied. Vessels wall has smaller blood vessels inside, called *vasa vasorum* that provide nourishment to it (Bit *et al.*, 2020).

The *tunica intima* of the entire pulmonary vascular bed consists of a non-fenestrated monolayer endothelium, whose epithelial cells are connected by tight junctions, which limit the exchange between the vascular lumen and the interstitium. The number of tight junctions is greater in the *tunica intima* of the arteries than of the capillaries or veins. Moreover, the endothelium is also characterized by the presence of myo-endothelial junctions which, thanks to the discontinuity of the internal elastic lamina, allow contact and communication between the smooth muscle cells of the *tunica media* and the endothelium (Townsley, 2013).

On the base of the presence of elastic lamina and the degree of muscularity of the *tunica media*, pulmonary arteries and arterioles are classified as elastic, transitional and muscular. The pulmonary trunk and the main pulmonary arteries are predominantly elastic and give rise to

arteries of lower caliber, in which the muscular component predominates. Nevertheless, it is not possible to predict the classification of the pulmonary arterial component solely on the basis of its location within the respiratory tree or on the vascular diameter. Throughout much of the pulmonary vascular bed, the internal elastic lamina is characterized by frequent gaps, which enable endothelial projections into the *tunica media* and facilitate myo-endothelial communication. Considering the venous component, on the other hand, the large caliber pulmonary veins are muscular and non-elastic, unlike what happens for the arteries (Townsley, 2013).

In the lungs, the *tunica adventitia* is shared with the connective tissue of the adjacent airways and has a variable thickness. The *tunica adventitia* of larger arteries consists of a large amount of tissue, which allows limiting the mechanical forces that are discharged on the vessels during pulmonary expansion. On the other hand, the smaller extra-alveolar vessels, located more deeply within the respiratory parenchyma, have a thin *tunica adventitia* that is almost completely fused with the alveolar septa. Consequently, these vessels are more affected by the forces generated during breathing and undergo expansion during lung distension. Fibroblasts sparsely populate the *tunica adventitia* of the pulmonary arteries and are mainly responsible for the synthesis of collagens, fibronectin, proteoglycans and elastin. The elastic fibers and collagen in the outermost lamina of small vessels are continuous with those of the alveolar septal wall, forming a dense network that reaches the pleural surface and constitutes an important source of stability for the architecture of the organ (Townsley, 2013).

In contrast to the dynamism of the collagen, which undergoes production and remodeling during life, the elastic fibers present a relative stability and they are not subject to renewal. Nonetheless, it is known that the elasticity of the pulmonary arteries in human lungs decreases with age (Castillo *et al.*, 1967; Townsley, 2013).

1.3 Physiology

The main function of pulmonary circulation is to move blood to and from blood-gas barrier for gas exchange. Moreover, it has other two functions: to act as reservoir of blood and to filter blood (West, 2008).

1.3.1 Gas exchange

Gas exchange that occurs incessantly between alveoli and capillaries is possible due to the difference in partial pressure of oxygen (pO2) and carbon dioxide (pCO2) existing between these two compartments. Venous blood has a pO2 of about 40 mmHg and a pCO2 of about 46 mmHg, while in the pulmonary alveoli these two pressures are, respectively, of 100 mmHg and 40 mmHg. This pressure gradient allows the passive diffusion of oxygen towards capillaries and that of carbon dioxide towards the alveoli (Boiti, 2010). In healthy horses at rest, arterial partial pressure of oxygen (PaO2) is 85–100 mmHg and arterial partial pressure of carbon dioxide (PaCO2) is 40–45 mmHg (Art and Bayly, 2014).

Ventilation has the purpose of conveying air rich in oxygen and low in carbon dioxide at the alveolar level, in order to maintain the pressure gradient between the alveoli and the blood that allows gas exchange. In horses, approximately only the 50% of inspired gas reaches the alveolar space; the remaining portion of the tidal volume remains in the upper airways, forming the so-called "anatomic dead space", and it is subsequently exhaled unchanged with the next breath. Ventilation of dead space is probably a way to dissipate heat during exercise. Similarly, the portion of inspired gas that inflates non-perfused alveoli does not contribute to gas exchange and it is called “alveolar dead space”. The association of anatomic dead space and alveolar dead space is known as “physiological dead space” (Clutton, 2007).

Oxygen can be carried in the blood either dissolved in the plasma or bound to haemoglobin. However, the solubility coefficient of O_2 is low and, thus, plasma carries only small volume of O_2 that are not sufficient for the request of the body. Therefore, the presence of haemoglobin inside the erythrocytes is essential for survival. It increases the O_2 carrying capacity of blood 65-fold (Clutton, 2007).

Haemoglobin is a protein containing four heme groups, each capable of binding an oxygen molecule. It can be present in two different forms: oxyhaemoglobin and deoxyhaemoglobin. When blood flows inside the pulmonary capillaries, the pO_2 is higher within the alveolar compartment and, therefore, there is a diffusion of oxygen from the lung parenchyma to the blood, with the formation of oxyhaemoglobin. In the tissue capillaries, on the other hand, the plasma pO_2 is higher than that in the tissues and therefore there is a release of the oxygen molecules linked to haemoglobin, which are transferred to the body's cells (Boiti, 2010).

A process similar to that involved in oxygen uptake, allows the elimination of carbon dioxide generated in body tissues. Carbone dioxide represents the final product of cells metabolism. It

diffuses from active cells into interstitial fluid and then across capillary walls into plasma. Its transport can take place in three different ways: as carbon dioxide in solution, as carbamin compounds, and as carbonic acid. In lungs, pCO_2 within alveoli is less than that of capillaries and, therefore, CO_2 diffuses from blood to alveolar space and then it is eliminated with the expiration (Clutton, 2007).

1.3.2 Lungs as blood reservoir

In rest horses, pulmonary circulation is typically considered a low-pressure, high-conductance system due to the characteristics of its vessels, in particular of artery and arterioles, that are far less muscular and more elastic than those of systemic circulation (Poole and Erickson, 2008).
These characteristics allow the pulmonary circulation to adapt to even significant changes in blood volume, without leading to an increase in pulmonary pressure. However, during exercise the situation is in part different; in fact, the mechanisms of capillary distension and recruitment that normally occur when the blood volume present in pulmonary circulation increases, are able to limit a possible increase in pulmonary pressure only in conditions of mild or moderate exercise. In horses, maximal exercise can lead to such an important increase in cardiac output as to exceed the adaptability of the alveolar capillaries. Therefore, at particularly high values of heart rate, it is possible to observe physiological increases in pulmonary pressure (Poole and Erickson, 2008). Unlike other organs, lungs have a rather marked perfusion heterogeneity, both in humans and in horses; this means that some lung regions tend to receive a greater amount of blood than others (Art and Bayly, 2014).
In the human lung, the basal portions receive about eight times more blood than the apical ones due to the presence of a pressure gradient that limits the blood flow that reaches the lung. The pressure inside the alveoli corresponds to the atmospheric pressure and it is identical throughout the organ, regardless of the portion considered. On the other hand, arterial and venous blood pressures are lower in the most apical portions than in the basal ones, due to gravity. Therefore, in the apical portions, the alveolar pressure exceeds the venous pressure (and, in pathological conditions, also the arterial pressure), causing compression of the capillary bed and severely limiting the perfusion of these regions. Proceeding more distally, on the other hand, both arterial and venous pressure are higher than the alveolar one, thus determining the presence of a continuous blood flow in the more basal lung regions (West, 2008).

Conversely, in horses it has been shown that lung perfusion is not affected by the gravity. Mechanisms that are not yet fully known determine a greater distribution of blood in the more dorsal lung areas (Figure 1.2) (Hlastala *et al.*, 1996). The most accredited hypothesis regards a different production of nitric oxide by the capillaries of the dorsocaudal lung regions, a lower affinity for vasoactive substances by the capillaries of the cranioventral regions, and the contrasting role of some substances with cholinergic action, such as methacholine, that determine vasoconstriction of capillaries of cranioventral portions but vasodilation of those in the dorsocaudal ones (Pelletier *et al.*, 1998).

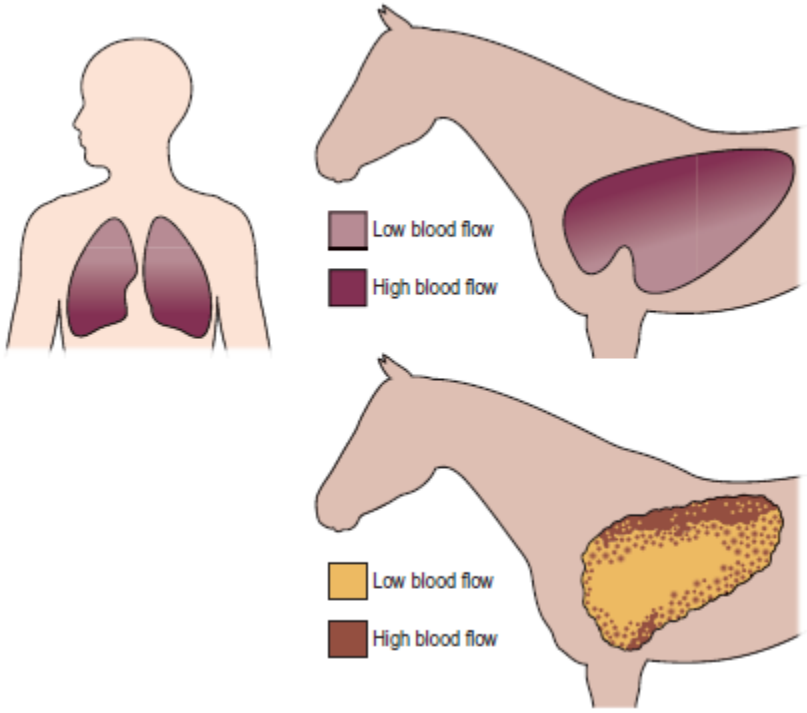

Fig. 1.2 – Pulmonary blood distribution according to vertical perfusion gradient in resting human (top left) and caudo-dorsal gradient in resting horse (top right). In the horse, the caudo-dorsal gradient is exacerbated by exercise as is evident from the schematic representation of a post-exercise scintigraphic image (bottom) (Art and Bayly, 2014).

In humans, exercise and the consequent increase in heart rate leads to greater homogeneity of lung perfusion. Conversely, in horses, exercise exacerbate the differences already present at rest (Figure 1.2). Moreover, the significant increase in blood flow that occurs in the more dorsal portions is greater in the transition phase from rest to trot rather than during the subsequent phases of progressive increase in speed (Bernard *et al.*, 1996).

Both in humans and horses, the pulmonary vascular bed must constantly be able to adapt to variations in cardiac output, accepting all the blood pumped by the right heart. Although the pulmonary circulation normally contains a considerably lower amount of blood than the systemic circulation, this situation can change due to exercise or conditions that lead to peripheral

vasoconstriction, resulting in an increase in blood flow that reaches the lung (Poole and Erickson, 2008; West, 2008). If the pulmonary capillaries are unable to adapt to these situations, we would periodically see dangerous increases in lung pressure, even under mild exercise conditions. The reason why this does not happen is linked to the presence of two homeostatic compensatory mechanisms of fundamental importance: recruitment and distension. Elevated pulmonary arterial pressure forces the perfusion of previously non-perfused vessels (recruitment) and distends those vessels already recruited (distension) (Figure 1.3) (Poole and Erickson, 2008; West, 2008).

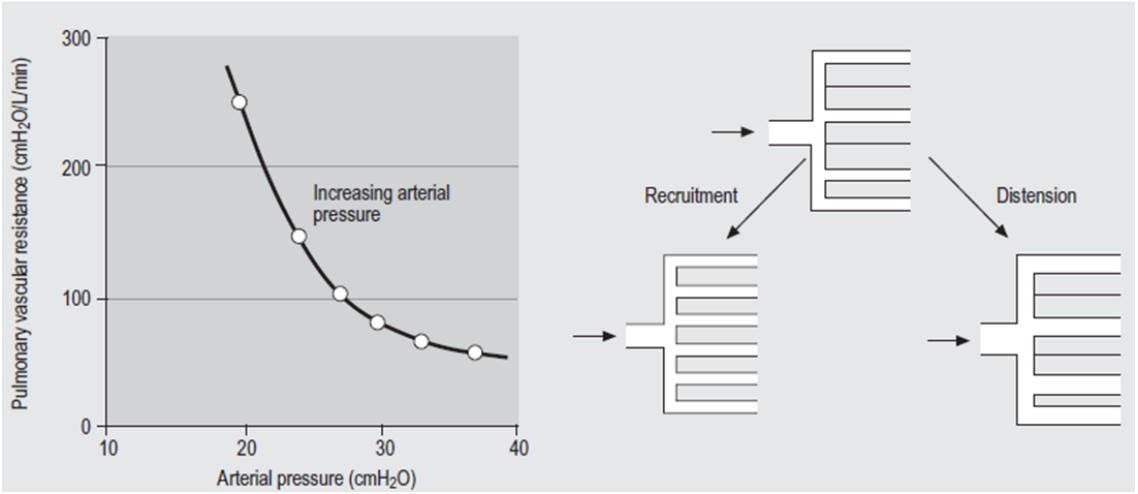

Fig. 1.3 – As the pressure in the pulmonary circulation increases, there is a reduction in pulmonary vascular resistance (left), through mechanisms of recruitment and distension of pulmonary capillaries (right). This scheme refers to equine (Poole and Erickson, 2008).

1.3.3 Lungs as a filter

Another fundamental function of the pulmonary circulation is its ability to trap and eliminate thrombi and emboli present in the systemic venous compartment (Marlin and Vincent, 2007). The size of the particles that can be filtered in the lungs differs substantially according to the size of the capillaries considered, which vary according to the animal species. Regardless of this, the pulmonary circulation is, therefore, able to prevent thrombi or emboli from reaching the brain or other organs, causing ischemic phenomena, potentially fatal. The particles present in the circulation can be represented by aggregates of leukocytes, erythrocytes, fibrin clots and tumour cells. These substances are stopped at the level of the capillaries which have a smaller diameter than the substances themselves and are mainly broken down by proteolytic enzymes or by phagocytosis (Heinemann and Fishman, 1969).

Chapter 2

PULMONARY PRESSURE

The term “pulmonary pressure” means the value of blood pressure within the pulmonary artery system. It is influenced by several factors, such as pressure of right and left heart, proportion of the vascular bed perfused, pulmonary arterial smooth muscle tone, circulatory volume, relative and absolute lung volume, and changes in intrathoracic pressure that occur with ventilation. Moreover, also some variables related to the animal can influence pulmonary pressure, such as the size of the subject, age, physical exercise and the presence of diseases (Marlin and Vincent, 2007).

Pulmonary pressure in standing adult horses is considerably lower than systemic pressure (around 1/5 to 1/6) because of the lower resistance encountered in the pulmonary vascular tree. In the new-born foal, the pulmonary pressure is higher than in adult horses; then it decreases significantly during the first two weeks of life as pulmonary arteriolar resistance falls (Schwarzwald, 2018).

Pulmonary pressure in the resting horse is slightly higher than in humans and small mammals. The normal systolic pulmonary pressure in horses is around 35-45 mmHg while the diastolic pulmonary pressure is approximately 20-25 mmHg. The mean pulmonary pressure in horses at rest ranges between 20 and 30 mmHg. During high-intensity exercise, the mean pulmonary pressure can easily reach 100 mmHg (Marlin and Vincent, 2007; Schwarzwald, 2018).

The factors that influence pulmonary pressure are grouped under the name of "pulmonary vascular resistance" (PVR). PVR is the force that opposes the passage of blood within the pulmonary circulation. PVR is an extremely dynamic value, capable of changing not only due to changes in circulatory volume or vascular tone, but also simply with the succession of respiratory cycles that causes changes in the intrathoracic pressure. During inspiration and the expansion of lungs, the attachment between the parenchyma and the vasculature determines a radial expansion of the blood vessels, thereby decreasing PVR; instead, during expiration, the vessels diameter is reduced due to the compression by the parenchyma and thereby PVR increases. Moreover, PVR is influenced by absolute lung volume. At very low lung volumes, the PVR is maximal because the support of parenchyma may become negligible, and the caliber of the vessels is determined by their wall stiffness. In addition, at high lung volumes and up to total lung

capacity, PVR becomes elevated because of longitudinal stretching of small blood vessels in the alveolar septa (Marlin and Vincent, 2007).

PVR in horses is low, with values of about 0.14 and 0.06 mmHg/ml/min.kg at rest and during exercise, respectively. The majority of PVR at rest is due to precapillary sphincters while the post-capillary resistance is very low. During exercise, the two homeostatic mechanisms of dilation and recruitment make the previous non-perfused or minimally perfused vessels functional; probably this is the major contribution to a decreased PVR during exercise (Art and Bayly, 2014).

2.1 Pathophysiology: pulmonary hypertension

The term pulmonary hypertension (PHT) indicates an hemodynamic situation in which pulmonary pressure exceed the upper normal limit by more than 10 mmHg (Schwarzwald, 2018). PHT implies an increase of cardiac output and/or pulmonary vascular resistance (Bonagura, 2019). Precapillary pulmonary hypertension is caused by a pulmonary vascular remodeling, upstream of the capillary network, that leads to an increase in pulmonary vascular resistance. Instead, the term postcapillary pulmonary hypertension refers to a condition in which the increase in blood pressure is linked to blood stasis that occurs downstream of the capillary network (Naeije and Chin, 2019). In human medicine, PHT is classified in five groups according to aetiology. The first group is represented by pulmonary arterial hypertension including idiopathic PHT, drug and toxin induced PHT, and persistent PHT of the new-born. The second group includes pulmonary hypertension due to left-side heart disease due to congenital and acquired cardiac diseases. The third group includes PHT due to lung diseases and/or hypoxia, such as chronic obstructive pulmonary diseases, interstitial lung diseases and sleep-disordered breathing. Group four is represented by chronic thromboembolic pulmonary hypertension and other pulmonary artery obstructions. Finally, in the fifth group are included PHT due to unclear and/or multifactorial mechanisms such as hematologic, systemic and metabolic disorders (Galiè *et al.*, 2016). According to this classification, some of the potentially relevant causes for horses are showed in the Table 2.1. In veterinary medicine there is no consensus about the classification of PHT; however, it may be practical to use a pathophysiologic or functional classification, like exercise-induced PHT, postcapillary PHT, PHT due to advanced pulmonary diseases and precapillary pulmonary arterial hypertension (PAH) (Bonagura, 2019).

Table 2.1 - Potential aetiologies of pulmonary hypertension

Exercise-related PH
High cardiac output-related PH
PAH
Congenital heart disease (Eisenmenger's pathophysiology) Pulmonary veno-occlusive disease Drug or toxin induced Persistent PH of the newborn Pulmonary arteriopathy Idiopathic PAH (?)
PH owing to left heart disease
Left ventricular dysfunction Valvular heart disease Congenital disease
PH owing to lung disease or hypoxia
Chronic reactive (obstructive) pulmonary disease (asthma) Interstitial lung disease Pleuropneumonia (?) Chronic exposure to high altitude (?)
Chronic thromboembolic PH (?)

This table showed some of the potentially relevant causes of pulmonary hypertension in horses. PAH: pulmonary arterial hypertension; PH: pulmonary hypertension; (?): uncertain relevancy to horses (Bonagura, 2019).

2.1.1 Exercise-induced pulmonary hypertension

The significant increase in pulmonary pressure that occurs during maximal exercise is a typical characteristic of the equine, and the mean pulmonary pressure can easily reach 100 mmHg due to the large cardiac output achieved by exercising horses (Art and Bayly, 2014; Schwarzwald, 2018).

Pulmonary hypertension that physiologically develops during exercise is considered one of the main causes of exercised-induced pulmonary haemorrhage (EIPH), typical of racehorses. The rupture of alveolar capillaries occurs when the transmural pressure (pressure difference between the luminal capillary and intra-alveolar pressure) increases excessively, exceeding the tensile strength of the pulmonary capillaries wall with consequent release of blood inside the alveoli (Hinchcliff, 2014).

In horses, during maximal exercise, heart rate increases six- to eight-fold, cardiac output increases eight- to ten-fold, and packed cell volume increases nearly two-fold the resting values. These factors contribute to the increase in pulmonary pressure during exercise (Erickson *et al.*, 1990).

Maximal exercise causes an increase in blood volume that reaches the lung by about 10-fold, without however an equivalent increase in pulmonary pressure, which increases a maximum of three-fold compared to that at rest (Marlin and Vincent, 2007). This is possible because the increased blood flow in pulmonary circulation induces recruitment and distension of the pulmonary capillaries, resulting in a reduction in PVR that reaches minimum values during moderate exercise (0.06 mmHg/ml/min.kg). Once the minimum PVR value has been reached, the pulmonary capillaries are no longer able to compensate for a further increase in pulmonary pressure during maximal effort (Manohar and Goetz, 1999; Art and Bayly, 2014).

On the other side, during maximal exercise, there is a marked decrease in pleural and therefore intra-alveolar pressure; it decreases from -0.7 kPa at rest to -8.5 kPa during strenuous exercise. The combination of increased intra-capillary pressure and decreased intra-alveolar pressure contributes to markedly increase the transmural pressure that stresses alveolar wall leading to rupture (Hinchcliff 2014).

2.1.2 Postcapillary pulmonary hypertension

Postcapillary PHT can be caused by left heart diseases that lead to left heart failure (Bonagura, 2019). Left heart failure refers to the inability of the left compartment of the heart to effectively pump blood within the systemic circulation. The causes of heart failure can be different; in horses the most common are mitral or aortic valve insufficiencies, valve endocarditis, congenital cardiac diseases (ventricular septal defect, patent ductus arteriosus), myocarditis and rupture of chorda tendinea (Marr, 2010). Depending on the primary cause and its severity, congestive heart failure can occur more or less quickly; this condition is characterized by stagnation of blood inside the heart chambers. This causes an increase in ventricular and atrial pressure and limits pulmonary venous drainage, resulting in congestion of pulmonary circulation, increase in capillary hydrostatic pressure and consequently, development of pulmonary oedema (Marr, 2010). The pulmonary hypertension is due to processes of vasoconstriction and vascular remodeling that occur in order to overcome the high hydrostatic pressure present in the pulmonary capillaries, in

order to guarantee an unidirectional flow of blood from the pulmonary artery towards the left atrium (Schwarzwald, 2018; Bonagura, 2019).

Another cause of postcapillary PHT is the regional pulmonary veno-occlusion (Bonagura, 2019) that is very similar to the human pulmonary veno-occlusive disease (PVOD). In humans, PVOD is an uncommon cause of PHT, characterized by pulmonary venous sclerosis, interstitial and septal fibrosis, marked hemosiderin accumulation, and proliferation of the bronchial circulation (Williams *et al.*, 2008).

2.1.3 Pulmonary hypertension due to advanced pulmonary diseases

In the systemic circulation, hypoxia induces vasodilation and consequent increase in blood supply to the tissue. Conversely, hypoxia in the pulmonary circulation causes vasoconstriction; this phenomenon is called "hypoxic pulmonary vasoconstriction". This adaptation limits the vascularization of the unventilated or poorly ventilated lung regions maintaining a correct ventilation/perfusion ratio (V/Q ratio), which is obtained by spreading the blood flow towards the lung portions that are able to determine a correct oxygenation of the blood. Hypoxic pulmonary vasoconstriction can occur in physiological conditions, such as during exercise and at altitude, or as a consequence of pulmonary diseases (Marlin and Vincent, 2007). Chronic pulmonary diseases are associated with a persistent and initial hypoxic vasoconstriction, followed by phenomena of vascular remodeling. Both hypoxic vasoconstriction and vascular remodeling are the triggering causes of pulmonary hypertension that develops during severe chronic lung diseases (Stenmark and McMurtry, 2005).

One of the most frequent pulmonary diseases in horses is the severe equine asthma. Its association with pulmonary hypertension is known since 1978 with a study that measured pulmonary pressure by right cardiac catheterization in healthy and asthmatic horses. The study showed that both symptomatic (44.56 ± 13.84 mmHg) and asymptomatic (28.13 ± 4.37 mmHg) asthmatic horses had significantly higher pulmonary pressure than healthy horses (23.54 ± 2.98 mmHg) (Dixon, 1978).

Since oxygen administration only partially reversed the pulmonary hypertension in these patients, the involvement of some factors other than hypoxia was hypothesized (Dixon, 1978). In human medicine, it has been demonstrated that chronic obstructive pulmonary diseases can induce remodeling of the pulmonary arteries structure which is at the basis of persistent pulmonary hypertension (Harkness *et al.*, 2014). In these patients, recurrent episodes of

hypoxemia and hypercapnia, associated with inflammatory mediators and cytokines released due to chronic inflammation, cause structural modifications of the pulmonary vascular bed. These changes consist in muscularization of pulmonary arterioles and excessively deposition of matrix proteins in the wall of large pulmonary arteries. The excess of collagen compromises the elasticity of pulmonary arteries. The stiff pulmonary artery causes elevated pulmonary vascular resistance and contributes to the raised oscillatory load that increases right ventricular systolic pressure. Moreover, this stiffness itself leads to further damages to the pulmonary vascular bed over time, ultimately leading to pulmonary hypertension (Harkness *et al.*, 2014; Altiparmak *et al.*, 2016).

The same mechanism has recently been confirmed also in horses, in which histomorphometry, performed both in vivo (through peripheral biopsies of the caudodorsal lung) and post mortem, has shown the presence of a particularly important and hardly reversible vascular remodeling of the pulmonary vessels wall in patients with severe equine asthma. The hypoxic vasoconstriction and the inflammation represent the starting point for the development of vascular modifications, such as thickening of the vessel wall, with significant proliferation of the smooth muscle component and consequent reduction of the vessel lumen (Fig. 2.1). This remodeling involves both the lung apex and the caudodorsal lung region. The resulting narrowing of the artery lumen could enhance hypoxic pulmonary vasoconstriction. This justifies the fact that hypoxemia determines a transient increase in mean pulmonary pressure in healthy horses, but not a real condition of pulmonary hypertension which is instead present only in pathological patients with pulmonary vascular remodeling (Ceriotti *et al.*, 2020).

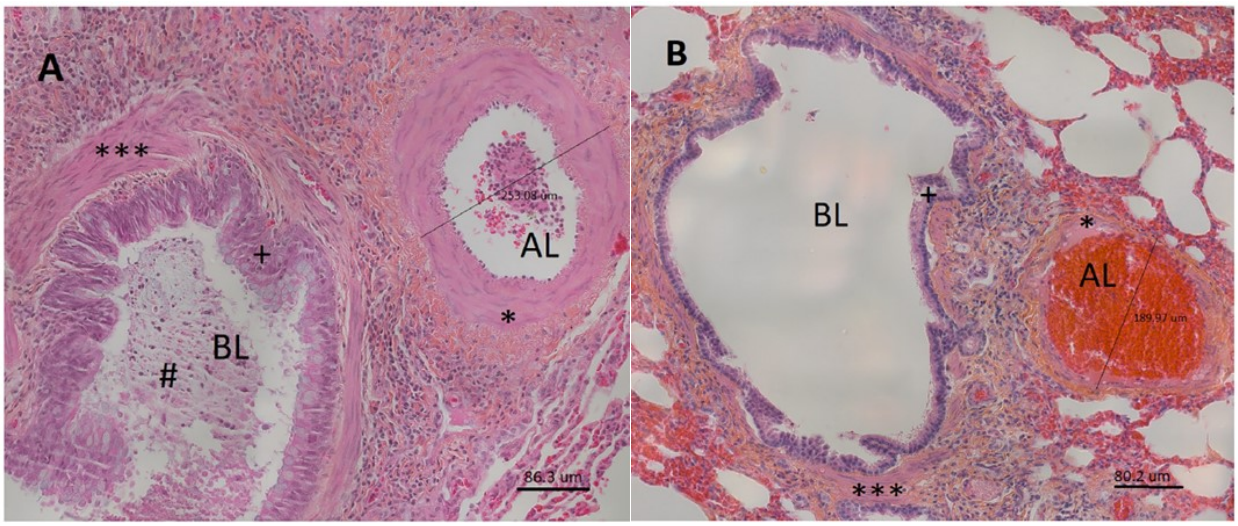

Figure 2.1 – Histological section (40X, hematoxylin eosin saffron) of pulmonary artery and annexed bronchus of an asthmatic horse in exacerbation (A) and of a control horse (B). Notice the thickening of the muscular pulmonary artery wall (*), the mucus

accumulation in the airway lumen (#), the epithelial thickening (+) and the increased airway smooth muscle mass (***) in the asthmatic subject (A) compared to the healthy one (B). BL: bronchus lumen; AL: artery lumen (Ceriotti *et al.*, 2020).

The same pathophysiological modifications can develop in other chronic pulmonary diseases that cause hypoxia and consequent pulmonary hypertension in horses, such as granulomatous pneumonia (Schwarzwald *et al.*, 2006) and pulmonary fibrosis (Le Corre *et al.*, 2019).

2.1.4 Precapillary pulmonary arterial hypertension

Chronic thromboembolic pulmonary hypertension is a rare but severe consequence of acute pulmonary embolism. It is a form of precapillary PHT that results from an incomplete resolution of pulmonary thromboembolism and consequent formation of a chronic, fibrotic, organized thrombus within the pulmonary vascular bed that can limit the normal flow and lead to hypertension (Mahmud *et al.*, 2018).

Pulmonary thromboembolism is largely unrecognized in horses (Bonagura, 2019). However, it is possible that its real prevalence is higher than what it is effectively diagnosed. In equine medicine, cases of pulmonary thromboembolism have been reported in patients affected from different severe systemic diseases of an infectious and neoplastic aetiology or in patients undergoing abdominal surgery. Although the triggering causes are not fully known, it seems that the formation of thrombi is connected to the presence of systemic inflammation capable of leading to hypercoagulability (Norman *et al.*, 2008; Bryan *et al.*, 2009).

2.1.5 Clinical signs and cor pulmonale

Regardless of the cause, the symptoms of pulmonary hypertension are nonspecific and mainly related to a decrease in athletic performance, which may be due to the primary cause of PHT (left heart failure, pulmonary diseases) or may be a consequence of that (Bonagura, 2019).

Pulmonary hypertension, if severe and permanent, can have devastating effects on the body, especially on the right heart, which can undergo remodeling. The development of right ventricular hypertrophy and/or dilation that occurs as a consequence of PHT due to lung disease, in absence of primary cardiac diseases, is defined "*cor pulmonale*" (Schwarzwald, 2018; Bonagura, 2019). This occurs because PHT increases afterload on the right ventricle (RV) and leads to elevated end-diastolic and central venous pressure. In case of acute PHT, the right ventricle does not have time to develop adaptive mechanisms, such as RV hypertrophy, and therefore it undergoes enlargement. On the other hand, in case of chronic PHT, the increased

afterload is associated with hypertrophy of the RV with a consequent increase in the force of contraction and intraventricular pressure. Cardiac remodeling and increased RV pressure can lead to tricuspid regurgitation. In most severe cases, the interventricular septum bulges into the left ventricle (LV), interfering with the ventricular filling and therefore leading to diastolic LV dysfunction and consequent right-sided or biventricular heart failure (Schwarzwald, 2018).

Cor pulmonale has always been considered uncommon in horses. Conversely of what happens in humans, chronic obstructive pulmonary diseases can lead to pulmonary hypertension and cause transient cardiovascular alterations, which however seem to resolve once the event is over (Johansson *et al.*, 2007). A more recent study on asthmatic horses has confirmed that, during clinical symptoms, alterations of the right heart function, increased right heart pressure and increased pulmonary artery diameter may be observed, despite no significant alterations in RV diameters. However, even in remission, asthmatic horses have a thicker RV wall, a higher left ventricular end-systolic eccentricity index, a longer RV pre-ejection period and a lower RV contractile function than healthy horses. These findings suggest the presence of a subclinical permanent myocardial remodeling, possibly due to successive episodes of hypoxemia and pulmonary hypertension. However, these alterations cannot be observed using merely crude RV size measurements and the authors claimed that a proper evaluation of cardiac function is not routinely performed in asthmatic horses; therefore, it is possible that the true prevalence of cardiac abnormalities that develop in these patients is actually greatly underestimated (Decloedt *et al.*, 2017a).

Real cases of *cor pulmonale* have been documented in horses, associated with severe chronic respiratory diseases such as granulomatous pneumonia (Schwarzwald *et al.*, 2006), severe equine asthma (Sage *et al.*, 2006; Hanka *et al.*, 2015) and pulmonary fibrosis (Le Corre *et al.*, 2019).

2.2 Evaluation of pulmonary pressure

The pulmonary pressure can be measured directly by right cardiac catheterization or indirectly through different methods, such as magnetic resonance imaging, computed tomography or ultrasonography; the indirect methods give an estimate of the real value.

The mean pulmonary pressure in resting horses is about 20-30 mmHg; if the pulmonary pressure exceed the upper normal limit by more than 10 mmHg there is pulmonary hypertension (Schwarzwald, 2018).

2.2.1 Right cardiac catheterization

The right cardiac catheterization is the gold standard for the measurement of pulmonary pressure both in human and in veterinary medicine (Noble and Kay, 1974; Dixon, 1978; Johnson, 1999; Galiè *et al.*, 2016). In equine medicine, this technique was used both in resting horses (Dixon, 1978) and during exercise (Erickson, Erickson and Coffman, 1990; Manohar *et al.*, 1998). The right cardiac catheterization is typically performed in standing horses using a percutaneous technique; the catheter placement in the recumbent horse is significantly more difficult. The correct positioning of the catheter is guided by pressure measurements and occasionally by ultrasonography. After surgical preparation and local anaesthesia, an 8-French introducer sheath is placed in the jugular vein and a 7-French balloon tipped (Swan-Ganz) catheter of 110-120 cm in length is inserted through the jugular vein and the cranial vena cava into the right atrium (RA), the right ventricle and the pulmonary artery. The Swan-Ganz catheter is equipped with a terminal balloon that is blown during advancement and therefore, it is able to record intra-vascular and intra-cardiac pressures (Schwarzwald, 2018) (Figure 2.2).

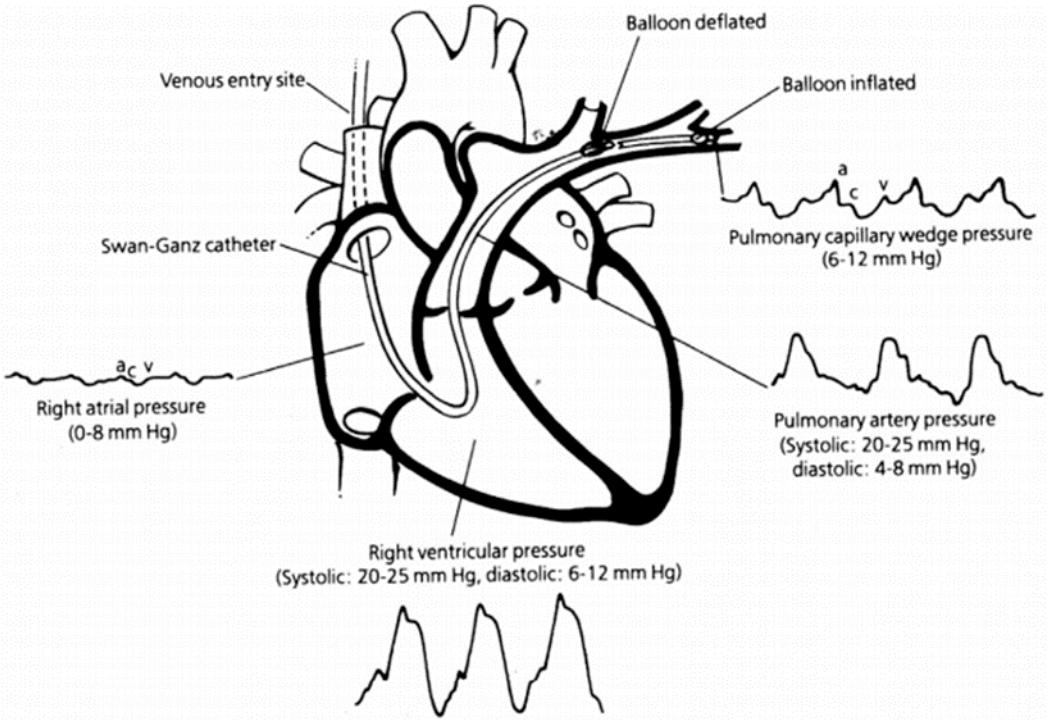

Figure 2.2 – Schematic representation of hemodynamic waveforms in right atrium, right ventricle, pulmonary artery and pulmonary capillary wedge detected by right cardiac catheterization in humans (Gidwani *et al*., 2013).

Although right cardiac catheterization is considered the gold standard for the pulmonary pressure measurement, it is an invasive procedure with potential risks. In human medicine, it is known that this technique can lead to complications related to venous access, cardiovascular

complications and rarely also to fatal events (Hoeper *et al.*, 2006). The former include hematoma at puncture site, vagal reaction with bradycardia and hypotension, pneumothorax, arteriovenous fistula and puncture of the carotid artery. Among cardiovascular complications are described supraventricular tachycardia, vagal reaction with bradycardia and hypotension, ventricular tachycardia, systemic hypotension, transient ischemic attack, hypertensive crisis, chest pain and haemoptysis after balloon inflation and new right bundle branch block. Moreover, it is necessary to consider that right cardiac catheterization is a technique that requires specialized and adequately trained operators for its execution (Hoeper *et al.*, 2006).

In horses, it has been reported the onset of cardiac arrhythmias (second-degree atrioventricular block, supraventricular and ventricular premature complexes, ventricular tachycardia), transient weakness, ataxia and syncope during the procedure (Bueno *et al.*, 1999; De Almeida Silva *et al.*, 2017). Furthermore, the probability of jugular thrombophlebitis due to the placement of venous catheter is common in horses (Dias and de Lacerda Neto, 2013).

In conclusion, the possible complications, the necessity of trained operators, an adequate restraint of the patient and his hospitalization make cardiac catheterization a difficult technique in the equine practice, unless for research purposes.

2.2.2 Computed Tomography

Computed tomography (CT) is an advanced technique of radio-diagnosis that is frequently used in human medicine to evaluate the thorax; it is a valid method to diagnose pulmonary hypertension (Alhamad *et al*., 2011; Kam *et al.*, 2013; Shen *et al.*, 2014).

Through the evaluation of vascular anatomic characteristics detected by thoracic CT, it is possible to estimate pulmonary pressure. The parameters that are taken into consideration to detect the presence or the absence of pulmonary hypertension are mainly two: the diameter of the pulmonary artery (PAD) and the ratio of PAD to ascending aorta diameter (PAD/AOD). Patients with PHT have evident anatomical alterations of the pulmonary artery, increased PAD (higher than in healthy people), and PAD/AOD ratio greater than 1 *(*Alhamad *et al*., 2011). However, these parameters showed a medium sensitivity (PAD: 79%; PAD/AOD: 74%) and specificity (PAD: 83%; PAD/AOD: 81%) suggesting a relative high rate of missed diagnosis (PAD: 21%; PAD/AOD: 26%) and misdiagnosis (PAD: 17%; PAD/AOD: 19%). Moreover, it has been reported that pulmonary artery dilation can occur also in absence of PHT in patients with some pulmonary diseases, such as pulmonary fibrosis. These considerations, associated with the lack of an

universally recognized PAD cut-off value to define the presence of PHT, demonstrate that CT cannot be considered a sufficiently reliable diagnostic technique, but it must be used in association with other methods (Shen *et al.*, 2014).

Independently of these limits known in human medicine, it must be considered that it is not possible to perform a thoracic CT in adult horses due to dimensions. In literature, there are studies regarding thoracic CT only in healthy neonatal foals (Lascola *et al.*, 2013; Schliewert *et al.*, 2015; Arencibia *et al.*, 2020).

2.2.3 Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) is an advanced diagnostic imaging technique capable of performing a highly detailed representation of different anatomical sections using magnetic fields. In human medicine, cardiac MRI (CMRI) provides detailed morphology of the cardiac chambers and accurate quantification of chamber volumes, myocardial mass and transvalvular flows; it is considered the gold standard for the measurement of ventricular volume, mass and structure (Grünig and Peacock, 2015).

An indirect estimate of pulmonary pressure can be obtained with MRI through an assessment of the cardiovascular structures (Marrone *et al.*, 2010; Johns *et al.*, 2019) or an evaluation of the motion of blood in the main pulmonary artery (Reiter *et al.*, 2013).

CMRI is based on the acquisition of a series of cross-sectional and longitudinal sequences during the entire cardiac cycle, from the apex to the base of the heart. The resulting images allow an in-depth study of the morphology of the atria and ventricles, as well as of the first outflow tract of the right heart. The alterations detectable in patients with PHT can be divided into morphological signs (RV hypertrophy or dilation, RA dilation, LV morphology alteration, pulmonary artery dilation) and functional findings (RV hypokinesis, paradoxical movement of the interventricular septum associated with the leftward deviation of the interatrial septum, RV dysfunction, pulmonary and tricuspid insufficiency, decreased pulmonary artery flow velocity, decreased right coronary artery blood flow) (Marrone *et al.*, 2010). Some of these alterations can be measured and can be used within specific formulas that allow a particularly accurate indirect estimate of the real value of pulmonary pressure, thus allowing to distinguish patients with and without PHT (Johns *et al.*, 2019).

Although the high accuracy of CMRI (sensibility 94%; specificity 79%) (Johns *et al.*, 2019), it is not routinely used in the diagnosis of PHT due mainly to high cost and poor availability of adequate machines (Wessels *et al.*, 2020).

In addition to these obvious limitations also in veterinary medicine, the use of MRI for the evaluation of patients with PHT does not appear to be a feasible diagnostic technique in equine patients since, as for CT, there are limits due to the size of adult horses. MRI is a highly useful diagnostic technique in horses but its use is limited to the head and the distal portion of limbs, arriving more proximally only in exceptional cases, with particularly small patients (Murray, 2011). As for CT, the evaluation of chest by means of MRI in horses is possible only in new-born foals (Arencibia *et al.*, 2015).

2.2.4 Ultrasonography

Ultrasonography is a diagnostic technique based on the interpretation of images that are produced by the interaction between ultrasound waves and body tissues. Compared to more advanced diagnostic techniques (CT and MRI), ultrasonography does not use ionizing radiation; moreover, it is a relatively inexpensive and easily applicable procedure in the field. Therefore, it is frequently used as the method of choice for the screening or the diagnosis of different diseases in the equine medicine (Palgrave and Kidd, 2014).

The same advantages allow ultrasonography to be considered the method of choice for the screening of pulmonary hypertension in human medicine. The detection of patients affected by PHT is possible through the evaluation of different echocardiographic parameters: first of all the tricuspid regurgitation velocity (TRV) (Janda *et al.*, 2011).

The maximum velocity of the tricuspid regurgitant jet can be measured using continuous-wave Doppler and this value can be used to estimate the transtricuspid pressure gradient (ΔP) by means of simplified Bernoulli equation:

$$\Delta P = 4\,x\,TRV^2$$

Transtricuspid pressure gradient corresponds to the difference between right ventricular systolic pressure (RVSP) and right atrial pressure (RAP). Moreover, during systole, the pulmonary valve is open, and in the absence of valvular or subvalvular obstruction, the RVSP is transmitted directly to the pulmonary artery and so can be equated to the systolic pulmonary artery pressure (sPAP). Therefore, sPAP can be estimate using this formula:

$$sPAP \approx RVSP = (4\,x\,TRV^2) + RAP$$

The RAP can be measured by means of different methods, such as the collapse of *inferior vena cava* during respiration (Janda *et al.*, 2011; Rudski and Afilato, 2016).

The mean pulmonary artery pressure (mPAP) can be approximately calculated using the echo-derived value of sPAP through this formula (Rudski and Afilato, 2016):

$$mPAP = 0.61\ x\ sPAP + 2mmHg$$

Finally, diastolic pulmonary artery pressure (dPAP) can be estimated employing the same principles used for sPAP. During diastole, the pulmonary regurgitant jet represents the pressure gradient between the pulmonary artery and the right ventricle that, therefore, can be estimated by the simplified Bernoulli equation using the end-diastolic pulmonary regurgitation velocity (PRV). Moreover, during diastole, the tricuspid valve is open and so the RV pressure and RAP can be equated. Therefore, dPAP can be estimate using this formula (Rudski and Afilato, 2016):

$$dPAP = (4\ x\ end\_diastolicPRV^2) + RAP$$

Although the TRV has long been considered the most reliable method to obtain an estimate of pulmonary pressure, the agreement between the estimated pressures derived by this method and those measured invasively is poor. Minimal errors in the measurement of TRV can result in underestimating or overestimating the real pressure values in a significant way because in the Bernoulli equation, the measured value is squared and multiplied by 4. Other limitations of the method relate to the difficulty in measuring changes in the diameter of the *inferior vena cava*, to the mismatch between TRV and right ventricular systolic pressure in patients with severe tricuspid insufficiency and to the impossibility of excluding PHT in patients without tricuspid regurgitation. For these reasons, in recent years, echocardiography is considered solely as a tool for identifying the presence or absence of pulmonary hypertension, without attempting to estimate a value (Augustine *et al.*, 2018).

For the assessment of the probability of PHT, the measurement of TRV should be used in association with other echocardiographic parameters, such as PAD, acceleration time of the RV outflow tract, early diastolic PRV, pulmonary systolic notch, eccentricity index, RV/LV basal diameter ratio, right atrial area and *inferior vena cava* diameter. Based on echocardiographic findings, the probability of PHT can be classified as low, intermediate and high (Figure 2.3) (Augustine *et al.*, 2018).

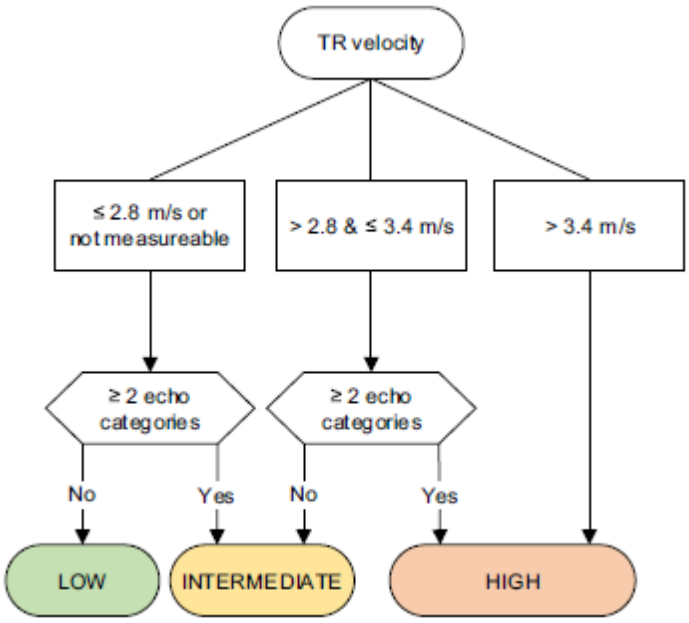

Figure 2.3 – Flow chart to assess the probability of pulmonary hypertension using tricuspid regurgitation (TR) velocity associated with other echocardiographic parameters. This flow chart refers to human medicine (Augustine *et al.*, 2018)

In dogs, TRV associated with other echocardiographic changes secondary to PHT is used to classified the probability of PHT as low, intermediate and high using a flow chart similar to the human one (Figure 2.3) (Reinero *et al.*, 2020).

In equine medicine, to date, there are no officially recognized echocardiographic parameters to obtain an estimate of pulmonary pressure. If tricuspid or pulmonary regurgitation are present, continuous-wave Doppler can be used to measure the peak regurgitant velocity and estimate an elevated pulmonary pressure. As rough guidelines, in resting horses, a TRV greater than 3.5 m/s or a PRV of exceeding 2.5 m/s are quite suggestive of PHT. However, tricuspid and pulmonary regurgitation are not present in all horses with PHT (Bonagura, 2019). Moreover, the impossibility to obtain a right alignment between the regurgitant flow and the ultrasound beam can underestimate the peak velocity. Finally, the presence of mild or moderate tricuspid regurgitation is frequent in old horses and it is not necessarily associated with diseases (Reef, 1998).

Currently, the most reliable echocardiographic parameters to assess the presence or absence of PHT in horses seem to be the measurement of the pulmonary artery diameter and the relative relationship between the pulmonary artery and aortic root diameter. A pulmonary artery dilation and a pulmonary artery to aortic root ratio (PAD/AOD) greater than 1 are probably indicative of PHT (Reef *et al.*, 1998; Marr, 2010) (Figure 2.4).

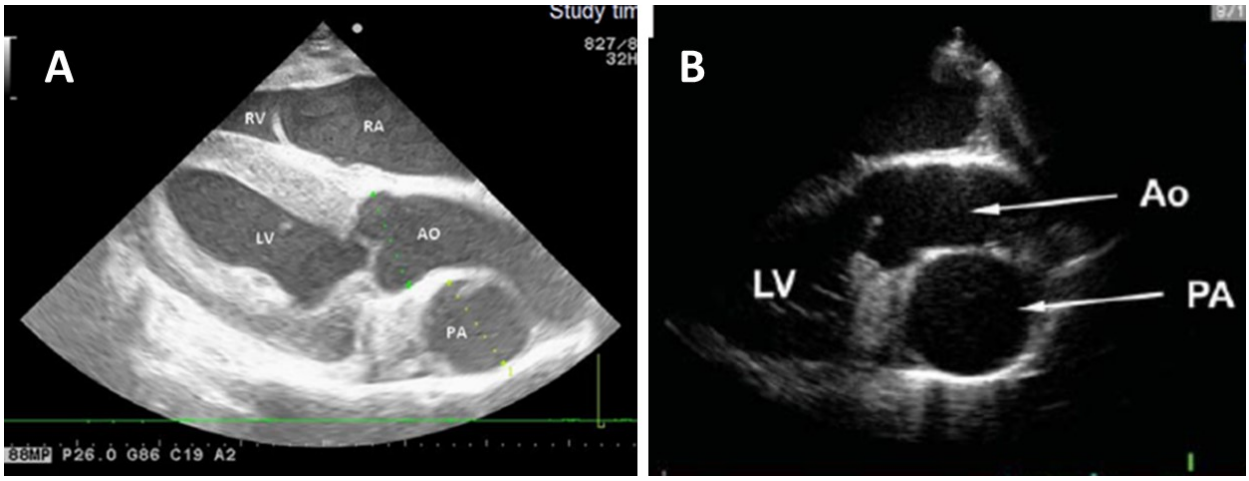

Figure 2.4 - Right parasternal long-axis view of the left ventricular outflow tract showing dilatation of the pulmonary artery in two horses.
A) Moderate dilation of pulmonary artery which diameter is equivalent to the diameter of the aorta (6 cm) (Le Corre *et al.*, 2019). *B) Severe dilation of pulmonary artery which diameter is larger than aortic diameter* (Schwarzwald *et al.*, 2006).
RV: right ventricle; RA: right atrium; LV: left ventricle; AO: aorta; PA: pulmonary artery.

Other echocardiographic signs suggestive of PHT are morphological or functional modifications of different structures of the right heart; these alterations may be transient and reversible during acute exacerbation of some diseases (Johansson *et al.*, 2007; Decloedt *et al.*, 2017a) or may be permanent as a consequence of serious chronic pathologies (Schwarzwald *et al.*, 2006; Le Corre *et al.*, 2019).

During exacerbation of severe equine asthma, it is possible to observe mild hypertrophy of RV, flattening and abnormal motion of the interventricular septum, increased PAD, increased PAD/AOD, increased ratio of RV area to LV area, prolonged pre-ejection period and decreased RV contractile function. These modifications are reversible and the echocardiographic parameters return normal when the offending allergens are removed from the horses' environment. This demonstrates that PHT is transient in these patients and that it is important to avoid or minimize the exposure to the offending allergens to limit both the inflammatory process and the hypoxic vasoconstriction, which would lead to a vascular remodeling causing permanent PHT (Johansson *et al.*, 2007; Decloedt *et al.*, 2017a).

Cases of permanent PHT are described in horses affected by different severe chronic pulmonary diseases. In these patients, the morphological and functional modifications of the right heart, detected by echocardiography, result to be permanent. In addition to the aforementioned alterations, these horses present right ventricular dilation and, in severe cases, also enlargement of right atrium (Figure 2.5). The irreversibility of the phenomenon in these subjects is due to the establishment of a real modification of the cardiac structures defined *cor pulmonale* (Schwarzwald *et al.*, 2006; Le Corre *et al.*, 2019).

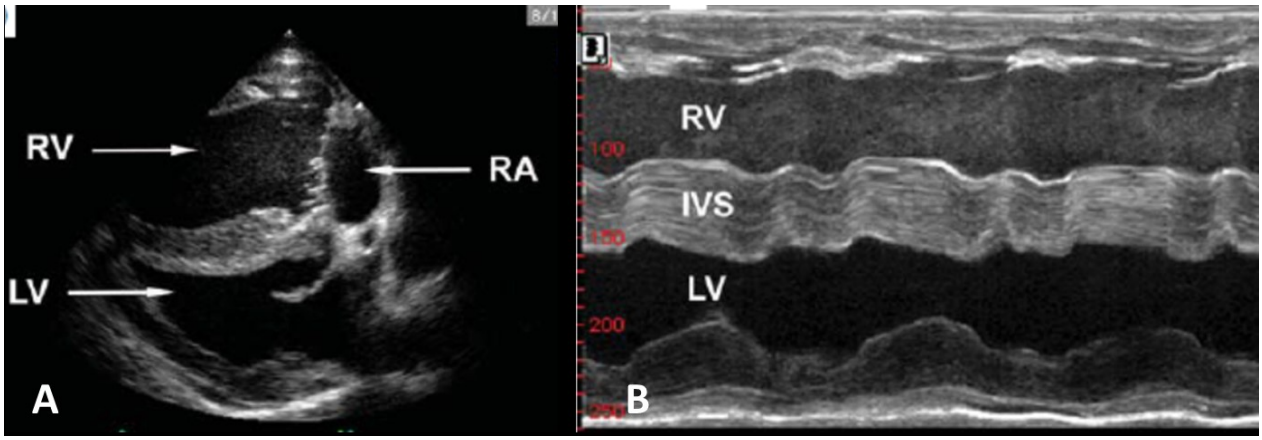

Figure 2.5 – Echocardiographic images of a horse with cor pulmonale.
A) Right parasternal 4-chamber view that shows right ventricular enlargement with inversion of the septum into the left ventricle.
B) M-mode of the right parasternal short-axis view at the level of chorda tendinea that shows enlargement of the right ventricle, small left ventricle, and flattened ventricular septal motion.
RV: right ventricle; LV: left ventricle; RA: right atrium; IVS: interventricular septum (Schwarzwald *et al.*, 2006)

Recently, a study assessed the feasibility of measuring right pulmonary artery fractional dimensional change (ΔRPA) in healthy horses. This parameter corresponds to the fractional dimensional change of the right pulmonary artery (RPA) throughout a single cardiac cycle and it is calculated using the following formula (Caivano *et al.*, 2019):

$$\Delta RPA = \frac{RPAmax - RPAmin}{RPAmax}\ x\ 100$$

The maximal (RPAmax) and the minimal (RPAmin) diameters of the right pulmonary artery were measured from the M-mode echocardiographic image of the third pulmonary vein ostium and right pulmonary artery ostium obtained from a modified right parasternal long axis view. This study reported a median ΔRPA value of 22% and a reference interval of 11-37%. Moreover, the authors did not find any relationship between this index and heart rate or bodyweight (Caivano *et al.,* 2019).

It is demonstrated that ΔRPA represents an index of the right pulmonary artery distensibility and that it is inversely related to pulmonary arterial pressure in humans (Pasierski *et al.*, 1993; Ertan *et al.*, 2013) and dogs (Venco *et al.*, 2014; Visser *et al.*, 2016).

Chapter 3

ECHOCARDIOGRAPHIC PARAMETERS

The pulmonary outflow can be studied by pulsed-wave (PW) Doppler echocardiography. Pulsed-wave Doppler trace across the pulmonary valve can be obtained from the left parasternal short-axis view in humans (Kitabatake *et al.*, 1983) and from the right parasternal short-axis view at the level of pulmonary artery in horses (Blissitt and Bonagura, 1995).

The normal profile of the PW Doppler waveform of the pulmonary flow is symmetrical in shape because, in normal conditions, lung has a low vascular resistance and so, peak velocity occurs in mid-systole; this pattern is defined "dome-like". The increased pulmonary vascular resistance and pulmonary pressure cause a change in the blood flow velocity, which is reflected in a modification of the profile of the PW Doppler waveform. Due to the decreased compliance of pulmonary vascular bed, the peak velocity occurs early in the ejection phase, resulting in a short pulmonary acceleration time. This could be associated with a later and slower acceleration of blood into the pulmonary artery, resulting in mid-systolic notching of the Doppler trace. In summary, it is known that, in humans (Celermajer and Playford, 2017; De-Paula *et al.*, 2018) and dogs (Uehara, 1993; Schober and Baade, 2006) with elevated pulmonary vascular resistance and pulmonary pressure, a sharp peak and a mid-systolic notch could be noted on the PW Doppler trace of the pulmonary flow (Figure 3.1). A profile of pulmonary flow waveform with a steep ascending phase and prolonged notched descending phase is reported also in a pony with severe equine asthma and PHT (Lightowler *et al.*, 2009).

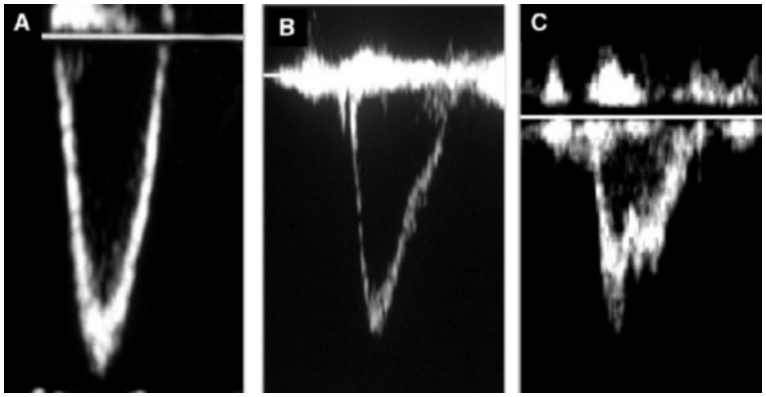

Figure 3.1 - Pulsed-wave Doppler waveform of the pulmonary flow in dogs (Schober and Baade, 2006).
A) Normal pulmonary pressure. Notice the symmetrical shape of the waveform.
B) Increased pulmonary pressure. Notice the sharp peak.
C) Severe pulmonary hypertension. Notice the sharp peak and the mid-systolic notch.

On the pulmonary PW Doppler trace, it is possible to calculate different parameters, such as the right ventricular systolic time intervals (RVSTIs) and the pulmonary artery stiffness (PAS), which provide information regarding the pulmonary vascular bed (Kitabatake *et al.*, 1983; Görgulü *et al.*, 2003).

3.1 Right ventricular systolic time intervals

The right ventricular systolic time intervals are pulsed-wave Doppler echocardiographic parameters that include acceleration time, right ventricular ejection time and acceleration time index (Schober and Baade, 2006).

In human medicine, RVSTIs provide useful indications regarding pulmonary pressure and their reliability in the diagnosis of PHT appears to be high (Chan *et al.*, 1987; Yared *et al.*, 2011; Habash *et al.*, 2019). In fact, it is demonstrated that RVSTIs strongly correlate with TRV (Yared *et al.*, 2011) and invasively measured pulmonary pressure (Kitabatake *et al.*, 1983). These parameters represent a particularly attractive alternative to the TRV method, because they do not rely on the presence of valvular regurgitation and, therefore, they are measurable in the vast majority of individuals (Yared *et al.*, 2011). Consequently, they were studied as useful parameters for the screening of PHT in adult patients affected from different diseases, such as mitral and aortic valve diseases, atrial septal defect, constrictive pericarditis, dilated cardiomyopathy, ischemic heart diseases and primary pulmonary hypertension (Kitabatake *et al.*, 1983; Yared *et al.*, 2011). Moreover, RVSTIs have been studied in children and adolescents and their reliability has been demonstrated in predicting the PHT even in paediatric subjects (Habash *et al.*, 2019).

In veterinary medicine, RVSTIs are well studied in dogs with cardiac and pulmonary diseases that lead to pulmonary hypertension and their reliability in predicting PHT has been demonstrated (Schober and Baade, 2006; Akabane *et al.*, 2019).

In 1995, Blissitt and Bonagura evaluated a series of PW Doppler echocardiographic parameters, including RVSTIs, in 40 healthy Thoroughbreds and reported their normal ranges (Blissitt and Bonagura, 1995). In literature, two studies reported RVSTIs in horses with elevated pulmonary pressure. The first is a case report that investigated RVSTIs in a pony with severe equine asthma and pulmonary hypertension; in this case, PHT was diagnosed based on echocardiographic features of *cor pulmonale* and an estimation of pulmonary pressure by TRV method (Lightowler *et al.*, 2009). The second study, evaluating the right ventricular structure and function during acute exacerbation of severe equine asthma, reported also AT and ET values; in this case the

transient increase in pulmonary pressure during the clinical episode was confirmed by cardiac catheterization (Decloedt *et al.*, 2017a)

3.1.1 Acceleration time

The time-to-peak pulmonary artery flow velocity, called briefly as acceleration time (AT), is defined as the interval between the beginning of the ejection of pulmonary flow and the peak flow velocity. On the PW Doppler trace (Figure 3.2), it is measured from the onset of the Doppler waveform to peak flow velocity (Kitabatake *et al.*, 1983).

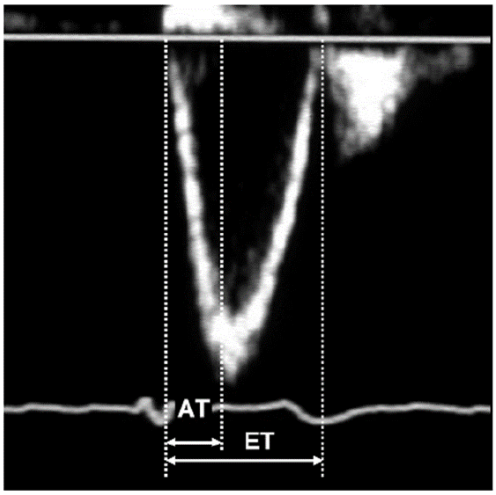

Figure 3.2 – Measurement of acceleration tine (AT) and ejection time (ET) from a normal Doppler flow pattern of pulmonary artery flow in a healthy dog (Schober and Baade, 2006).

In human medicine, it is well known that AT is inversely correlated to pulmonary pressure measured invasively by right cardiac catheterization. Therefore, a decreased AT is indicative of high vascular resistance and pulmonary hypertension (Kitabatake *et al.*, 1983; Görgulü *et al.*, 2003; Celermajer and Playford, 2017; Grapsa and Tzemos, 2017; De-Paula *et al.*, 2018).

The same relationship has been demonstrated also in dogs (Uehara, 1993; Schober and Baade, 2006; Visser *et al.*, 2016; Serres *et al.*, 2017; Reinero *et al.*, 2020).

Under normal conditions, the pulmonary vascular bed has a high compliance and thus the peak flow velocity occurs in mid-systole. Conversely, in the presence of PHT, there is high impedance to flow due to a decreased distensibility of the pulmonary vascular bed; this high impedance is more manifested during the earliest phase of the ejection and, therefore, the acceleration time decreases (Schober and Baade, 2006).

In humans, the AT can be used to calculated the mean pulmonary artery pressure using the "Mahan formula"(Ozkececi *et al.*, 2016):

$$mPAP = 90 - (0.62\ x\ AT)$$

However, despite a good correlation between mPAP and AT, significant errors in non-invasive estimation of mPAP using this formula are still recognised, especially in mild PHT (Görgulü *et al.*, 2003).

In humans, the normal value of AT is 137 ± 24 msec and it decreases significantly with increasing pulmonary pressure, resulting in a mean value of 65 ± 14 msec in subjects with a pulmonary pressure greater than 40 mmHg (Kitabatake *et al.*, 1983). An acceleration time less than 90 msec is considered indicative of PHT in humans (Celermajer and Playford, 2017).

The mean AT in healthy dogs is 79 ± 17 msec (Kirberger *et al.*, 1992); instead an acceleration time lower than 52-58 msec is indicative of PHT (Reinero *et al.*, 2020).

In healthy horses, the mean value of AT is 208 ± 27 msec, with minimum and maximum values of 160 msec and 270 msec, respectively (Blissitt and Bonagura, 1995). During an acute exacerbation of severe equine asthma in 6 horses was reported a mean AT (right parasternal measurement) of 139 ± 29 msec (Decloedt *et al.*, 2017a). Moreover, in a pony with severe equine asthma and pulmonary hypertension was reported a AT of 60 msec (Lightowler *et al.*, 2009).

In humans, AT could be influenced by heart rate. A study in which patients' heart rate (HR) ranged widely (38-180 bpm) showed that the correlation between AT and mPAP improved when patients with extreme values of HR were excluded or when AT was corrected for HR (Chan *et al.*, 1987). However, other studies found that this correction was not necessary (Dabestani *et al.*, 1987; Yared *et al.*, 2011). Moreover, it is known that AT in children correlates positively to age and body surface area and negatively to HR; however, these correlations become weaker with the increasing age. Conversely, AT is not influenced by gender (Habash *et al.*, 2019). In dogs, it is demonstrated that AT is not influenced by breed, age, bodyweight, heart rate and right ventricular segmental shortening fraction (Kirberger *et al*., 1992; Schober and Baade, 2006). Similarly, no correlation between AT and age, sex or bodyweight were found in horses (Blissitt and Bonagura, 1995).

3.1.2 Right ventricular ejection time

The right ventricular ejection time (ET) was defined as the interval between the onset of right ventricle ejection and the point of systolic pulmonary arterial flow cessation (Yared *et al.*, 2011). It is measured from the onset to the end of the Doppler waveform of the pulmonary flow (Figure 3.2) (Schober and Baade, 2006).

Like the acceleration time, ET is inversely correlated to pulmonary pressure. In humans, the normal value of ET is 304 ± 38 msec and it decreases significantly with increasing of the pulmonary pressure, with a mean value of 256 ± 53 msec in subjects with a pulmonary pressure greater than 40 mmHg (Kitabatake *et al.*, 1983).

The mean ET in healthy dogs is 184 ± 28 msec (Kirberger *et al*., 1992); instead a study that evaluates RVSTIs in dogs with PHT reported a median ET of 170 msec (Schober and Baade, 2006). In healthy horses, the mean value of ET is 501 ± 30 msec, with minimum and maximum values of 450 msec and 580 msec, respectively (Blissitt and Bonagura, 1995). During an acute exacerbation of severe equine asthma in 6 horses was reported a mean ET (right parasternal measurement) of 437 ± 53 msec (Decloedt *et al.*, 2017a). Moreover, in a pony with severe equine asthma and pulmonary hypertension was reported a ET of 240 msec (Lightowler *et al.*, 2009).

Despite the correlation between ET and pulmonary pressure, this parameter is considered a poor predictor of PHT in dogs, because influenced by different factors (Schober and Baade, 2006). Moreover, in humans its measurement may be inaccurate because the end of the pulmonary flow can be difficult to determine, compared with the onset and the peak flow (Chan *et al.*, 1987). In children, it is demonstrated that ET is positively correlated to age and body surface area while it is negatively correlated to HR; however, these correlations become weaker with increasing age. Conversely, ET is not influenced by gender (Habash *et al.*, 2019). Moreover, it is known that, in dogs, ET is influenced by age, HR and right ventricular segmental shortening fraction; while it is not correlated to bodyweight (Schober and Baade, 2006). Finally, no correlation between ET and age, sex or bodyweight were found in horses (Blissitt and Bonagura, 1995).

3.1.3 Acceleration time index

The acceleration time index is the ratio of acceleration time to right ventricle ejection time (AT/ET) (Schober and Baade, 2006). As acceleration time, this parameters is inversely correlated to pulmonary pressure; in fact, a reduced AT/ET is reported in humans (Kitabatake *et al.*, 1983; Celermajer and Playford, 2017) and dogs (Schober and Baade, 2006; Serres *et al.*, 2017) with PHT. In healthy people, AT/ET is 0.45 ± 0.05 while in subjects with a pulmonary pressure greater than 40 mmHg, it is reported an AT/ET of 0.26 ± 0.02 (Kitabatake *et al.*, 1983). Moreover, an acceleration time index less than 0.35 is considered indicative of PHT in adult people (Wong and Otto, 2002). In children, AT/ET less than 0.29 had a 100% sensitivity and a 95.8% specificity to predict PHT (Habash *et al.*, 2019).

Healthy dogs showed a AT/ET of 0.43 ± 0.7 (Kirberger, Bland-vanden Berg and Grimbeek, 1992); while an AT/ET lower than 0.30 is indicative of PHT (Reinero *et al.*, 2020).

In healthy horses, AT/ET calculated using the mean values of AT and ET reported in the literature (Blissitt and Bonagura, 1995), turns out to be of 0.41. In a pony with severe equine asthma and pulmonary hypertension AT/ET was 0.25 (Lightowler *et al.*, 2009).

It is know that, in children, AT/ET is weakly correlated to age, body surface area and HR but it is not gender dependent (Habash *et al.*, 2019). It is demonstrated that AT/ET in dogs is influenced by age but not by HR, bodyweight and right ventricular segmental shortening fraction; however, authors considered this influence negligible (Schober and Baade, 2006).

3.2 Pulmonary artery stiffness

The pulmonary artery stiffness (PAS) is an index of pulmonary artery elasticity that permits to evaluate the structural features and function of the pulmonary vascular bed in humans (Baysal and Has, 2019). This parameter could be evaluated non-invasively by CT or MRI; however, these methods have some disadvantages, such as radiation exposure, potential complication of contrast agents and high costs (Altiparmak *et al.*, 2016; Baysal and Has, 2019). Moreover, PAS can be calculated non-invasively using echocardiographic parameters. By means of this method, PAS can be assessed from the PW Doppler waveform of the pulmonary artery flow (Figure 3.3) using the following formula (Görgulü *et al.*, 2003):

$$PAS = \frac{MFS}{AT}$$

where MFS is the maximal frequency shift and AT is the acceleration time. The pulsed-wave Doppler trace of the pulmonary artery flow is obtained from the parasternal short-axis view with the sample volume placed in the pulmonary artery just 1cm distal to the pulmonary valve annulus. The maximal frequency shift (MFS) is measured as the peak systolic frequency shift and it is expressed in kilohertz. The acceleration time (AT) is expressed in seconds and therefore, PAS is expressed as kHz/sec (Görgulü *et al.*, 2003).

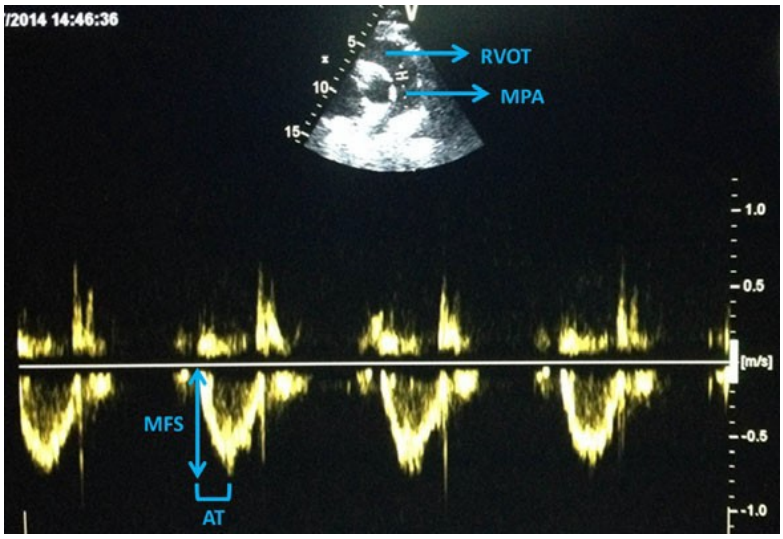

Figure 3.3 - Echocardiographic calculation of PAS in humans from the pulsed-wave Doppler trace of pulmonary flow. RVOT: right ventricle outflow tract; MPA: mean pulmonary artery; MFS: maximal frequency shift; AT: acceleration time (Görgulü *et al.*, 2006)

In human medicine, PAS is mainly used to early detect an increase in pulmonary artery stiffness, as a consequence of a remodelling of the pulmonary vessel wall (Altiparmak *et al.*, 2016; Baysal and Has, 2019). During different chronic diseases, the pulmonary vascular bed can undergo structural modifications caused by recurrent episodes of hypoxemia and hypercapnia, associated with inflammatory mediators and cytokines released due to chronic inflammation (Görgulü *et al.*, 2003; Altiparmak *et al.*, 2016). These changes consist in an increase in smooth muscle mass of the pulmonary arterioles and excessive deposition of matrix proteins in the wall of large pulmonary arteries leading to a thickening of the pulmonary artery wall. These structural changes compromise the elastic properties of pulmonary arteries leading to a loss of compliance. These changes in the elastic properties of pulmonary valve can be recognised and measured by PAS. Moreover, the loss of compliance leads to an increase in pulmonary vascular resistance and contributes to the elevated oscillatory load that increases right ventricular systolic pressure. This stiffness itself causes further damages to the pulmonary vascular bed over time, ultimately leading to PHT (Görgulü *et al.*, 2003; Altiparmak *et al.*, 2016; Baysal and Has, 2019).

It is known that the reduction of pulmonary artery elasticity shortens the duration of right ventricular systolic ejection time primarily due to a decrease in the acceleration time of the pulmonary flow trace. To maintain a constant flow within a stiff pulmonary artery, AT decreases while the velocity and MFS increase. For this reason, PAS has been developed as an echocardiographic parameter calculated with a formula associated with AT (Görgulü *et al.*, 2003; Altiparmak *et al.*, 2016).

In human medicine, different studies showed an increase of PAS value in patients with congenital heart diseases (Görgulü *et al.*, 2003; Görgulü *et al.*, 2006), in asthmatic patients (Baysal and Has, 2019), in people with obstructive sleep apnoea syndrome (OSAS) (Altiparmak *et al.*, 2016; Ozkececi *et al.*, 2016) and in patients affected by chronic obstructive pulmonary disease (Weir-Mccall *et al.*, 2015). In patients with OSAS and chronic obstructive pulmonary diseases, the PAS values also seem to increase with the severity of the disease (Weir-Mccall *et al.*, 2015; Altiparmak *et al.*, 2016).

It is also demonstrated a correlation between PAS and indices of right ventricular dysfunction, suggesting that PAS can be an index of subclinical right ventricular dysfunction in patients with chronic pulmonary diseases (Baysal and Has, 2019). Moreover, a study that evaluated PAS in people with heart failure (HF) and reduced ejection fraction without echocardiographic evidence of pulmonary hypertension or right heart failure, reported that a high PAS value was constantly observed in those patients. The same study demonstrated an association between PAS and NYHA (New York Heart Association) functional class. NYHA classify the HF based on the activity that the patient can perform without the manifestation of symptoms such as dyspnoea (Yildirim *et al.*, 2017). Furthermore, PAS seems to be a useful marker of early subclinical myocardial dysfunction in children at risk for obesity; in fact, in these patients, atherosclerosis and the consequent modifications in artery elasticity, begin early in childhood (Mahfouz *et al.*, 2012).

Despite the use of PAS as an early index of PHT in humans, and the numerous reports about its value in different pathologic conditions in the literature, the reference range of PAS measured in healthy people by echocardiography are not uniquely established (Yildirim *et al*., 2017). In fact, the value of PAS reported for healthy controls varies widely between different studies. A study that evaluate differences between recently diagnosed asthmatic patients and healthy people, reported a PAS of 22.4 ± 4.1 kHz/sec in the latter (Baysal and Has, 2019). Another study investigating PAS in patients with heart failure with reduced ejection fraction, reported a PAS of 7.41 ± 1.32 kHz/sec in healthy people used as controls (Yildirim *et al.*, 2017). Furthermore, other two studies assessed PAS in patients with OSAS, indicating for the control group a PAS value of 18.0 ± 3.5 kHz/sec (Altiparmak *et al.*, 2016) and of 18.6 ± 6.3 kHz/sec (Ozkececi *et al.*, 2016).

Eventually, a study reported that PAS is affected by BMI (body mass index) and it is not influenced by age and sex in human patients (Ozkececi *et al.*, 2016).

In veterinary medicine, there are no studies regarding echocardiographic evaluation of PAS, both in horses and small animals.

Chapter 4

EQUINE ASTHMA

Equine asthma is a chronic inflammatory syndrome of the lower airways of adult horses. The term “equine asthma” has been introduced only in the last years (Bullone and Lavoie, 2015). Although, the presence of a chronic respiratory inflammation in stabled horses has been recognised for centuries (Markham, 1656), this condition has been defined with a large variety of names and its classification has been reviewed several times (Bullone and Lavoie, 2015).

Historically, the equine respiratory syndrome characterized by reversible bronchospasm was named “heaves” or “broken-wind”. In the half of XX century, Obel and Schmiterlow introduced the term “equine emphysema” based on the macroscopic appearance of the lung at necropsy: the lung of affected horses failed to collapse when the chest was opened (Obel and Schmiterlow, 1948). However, this term was abandoned because the hyperinflation and the failure to collapse are due to gas trapping behind obstructed airways rather than structural damage to the alveolar septa like in true emphysema (Thurlbeck and Lowell, 1964).

With the introduction of bronchoalveolar lavage (BAL) cytology, it became possible to identify the presence of a large number of neutrophils in the airways of affected horses. Therefore, the disease was named “chronic obstructive pulmonary disease” (COPD) due to the similarities with human COPD. However, a characteristic of human COPD is the presence of pulmonary emphysema that is not present in affected horses (Robinson *et al.*, 1996). Meanwhile, cytology of the BAL revealed also the presence of a mixed cell population (neutrophils, mast-cells and eosinophils) in horses with milder inflammatory condition, not characterized by bronchospasm; this condition was defined as “chronic bronchiolitis” (Nyman *et al.*, 1991).

In the last decades, the terms “recurrent airway obstruction” (RAO) and “inflammatory airway disease” (IAD) were introduced. RAO identified horses affected by reversible bronchospasm due to the inhalation of environmental antigens while IAD defined subjects with milder inflammation of the lower airways in absence of dyspnoea (Couëtil *et al.*, 2007).

However, more recent studies have demonstrated that RAO and IAD have several similarities with different phenotypes of human asthma (Leclere *et al*., 2011; Bullone and Lavoie, 2015; Lange-Consiglio *et al.*, 2019). For this reason, the term “equine asthma” has been introduced. Nowadays, IAD is defined as “mild/moderate equine asthma” and RAO is defined as “severe equine asthma” (Couëtil *et al.*, 2016).

4.1 Mild equine asthma vs severe equine asthma

Equine asthma can present different clinical features depending upon the severity of the disease and it is classified in mild/moderate equine asthma (MEA) and severe equine asthma (SEA) (Couëtil *et al.*, 2016).

4.1.1 Aetiopathogenesis

It is largely known that the inhalation of antigens, present in stable and hay, has an important role in SEA. These antigens are pro-inflammatory agents such as bacterial endotoxins, moulds, peptidoglycan, proteases, microbial toxins, forage mites, plant debris and inorganic dusts. Moreover, in poorly ventilated stable, also high levels of potentially toxic gases such as ammonia may be present (Pirie, 2014). It is also reported a different phenotype of SEA, known as summer pasture-associated severe equine asthma that presents the same clinical and pathological features of SEA, but symptoms are triggered by exposing subjects to pasture. The aetiological agents of this phenotype are unknown; however the response to stable housing suggests a role of inhaled seasonal pasture-associated pollen (Couëtil *et al.*, 2020).

It is known that hypersensitivity response plays a central role in the development of airways inflammation and bronchoconstriction in SEA; however, the complex immunological mechanisms are not still fully clarified (Robinson *et al.*, 1996).

It has been reported that IgE-mediated immediate-type allergic reactions may be associated with SEA (McPherson *et al.*, 1979; McGorum *et al.*, 1993). In fact, the increase in IgEs identified in the BAL fluid of the SEA affected horses are characteristic of a type I hypersensitivity response (Marti *et al.*, 2003). Furthermore, in vitro studies, demonstrated that histamine released by pulmonary mast-cells in response to allergens exposure is significantly greater in asthmatic horses compared to the healthy ones; this finding suggests the involvement of IgE-mediated reactions leading to increased mast-cell degranulation (Hare *et al.*, 1999). Mast-cell mediators, such as histamine and leukotrienes, can induce smooth muscle contraction and therefore, may contribute to bronchospasm in SEA affected horses (Olszewski *et al.*, 1999; Marti *et al.*, 2003).

Moreover, SEA is characterized by a delayed-onset type IV hypersensitivity, mediated by an increase in CD4+ T cells and the recruitment of neutrophils into the airways about 5-6 hours after allergens exposure (McGorum *et al.*, 1993). However, there is no agreement among the Th-type involved. Some studies demonstrated an increase in IL-4 and IL-5 level, and a decrease in IFN-γ, suggesting the activation of Th-2 mechanism. Conversely, other studies identified an increase in

IFN-γ, supporting a Th-1 or a mixed Th1/Th2 answer (Lavoie *et al.*, 2001; Giguère *et al.*, 2002; Cordeau *et al.*, 2004). This disagreement may reflects the complexity of the immune response in SEA affected horses and suggests that different T-cells subgroups can be involved in different phases of the disease (Pirie, 2014)

The most important features of SEA are airway inflammation, airway obstruction, mucus accumulation and tissue remodeling. Airway inflammation is characterized by intense neutrophilic chemotaxis, increased proteolytic activity and raised oxidative stress (Art *et al.*, 2006). Bronchospasm in SEA affected horses is related to dysfunction in the inhibitory non-adrenergic non-cholinergic systems, decreased response of smooth muscle to cholinergic activation, enhancement of the production of epithelial-derived relaxing factor and reduction of the inhibitory function of prostanoids (Yu *et al.*, 1994). In SEA affected horses, mucus hypersecretion is correlated to altered viscoelasticity and reduced mucociliar clearance (Lugo *et al.*, 2006). Finally, the peripheral airways wall of SEA affected horses undergo to an increase in thickness of the bronchial epithelium, lamina propria and smooth muscle; this contributes to airway obstruction (Leclere *et al.*, 2011; Bullone *et al.*, 2017).

Environmental antigens seem to play a central role also in the development of MEA (Holcombe *et al.*, 2001; Millerick-May *et al.*, 2011). However, the aetiology of MEA is multifactorial. It has been reported that also the intense training can trigger the onset of MEA in young racehorses. In fact, exercise-related stress, associated with cold air and the dust of the track, could be challenging for the immunity of the respiratory system. Furthermore, in case of exercise-induced pulmonary haemorrhage, the presence of blood in the airways can lead to inflammatory reaction of the lung and acts as pabulum for bacterial colonization (McKane and Slocombe, 2010). Some infectious agents also seem to play a role in the aetiology of MEA. It has been reported that the degree of pulmonary inflammation is directly related to the number of colony-forming unit (CFU) of *Streptococcus spp.* detected in the tracheal wash (TW). However, bacteria could derive from secondary colonization and are not a primary aetiological factor (Chapman *et al.*, 2000). Finally, the role of a variety of viral agents (equine influenza virus, equine herpesviruses type 1,2,4,5 and equine rhinitis viruses) has been investigated. However, even if some viruses seem to play a role in triggering or exacerbating MEA, their pathogenic role is still debated because they are ubiquitous both in healthy and in clinically affected horses (Couëtil *et al.*, 2020).

4.1.2 Epidemiology

Regarding MEA, several studies reported different prevalence depending upon the studied population. It is described that MEA affects 70-80% of racehorses (Nolen-walston *et al.*, 2013; Couëtil *et al.*, 2020). On the other hand, the prevalence in pleasure horses is around 17% if the diagnose is based on a combination of endoscopic findings and TW cytology; instead this prevalence rise to 70% if it is considered only the amount of neutrophils in the TW (Robinson *et al.*, 2006).

Regarding SEA, it has been reported a prevalence of 14-17% in the northern hemispheres where there is a cool climate and horses are stabled indoors during most of the year (Hotchkiss *et al*., 2007; Wasko *et al.*, 2011).

MEA can affect horses of any breed/discipline and any age; however, it is more commonly reported in young horses (<4 years old) and its incidence decreases with increasing in age. Conversely, SEA is usually documented in horses older than 7 years old (Couëtil *et al.*, 2016), of any breeds and sex (Hotchkiss *et al*.,2007). Moreover, stable in an urbanized environment, respiratory infections and exposure to dust/antigens early in life seem to be significant risk factors for developing SEA (Hotchkiss *et al.*, 2007). Finally, a genetic predisposition has been documented in German warmblood and Lipizzaner horses (Lavoie, 2007).

4.1.3 History and clinical signs

Horses with MEA can have a history of chronic (at least 4 weeks) occasional cough, nasal discharge and poor performance but sometimes the only sign could be the impaired athletic activity. At physical examination, these horses are mainly asymptomatic. The respiratory pattern is normal and the auscultation of the thorax usually does not reveal any abnormalities; however, increased breath sounds or subtle wheezes can be heard (Couëtil *et al.*, 2016).

SEA affected horses have commonly a history of regular to frequent coughing and nasal discharge; however, these are not specific signs of the disease (Dixon et al., 1995). The characteristic symptoms of SEA are exercise intolerance and increased respiratory efforts at rest but, depending upon the severity of the disease, patients can show only mild respiratory signs. Moreover, it is important to consider the season in which the horse shows symptoms. In fact, horses with SEA exhibit symptoms mainly in autumn-winter, when they are stabled for longer periods. Instead, if horses present symptoms during spring-summer, when they are kept in paddock, they are probably affected with summer pasture-associated severe equine asthma

(Seahorn *et al.*, 1996). In chronic patients, generalized cachexia may occur, due to significant mismatch between peripheral tissue oxygenation and breathing work energy consumption (Mazan *et al.*, 2004).

The physical examination of SEA affected horses reveals an abnormal breathing pattern at rest, characterized by increased respiratory effort with nasal flaring and recruitment of accessory abdominal muscles during expiration. The recruitment of these muscles, in chronic patients, leads to hypertrophy of the external abdominal oblique muscles resulting in the characteristic 'heave line'. End-expiratory wheezes and early inspiratory crackles can be detected at thorax auscultation; moreover, the auscultation area can be enlarged due to lung hyperinflation (Pirie, 2014).

Summarizing, the main difference between MEA and SEA is the absence (MEA) or presence (SEA) of laboured breathing at rest and exercise intolerance. Finally, it has been reported that risk-screening questionnaires, containing questions regarding historical information and clinical signs, to owners are effective in identifying cases of SEA by distinguishing them from MEA cases (Hotchkiss *et al.*, 2007; Wasko *et al.*, 2011).

4.1.4 Diagnosis

The diagnosis of equine asthma is based on the presence of clinical signs of lower airways inflammation, its confirmation by instrumental and laboratory findings, and the exclusion of infection or other respiratory diseases (Couëtil *et al.*, 2016).

Blood gas analysis

A marked hypoxemia during exacerbation of SEA can be detected at arterial blood gas analysis; this finding is due to the increased dead space ventilation and related pulmonary gas exchange impairment. The increased dead space results from alveolar hyperinflation and consequent compression on pulmonary capillaries that prevents adequate perfusion. Instead, due to the increase in respiratory rate and total ventilation, SEA affected horses, usually, does not present hypercapnia (Nyman *et al.*, 1991).

Conversely, horses with MEA, usually, present a normal PaO_2 and a high pCO_2, suggesting an alteration in ventilation/perfusion ratio (Couëtil and Denicola, 1999). Moreover, it has been reported these subjects have a more pronounced hypoxaemia during exercise compared to healthy horses (Sánchez *et al.*, 2005).

Thoracic ultrasonography

At thoracic ultrasonography, SEA affected horses show more ultrasound changes, such as comet tails, compared to MEA affected subjects. However, thoracic ultrasonography shows a low specificity because it does not allow to distinguish the nature of the lesions (Siwinska *et al.*, 2019; Lo Feudo *et al.*, 2021). Moreover, in SEA affected horses the surface of the visceral pleura may have irregular echogenicity (Couëtil, 2014).

Transendoscopic endobronchial ultrasonography has been validated in horses and seems to be promising for the estimation of airway smooth muscle remodeling in SEA affected horses. However, this technique is still limited to research purposes (Bullone *et al.*, 2015).

Airways endoscopy

Airways endoscopy is an important diagnostic test for the diagnosis of MEA and SEA that permits to evaluated different features. First of all, at pharyngeal level, it is possible to assess the presence of pharyngeal lymphoid hyperplasia (PLH) and classify its severity using a score from 0 to 4 (Baker, 1987). PLH is often correlated with cough and tracheal mucus in young horses affected by MEA (Christley *et al.*, 2001). Moreover, it is known that PLH is associated with stabling and exposure to antigens considered as risk factors for equine asthma. However, any direct relationship between equine asthma and PLH has not yet been demonstrated (Holcombe *et al.*, 2000).

Then, during airways endoscopy, mucus accumulation can be detected at tracheal level; this finding can be classify from 0 to 5 based on its quantity and it is frequently observed both in SEA and MEA affected horses (Gerber *et al.*, 2004). The detection of excess mucus in the tracheobronchial tree (grade ≥ 2/5 in racehorses or ≥ 3/5 in sport and pleasure horses) is indicative of equine asthma (Couëtil *et al.*, 2016). Furthermore, at endoscopic examination, the tracheal bifurcation can be evaluate and its oedema can be classify between 0 and 4 (Koch *et al.*, 2007). It is demonstrated that the tracheal mucus grade and tracheal bifurcation score are higher in SEA than in MEA affected horses and that they are positively correlated with neutrophils count in BAL cytology (Lo Feudo *et al.*, 2021).

Finally, during airways endoscopy, endobronchial biopsies of the large airways can be collected using transendoscopic forceps. In the last years, it was developed a semiquantitative histological scoring system that take into account the presence and severity of remodelling and inflammation at the level of bronchial epithelium, lamina propria, and smooth muscle layer. This scoring system

showed a good correlation with the degree of airway obstruction in SEA affected horses (Bullone *et al.*, 2016).

Bronchoalveolar lavage cytology

In the bronchoalveolar lavage cytology of healthy horses the most represented cell population are macrophages that constitute the 60-70% of inflammatory cells, followed by 30% of lymphocytes, ≤ 5% of neutrophils, ≤ 2% of mast-cells and ≤ 1% of eosinophils (Hare and Viel, 1998). The BAL cytology of SEA affected horses reveals moderate to severe neutrophilia (neutrophils > 25%) and decreased lymphocyte and alveolar macrophage counts (Couëtil *et al.*, 2016). However, BAL cytology and, in particular, neutrophils percentage do not correlate with the severity of clinical airway obstruction and lung dysfunction (Jean *et al.*, 2011).

Conversely, BAL cytology of MEA affected horses shows mild to moderate neutrophilia associated with increase in eosinophil and/or mast-cell percentages. In particular, values of > 10% neutrophils, > 5% mast-cells and > 5% of eosinophils are indicative of MEA (Couëtil *et al.*, 2016).

Moreover, microbiological examination can be performed on BAL sample, even if the best sample is tracheal wash. Bacterial isolation can be considered significant when CFU count is more the 10^3. The bacterial species that are more likely involved in the aetiology of MEA are *Streptococcus zooepidemicus*, *Streptococcus pneumoniae* and *Pasteurella spp.* (Chapman *et al.*, 2000; Christley *et al.*, 2001).

4.1.5 Treatment and prognosis

The treatment of equine asthma is based on a combination of management modifications for the control of antigens and pharmacological therapies for the control of symptoms. The medical treatment involves the systemic (intravenous, intramuscular or oral) and topical (inhalation) administration of corticosteroids, such as dexamethasone, prednisolone or fluticasone, and bronchodilators, such as clenbuterol, salmeterol or ipratropium bromide (Couëtil *et al.*, 2016).

The most effective long-term approach is antigens avoidance. This can be achieved maintaining horses at pasture as long as possible, reducing stable dust, improving stable ventilation and using low-dust feed and bedding materials (Lavoie, 2007).

The prognosis of MEA affected horses is good. The management changes and the pharmacological treatment can lead to a complete resolution of the syndrome and permit to return to the previous level of performance (Couëtil, 2014).

Conversely, SEA is a life-long disorder. After treatment, 79% of subjects have recurrent episodes of airways obstruction. A study reported a median survival time after the first diagnosis of 8 years, with a survivor percentage of 87% and 49% after three and eight years, respectively. A survey study reported that more than half of the owners thought that the athletic activity of affected horses had been compromised; in fact, 64% of horses after the diagnosis of SEA were used as pleasure horses and 21% had to be retired (Aviza *et al.*, 2001).

4.2 Cardiovascular effects

It is well known that SEA affected horses have increased pulmonary pressure, in particular during exacerbation of the disease and that a correlation between PHT and the severity of SEA exists. This PHT is due to increased vascular resistance (Dixon, 1978) caused by structural modification of the pulmonary vascular bed (Leclere *et al.*, 2011; Ceriotti *et al.*, 2020) (See chapter 2.1.3). The PHT during exacerbation of SEA is largely reversible during remission phase (Dixon, 1978); instead the pulmonary vascular bed remodelling is only reversible after long-term antigen avoidance (12 months) (Ceriotti *et al.*, 2020).

A study investigated the cardiovascular effects of an acute pulmonary obstruction in SEA affected horses, exposing subjects in remission to dust and moulds for 7 days in order to trigger an exacerbation of the disease. All the evaluations were made before the exposure (remission phase), at 7 days of acute pulmonary obstruction and after a month of antigens avoidance. It was reported that SEA affected horses during exacerbation showed detectable alteration in right and left ventricular internal diameter associated with breathing, and flattening and paradoxical motion of the interventricular septum. Moreover, during exacerbation, these subjects had larger pulmonary arteries and smaller left ventricle than during remission. Furthermore, the decreased LV leaded to a decrease estimated stroke volume; instead, cardiac output did not change due to the increased heart rate. All these echocardiographic alterations returned within limits with avoidance of antigens, suggesting that cardiac dysfunction is largely reversible. Finally, this study did not find any detectable difference between remission and exacerbation regarding the measured jet area of tricuspid regurgitation and the number of horses with tricuspid an pulmonary regurgitations (no horses had pulmonary regurgitation at any time) (Johansson *et al.*, 2007).

More recently, another similar study was conducted; this study, in addition to 2D and M-mode echocardiography, used the tissue Doppler imaging (TDI) and the 2D speckle tracking

echocardiography (STE) for the evaluation of RV function and compared also all the findings with a control group. TDI and STE showed altered RV functional measurements, such as a prolonged pre-ejection period, a prolonged isovolumic relaxation time and a decreased global peak longitudinal strain, during the exacerbation compared to the remission phase. The comparison between SEA affected horses in remission and healthy subjects showed differences of numerous echocardiographic parameters, such as thicker RV wall, higher LV end-systolic eccentricity index at chordal level and decreased segmental longitudinal strain and strain rate in affected horses (Decloedt *et al.*, 2017a). Similarly, other studies reported an impaired RV diastolic function in SEA affected horses compared to control group. They found a reduced early diastolic filling velocity and an elevated late diastolic filling velocity in affected subjects (Stahl and Gehlen, 2010; Gehlen and Neukirch, 2014; Gehlen and Stahl, 2014); moreover, differences in strain velocity and ratio at basal and mid segment of right ventricular outflow tract between controls and affected horses were found (Stahl and Gehlen, 2010). All these findings reflect a decreased RV contractile function suggesting a subclinical permanent myocardial remodeling in affected horses due to the recurrent episodes of PHT (Decloedt *et al.*, 2017a)

Cases of *cor pulmonale* in SEA affected horses (Sage *et al.*, 2006; Hanka *et al.*, 2015) are reported only occasionally; the low incidence of this condition can be explain by the intermittent and reversible nature of SEA, that causes intermittent bouts of PHT (Johansson *et al.*, 2007; Decloedt *et al.*, 2017a).

It has been demonstrated that cardiac troponin I (cTnI) and T (cTnT) do not increase in severe asthmatic horses at rest, neither in remission or during exacerbation (Johansson *et al.*, 2007; Decloedt *et al.*, 2017a). It has been reported an elevation of cTnI and cTnT only in one horse in remission that had been exercising (trotting up and down in paddock for 2 hours) in the hours before the sampling; since this horse had mild signs of SEA also during remission, authors hypothesized that this subject may have had extreme PHT during exercise leading to excessive RV wall stress. However, as the authors themselves claimed, the effects of PHT on RV function in asthmatic horses during exercise has not yet been investigated and further studies are necessary (Decloedt *et al.*, 2017a). The finding of normal cardiac troponin levels in SEA affected horses in remission and during exacerbation seems to suggest that myocardial tissue was not damaged by acute pulmonary obstruction; however, it has been hypothesized that a mild myocardial damage may occur, not sufficient to produce detectable increases in cTnI and cTnT (Johansson *et al.*, 2007). In fact, endomyocardial biopsies of RV and RA, performed in SEA affected horses during

exacerbation and in remission, showed some alterations, such as interstitial fibrosis and neutrophilic infiltration (Figure 4.1). The hypothesis is that an acute increase in right ventricle pressure leads to muscle stretch and increased wall tension; these could convert the myocardium to a pro-inflammatory phenotype and this inflammation contributes to right ventricle remodeling (Decloedt *et al.*, 2017a).

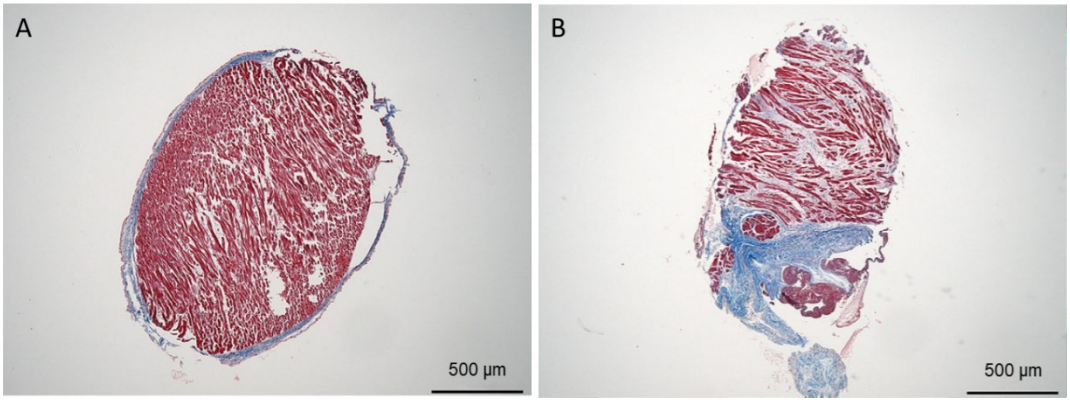

Figure 4.1 – Histological sections (Masson's trichrome staining) of equine right atrial endomyocardial biopsy, showing normal findings (A), and interstitial fibrosis (B) characterised by excess extracellular collagen (blue) between the myocardial fibres (red) (Decloedt *et al.*, 2017a).

4.3 Equine asthma and human asthma

Equine asthma and human asthma share numerous similarities regarding aetiology, clinical presentation, pathological changes and immunologic response (Leclere *et al.*, 2011; Bullone and Lavoie, 2015; Lange-Consiglio *et al.*, 2019; Bullone and Lavoie, 2020).

First of all, it must be said that human and equine lung share anatomic similarities, such as well-developed bronchial circulation, thick pleura, poorly-developed lobulation and respiratory bronchioles, presence of terminal bronchioles and bronchial artery-pulmonary shunts, and well-developed smooth muscle from the trachea to alveolar ducts (Leclere *et al.*, 2011). A summary of anatomical characteristic of human and equine lung is reported in Table 4.1.

Table 4.1 – Human and horse lung anatomy

	Human	Horse (500 Kg)
Weight	1,3 Kg (680 g right; 620 g left)	5 Kg
Number of lobes	3 at right 2 at left	3 at right 2 at left
Total lung capacity	6 L	50 L
Blood-gas barrier thickness	0.62 µm	0.6 µm
Respiratory rate	10-14 bpm	8-16 bpm
Sub-mucosal glands	Present	Present
Distribution of bronchial artery	All the lung	All the lung
Airway smooth muscle	Well-developed	Well-developed

Kg: kilograms; g: grams; µm: micrometres; bpm: breaths per minute (Lange-Consiglio *et al.*, 2019 modified)

Both human and equine asthma are naturally occurring, non-infectious, chronic syndromes of the lower airways caused by multifactorial interactions between genetic predisposition and environmental factors. They are heterogeneous diseases that can present different clinical presentations depending upon the severity of the disease, the chronicity of the condition and the pathogenetic pathway. Reversible bronchospasms, increased mucus production, airway hyperresponsiveness, and pulmonary remodeling are characteristics of both human and equine asthma (Leclere *et al.*, 2011; Bullone and Lavoie, 2015; Lange-Consiglio *et al.*, 2019). Moreover, SEA affected horses, like asthmatic people, have a history of repeated episodes of airway obstruction occurring over periods of years or sometimes even decades (Bullone and Lavoie, 2015). In fact, SEA and human asthma are life-long disorders (Lange-Consiglio *et al.*, 2019). MEA, conversely to its human counterpart, is considered a curable condition that may be transient. However, mild-moderate human asthma may be transient in some patients, especially during childhood (Bullone and Lavoie, 2020).

In SEA affected horses, as in asthmatic people, exacerbation of the disease, in genetically susceptible subjects, is triggered by exposure to antigens; therefore, an allergic response driven by a predominant Th-2 response is considered to have a pathogenic role in the development of SEA, as reported in humans. Moreover, in SEA affected horses and in people with neutrophilic asthma an increase in Th-17 expression is reported (Leclere *et al.*, 2011; Bullone and Lavoie, 2015; Lange-Consiglio *et al.*, 2019). Furthermore, SEA is characterized by pulmonary neutrophilia, and a neutrophilic inflammation is present also in human asthmatic patients, especially those with

severe and late-onset asthma. Whereas MEA is characterized by an increase in mast-cells and eosinophils; similarly, an accumulation of eosinophils in the bronchi of asthmatic people is commonly detected (Lange-Consiglio *et al.*, 2019).

In human asthma, remodeling of the airways is due to morphological alterations of all the structural cell types of the bronchi. Overall, the wall thickening is mainly due to increased deposition of submucosal extracellular matrix elements and increased airway smooth muscle. Moreover, the reduction of the airway lumen is also caused by the increased secretion of mucus from the epithelium and submucosal glands. Therefore, the airways of asthmatic subjects are characterized by subepithelial fibrosis, increased smooth muscle mass, gland enlargement, neovascularization and epithelial alterations (Bergeron *et al*., 2010). The same characteristics are described also in the airways of asthmatic horses (Bullone and Lavoie, 2020).

Furthermore, in human asthmatic patients recurrent hypoxemia and hypercapnia, associated with chronic inflammation, cause structural modification of the pulmonary vascular bed, such as increased smooth muscle and excessively deposition of matrix proteins. These modifications ultimately lead to PHT (Harkness *et al.*, 2014). Similarly, a particularly important reversible vascular remodeling is described in SEA affected horses (Decloedt *et al.*, 2017a; Ceriotti *et al.*, 2020) (See chapter 2.1.3).

Finally, equine and human asthma share similarities regarding treatment responses (Leclere *et al.*, 2011; Bullone and Lavoie, 2015; Lange-Consiglio *et al.*, 2019).

A summary of the similarities and differences between equine and human asthma is reported in Table 4.2.

Table 4.2 - Similarities and differences between equine and human asthma

		MEA	SEA	Mild human asthma	Severe human asthma
Epidemiology/aetiology	Naturally occurring disease				
	• Early in life	✓	†	✓	✓
	• Adult-onset	✓	✓	✓	✓
	Duration of disease	may be transient	all life	may be transient in childhood	all life
	Environmental component	✓	✓	✓	✓
	Genetic component		✓	✓	†
Pathophysiology	Airway hyperresponsiveness	✓	✓	✓	✓
	• Early phase		-	✓	✓
	• Late phase		✓	✓	✓
	Airway obstruction	sub-clinical	reversible (may be only partly)	reversible	partly reversible
	High temperature/ humidity induced exacerbations	?	✓	✓	✓
	Airway tissue remodelling	✓ *			
	✓ ↑ ASM mass		✓	✓	✓
	✓ ↑ ECM mass		✓	✓	✓
	✓ ↑ basal membrane thickness		†	✓	✓
	✓ ↑ mucous producing cells		✓	✓	✓
	✓ Associated bronchiectasis		†	†	†
	Remodelling sites				
	✓ Peripheral airways	?	✓	✓	✓
	✓ Central airways	?	†	✓	✓
	Hypercoagulability state	?	✓	✓	✓
Immunology	Airways				
	✓ neutrophilia	†	✓	†	†
	✓ eosinophilia	†	-	†	†
	✓ mastocytosis	†	† (rare)	†	
	Tissue inflammation	?	✓	✓	✓

Immunology	Inflammatory profile • Th2-mediated • Th1-mediated • Th17-mediated	 ✓ ✓ †	 ✓ ✓ ✓	 ✓ † ✓	 ✓ † ✓
	IgE mediated response	?	†	✓	✓
	Associated atopy	?	†	†	†
	Innate immune activation • systemic inflammation • ↑ endotoxin sensitivity	 † †	 ✓ ✓	 † ✓	 ✓ ✓
Treatments efficacy	Corticosteroids	✓	✓	✓	✓
	b2-agonists	✓	✓	✓	✓
	Anticholinergic agents	✓	✓	†	†
	Antioxidants	†	†	†	†
	Anti-leukotrienes	-	-	✓	✓
	Theophylline	✓	✓	✓	✓

✓: present; †: may be present; ?: not known; - : not present. MEA: mild/moderate equine asthma; SEA: severe equine asthma; ASM: airway smooth muscle; ECM: extracellular matrix (Bullone and Lavoie, 2015 modified using also information from Leclere et al., 2011 and Lange-Consiglio et al., 2019)

** : epithelial hyperplasia, extracellular matrix thickened, cellular infiltration in the lamina propria and fibrosis of the muscular layer* (Bessonnat *et al.*, 2021)

RESEARCH

PROJECT

Chapter 5

AIM OF THE PROJECT

Pulmonary artery stiffness (PAS) is a Doppler echocardiographic parameter useful in assessing the elasticity of the pulmonary artery and it is used as an early predictor of pulmonary hypertension in human medicine (Baysal and Has, 2019).

Other pulsed-wave Doppler parameters useful for the evaluation of pulmonary vascular bed are the right ventricular systolic time intervals (RVSTIs), such as acceleration time, right ventricular ejection time and acceleration time index (Celermajer and Playford, 2017; Grapsa and Tzemos, 2017). Moreover, the diameter of the pulmonary artery (PAD) and the ratio of PAD to ascending aorta diameter (PAD/AOD) may be indicative of pulmonary hypertension *(*Alhamad *et al*., 2011).

In human medicine, it has been reported an increase of PAS and a decrease of RVSTIs in people with chronic respiratory disease, such as asthma (Celermajer and Playford, 2017; Grapsa and Tzemos, 2017; De-Paula *et al.*, 2018; Baysal and Has, 2019).

Like human asthma, equine asthma induces thickening of the pulmonary vessel wall over time, leading to progressive increase of pulmonary vascular resistance and consequently pulmonary hypertension (Decloedt *et al.*, 2017a; Ceriotti *et al.*, 2020).

Given the aforementioned statements, the hypothesis of this project is that the evaluation of PAS and RVSTIs in asthmatic subjects could be useful also in horses. However, in literature, there are no studies regarding the evaluation of PAS in horses and only two studies reported RVSTIs in horses with increased pulmonary pressure (Lightowler *et al.*, 2009; Decloedt *et al.*, 2017a). Therefore, the aims of this project are 1) to assess the feasibility of PAS measurement in horses by the evaluation of intra-observer variability, inter-observer variability, variability of image acquisition and day-to-day variability, and 2) to evaluate possible influence of age, bodyweight, gender and heart rate on PAS and RVSTIs; 3) to investigate possible differences between healthy horses, subjects presenting mild equine asthma and those presenting severe equine asthma regarding PAS and RVSTIs. Furthermore, another purpose of this project is 4) to investigate the possible correlation between PAS values and PAD/AOD, as well as between RVSTIs and PAD/AOD. Finally, 5) specificity and sensitivity of PAS and AT measurement in the diagnosis of equine asthma were assessed.

Chapter 6

MATERIALS AND METHODS

6.1 Sample

This research was a prospective study performed on a convenience sample of client-owned horses admitted to the Equine Internal Medicine Unit of the University of Milan for diagnostic evaluation of non-infectious respiratory disease. Horses with primary cardiovascular or systemic diseases were excluded from the study. Horses were subdivided in MEA and SEA groups, according to history, clinical examination, endoscopic findings and cytology of the bronchoalveolar lavage fluid. Subjects were included in the MEA group on the bases of a history of occasional cough and poor performance, the absence of laboured breathing at rest and exercise intolerance, the presence of mucus accumulation and oedema of the tracheal bifurcation at endoscopic examination and a BAL cytology characterized by > 10% neutrophils, > 5% mast-cells and/or > 5% of eosinophils (Couëtil *et al.*, 2016). Instead, the criteria of inclusion in the SEA group were history of regular to frequent cough, exercise intolerance and increased respiratory efforts at rest, the presence of mucus accumulation and oedema of the tracheal bifurcation at endoscopic examination and a BAL cytology characterized by > 25% neutrophils (Couëtil *et al.*, 2016).

Moreover, healthy horses were selected according to the absence of cardiac and respiratory signs at history, clinical examination, echocardiography and thoracic ultrasonography.

6.2 Ethical statement

The research protocol was approved by the Institutional Animal Care and Use Committee of the University of Milan (OPBA_27_2020 and OPBA_54_2021). An informed client consent was signed by the owners, and the highest standard (best practice) of veterinary care was guaranteed.

6.3 Measurement of echocardiographic parameters

All horses underwent to a complete echocardiographic examination and measurement of pulmonary artery stiffness, right systolic time intervals and PAD/AOD.

Echocardiography was conducted in a quiet room, when the horse was acclimatized to the environment. The subject was held in a relaxed and standing position by means of halter and

lead rope. No other physical or pharmacological restraint were used to avoid any influence on cardiac function and measurements. Echocardiographic examination was performed using an ultrasound machine (MyLabOmegaVet, Esaote, Genoa, Italy) with a 2.5 MHz phased-array transducer (P1-5, Esaote, Genoa, Italy). An ECG was recorded simultaneously to timing the measurements within the cardiac cycles. Measurements were not taken from cycle immediately following sinus block, second-degree atrioventricular block and premature beats.

Standard parasternal long axis and short axis views in 2D and M-mode were performed as previously described (Reef, 1990; Long *et al*., 1992; Zucca *et al.*, 2008).

The diameter of the aorta at sinotubular junction (AOD) and the diameter of pulmonary artery (PAD) were measured from the right parasternal long-axis view of the left ventricle outflow tract (Marr and Patteson, 2010); then PAD/AOD was calculated and used as an indirect marker of pulmonary hypertension (Johansson *et al.*, 2007).

Moreover, PW Doppler of the pulmonary outflow was acquired from the right parasternal short-axis view at the level of the pulmonary artery (Long *et al*., 1992) (Figure 6.1) with the sample volume on the arterial side of the pulmonary valve (Blissit and Bonagura, 1995) (Figure 6.2 A). During PW acquisition, particular attention was paid to ensuring that the sample volume remained in the centre of artery during systole. A satisfactory alignment between blood flow and ultrasound beam was assumed when the audible signal was clear and the envelope of the waveform was complete. No correction of the sampling angle was made. When the point of baseline crossing was difficult to distinguish, due to low velocity signals, it was determined by extrapolation of the downstroke; to minimise the error, low velocity filters were set as low as possible (Blissitt and Bonagura, 1995). Moreover, echocardiographic device setting was arranged in order acquire the maximal frequency shift (MFS).

MFS, AT and ET were measured from the Doppler waveform. MFS (kHz) was measured as the peak systolic frequency shift (Görgulü *et al.*, 2003). Acceleration time (sec) was measured from the onset of the Doppler flow trace to the beginning of maximum velocity plateau and the ejection time (sec) was recorded from the onset to the end of the Doppler waveform (Figure 6.2 B) (Blissitt and Bonagura, 1995).

Pulmonary artery stiffness (KHz/sec) was then calculated as the ratio of MFS to AT (Görgulü *et al.*, 2003). Finally, AT/ET was calculated. Each parameter was calculated as average of measurements taken from 3 consecutive Doppler flow traces to minimise the influence of respiratory and cardiac cycles.

The heart rate during PW Doppler echocardiography was calculated measuring the RR distance on the simultaneous ECG tracing.

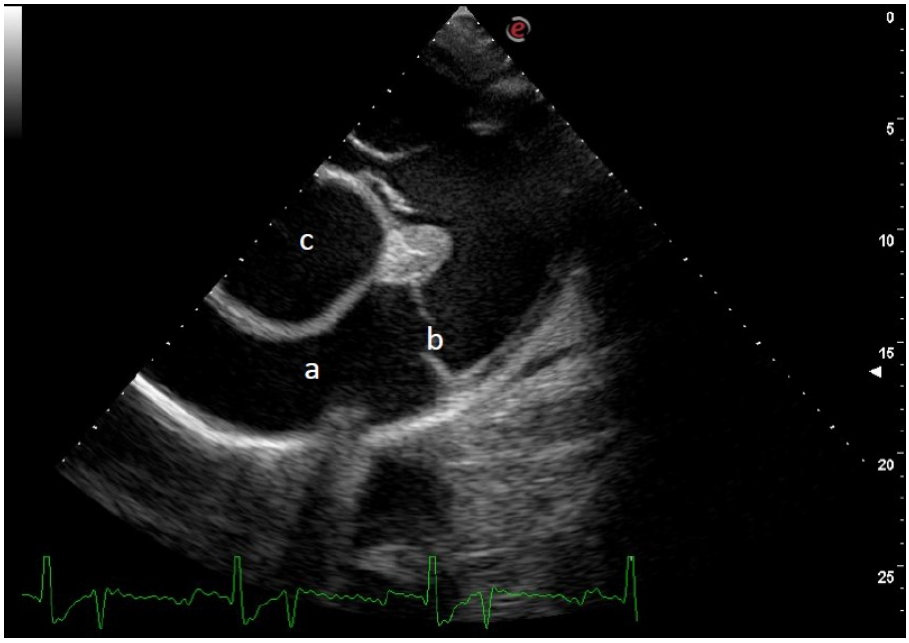

Figure 6.1 - Right parasternal short-axis view at the level of pulmonary artery in diastole. This view shows the pulmonary artery (a), the pulmonary valve (b) and the aortic valve (c).

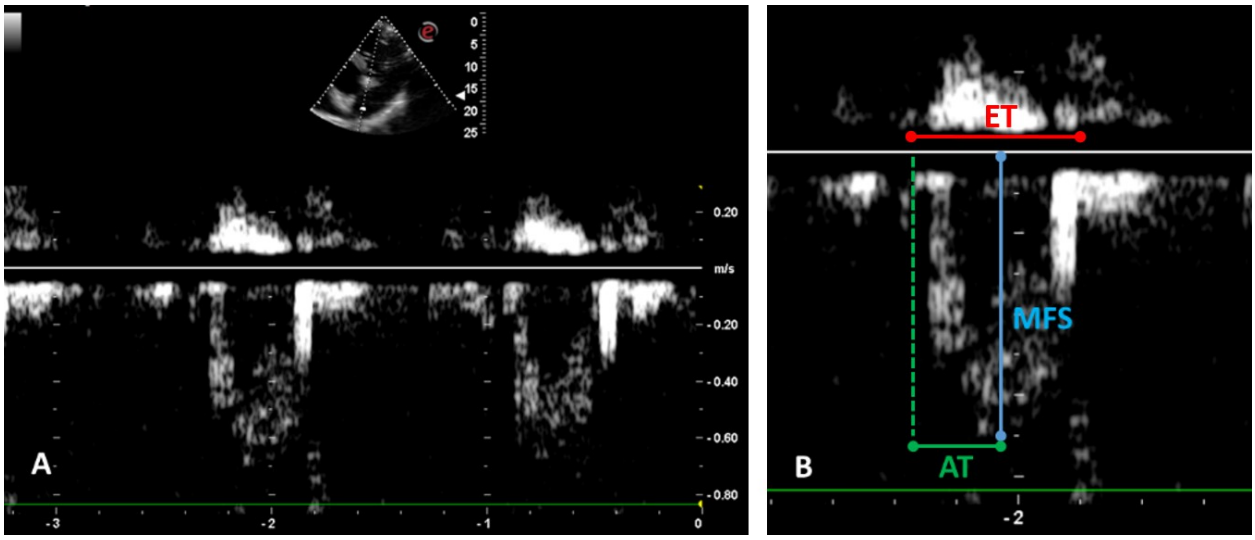

Figure 6.2 – Pulsed-wave Doppler of the pulmonary flow.
A) Right parasternal short-axis view at the level of pulmonary artery showing the positioning of the sample volume in proximal pulmonary artery and the simultaneous pulsed-wave Doppler trace
B) Zoom of the pulsed-wave Doppler waveform from which can be measured the maximal frequency shift (MFS), the acceleration time (AT) and the ejection time (ET).

6.4 Statistical analyses

All the following statistical analyses were performed using the statistical analysis software Graphpad Prism (version 9.1.2) and statistical significance was set at $p < 0.05$.

Data distribution

First of all, data distribution was tested for normality using Shapiro-Wilk test. Normally distributed data are presented as mean ± standard deviation while non-normally distributed data are presented as median and interquartile range (IQR).

Feasibility of PAS measurement

In order to assess the reproducibility of PAS measurement, the inter-observer variability, the intra-observer variability, the variability between different echocardiographic sessions (day-to-day variability) and the inter-observer variability of image acquisition were evaluated.

In 15 healthy horses two operators performed all the measurements independently on the same three cardiac cycles and one operator repeated all the measurements twice to assess inter-observer variability and intra-observer variability of the measurements, respectively.

In order to evaluate the day-to-day variability, one operator repeated the acquisition of the image and measurements in different days in 4 healthy horses. Finally, in the same 4 healthy horses the two operators acquired echocardiographic PW Doppler images and performed the measurements independently to assess the inter-observer variability of image acquisition.

Then, inter-observer variability, intra-observer variability, day-to-day variability and inter-observer variability of image acquisition were evaluated by Bland-Altman test and a simple linear regression analysis. Furthermore, a Pearson correlation was performed, and according to the *r* value, the correlation was classified as follows: $r = 0$ as no correlation, $r < 0.6$ as weak, $0.6 < r < 0.8$ as strong, $r > 0.8$ as very strong and $r = 1$ as perfect (Bunting *et al.,* 2019). Finally, the within-subject variance for repeated measurements (residual mean square) was determined by a one-way repeated-measures ANOVA and the within-subject standard deviation (s_w) was calculated as the square root of the residual mean square. The coefficient of variance (CV) was calculated dividing the s_w by the grand mean and multiplying by 100. According to the CV value, the variability was classified as follows: CV > 25% as high variability, 15% < CV < 25% as moderate variability and CV < 15% as low variability (Schwarzwald *et al*., 2007).

Influence of age, sex, bodyweight and heart rate

Spearman correlation was performed to evaluated the possible influence of age on PAS and RVSTIs. Moreover, possible differences in the measured parameters between females and males/geldings were evaluated using unpaired t test. Finally, simple linear regression analyses were performed to investigate possible influence of heart rate and bodyweight on parameters. These analyses were performed considering only healthy horses.

Differences between groups

Possible differences between healthy horses, subjects presenting mild equine asthma and those presenting severe equine asthma regarding PAS, RVSTIs, PAD, AOD, PAD/AOD, heart rate, PAS adjusted for HR (PAS_{HR}) and RVSTIs adjusted for HR (AT_{HR}, ET_{HR} and AT/ET_{HR}) were investigated. Pulmonary artery stiffness and RVSTIs were adjusted for heart rate dividing the parameter by the square root of the RR intervals (Chan *et al.,* 1987). A one-way analysis of variance (ANOVA) and a post hoc test for multiple comparison (Tukey's test) were used for normally distributed parameters while a Kruskal-Wallis test and Dunn's multiple comparison test were used for non-normally distributed parameters.

Correlation between PAS or RVSTIs and PAD/AOD

Spearman correlation was used to evaluated possible correlation between PAS values and PAD/AOD, as well as between RVSTIs and PAD/AOD. These analyses were performed considering the whole sample.

Sensitivity and specificity of PAS and AT

Sensitivity and specificity of PAS and AT were calculated for several cut-off values and a Receiving Operator Characteristics (ROC) Curve was elaborated to determine which cut-off value of PAS and AT represented the best compromise between sensitivity and specificity for the diagnosis of SEA.

Chapter 7

RESULTS

7.1 Sample

Fifty-three horses of different breed, age, sex and weight were included in this research project. Among them, 21 were geldings, 18 were males and 14 were females. The age ranged between 2 and 28 years old (median: 6 years old; IQR: 11 years old). The mean weight was 473 ± 54 kg (range: 346 - 600 kg). The most represented breed was Thoroughbred (17 subjects), follow by Standardbred (13 subjects) and other different breeds (Mixed breed, Quarter Horse, Paint Horse, Irish Sport Horse, Pura Raza Española, etc.).

According to criteria of inclusion, horses were subdivided in three groups: 15 healthy horses, 23 MEA affected horses and 15 SEA affected horses (Table 7.1).

Healthy horses

Fifteen horses were considered healthy according to the absence of cardiac and respiratory signs at history, clinical examination, echocardiography and thoracic ultrasonography. Among them, there were 8 geldings, 5 females and 2 males. The median age was 5 years old (IQR: 8 years old; range 3 - 16 years old). The bodyweight ranged between 400 and 600 kg (mean: 482 ± 57 kg).

MEA group

Mild/moderate equine asthma was diagnosed in 23 horses. Among these, 16 were males, 6 geldings and 1 female. The age ranged between 2 and 14 years old (median: 3 years old; IQR: 3 years old). The mean weight was 474 ± 44 kg (range: 379 - 557 kg).

SEA group

Fifteen horses were affected by severe equine asthma. Among these, there were 8 females and 7 geldings. The mean age was 17 ± 6 years old (range 5 - 28 years old). The bodyweight ranged between 346 and 525 kg (mean: 450 ± 63 kg).

Table 7.1 – Demographic data of healthy horses, mild/moderate equine asthma (MEA) affected horses and severe equine asthma (SEA) affected horses.

	HEALTHY (15 horses)	MEA (23 horses)	SEA (15 horses)
AGE (years old)	5 (8) 3 - 16	3 (3) 2 - 14	17 ± 6 5 - 28
GENDER **females** **males** **geldings**	 5 (33.3%) 2 (13.3%) 8 (53.4%)	 1 (4.3%) 16 (69.6%) 6 (26.1%)	 8 (53.4%) 7 (46.6%) 0 (0%)
WEIGHT (Kg)	482 ± 57 400 - 600	474 ± 44 379 - 557	450 ± 63 346 - 525
BREED **Thoroughbred** **Standardbreds** **Other breeds***	 6 (40%) 4 (26.7%) 5 (33.3%)	 11 (47.8 %) 8 (34.8%) 4 (17.4%)	 0 (0%) 1 (6.7%) 14 (93.3%)

*Continuous variables normally distributed are reported as: mean ± standard deviation and minimum value – maximum value. Continuous variables non-normally distributed are reported as: median (interquartile range) and minimum value – maximum value. Categorical variables are reported as: number (percentage). *Other breeds: healthy (1 Irish Sport Horse, 1 Koenigin Warmbloed Paard of Netherland, 1 Noriker, 1 Maremma Horse and 1 pony); MEA (1 mixed breeds, 1 Irish Sport Horse, 1 Pura Raza Española and 1 Oldenburg); SEA (3 mixed breeds, 2 Quarter Horses, 2 Paint Horses, 1 Pura Raza Española, 1 Italian Saddle Horse, 1 pony, 1 Camargue, 1 Haflinger, 1 Arabian thoroughbred and 1 Murgese Horse)*

7.2 Measurement of echocardiographic parameters

MFS, AT, ET, PAD and AOD were measured and PAS, AT/ET and PAD/AOD were calculated in all horses.

Healthy horses

All the parameters, except AT/ET_{HR}, were normally distributed. The heart rate ranged between 28 bpm and 44 bpm (mean: 36 ± 5 bpm). The mean MFS and the mean AT were 1.70 ± 0.21 kHz (range: 1.30 - 1.99 kHz) and 0.256 ± 0.047 seconds (range: 0.184 - 0.360 seconds), respectively. The minimum value of PAS was 4.89 kHz/sec while the maximum value was 7.94 kHz/sec (mean: 6.78 ± 0.97 kHz/sec). The mean ET value was 0.662 ± 0.068 seconds (range: 0.547 - 0.827 seconds). AT/ET ranged between 0.33 and 0.50 (mean: 0.39 ± 0.05). PAD ranged from 34.1 mm to 53.0 mm (mean: 43.2 ± 5.6 mm). The mean AOD was 57.7 ± 5.3 mm (range: 50.5 - 67.0 mm). PAD/AOD ranged from 0.61 to 0.85 (mean: 0.75 ± 0.07). A summary of these echocardiographic parameters is reported in Table 7.2. Instead, Table 7.3 shows data of parameters adjusted for heart rate.

MEA group

All the parameters were normally distributed. The mean heart rate was 36 ± 5 bpm (range: 27 - 46 bpm). The mean MFS and the mean AT were 1.62 ± 0.23 kHz (range: 1.23 - 2.00 kHz) and 0.224 ± 0.020 sec seconds (range: 0.181 - 0.265 seconds), respectively. The minimum value of PAS was 5.38 kHz/sec while the maximum value was 9.03 kHz/sec (mean: 7.30 ± 1.08 kHz/sec). ET ranged from 0.492 seconds to 0.766 seconds (mean: 0.620 ± 0.065 sec). The mean AT/ET was 0.36 ± 0.03 (range: 0.31 - 0.42). PAD ranged from 37.9 mm to 50.4 mm (mean: 44.2 ± 3.4 mm). The mean AOD was 59.9 ± 6.9 mm (range: 49.7 - 72.7 mm). PAD/AOD ranged from 0.65 to 0.87 (mean: 0.74 ± 0.07). A summary of these echocardiographic parameters is reported in Table 7.2. Instead, Table 7.3 shows data of parameters adjusted for heart rate.

SEA group

All the parameters, except ET_{HR}, were normally distributed. The heart rate ranged between 30 bpm and 57 bpm (mean: 43 ± 7 bpm). The mean MFS and the mean AT were 1.78 ± 0.29 kHz (range: 1.36 - 2.22 kHz) and 0.178 ± 0.032 seconds (range: 0.138 - 0.247 seconds), respectively. The minimum value of PAS was 7.30 kHz/sec while the maximum value was 14.74 kHz/sec (mean: 10.27 ± 1.99 kHz/sec). ET ranged from 0.477 sec to 0.723 seconds (mean: 0.565 ± 0.065 seconds). The mean AT/ET was 0.32 ± 0.05 (range: 0.23 - 0.39). PAD ranged from 41.8 mm to 66.2 mm (mean: 52.3 ± 8.1 mm). The mean AOD was 54.82 ± 7.1 mm (range: 42.5 - 67.6 mm). The mean PAD/AOD was 0.96 ± 0.10 (range: 0.77 - 1.13); one horse had PAD/AOD equal to 1 and 5 horses had PAD/AOD greater than 1. A summary of these echocardiographic parameters is reported in Table 7.2. Instead, Table 7.3 shows data of parameters adjusted for heart rate.

Table 7.2 – Echocardiographic parameters in healthy horses, mild/moderate equine asthma (MEA) affected horses and severe equine asthma (SEA) affected horses

	HEALTHY mean ± sd min -max	**MEA** mean ± sd min -max	**SEA** mean ± sd min -max
MFS (kHz)	1.70 ± 0.21 1.30 - 1.99	1.62 ± 0.23 1.23 - 2.00	1.78 ± 0.29 1.36 - 2.22
AT (sec)	0.256 ± 0.047 [ab] 0.184 - 0.360	0.224 ± 0.020 [ac] 0.181 - 0.265	0.178 ± 0.032 [bc] 0.138 - 0.247
PAS (kHz/sec)	6.78 ± 0.97 [a] 4.89 - 7.94	7.30 ± 1.08 [b] 5.38 - 9.03	10.27 ± 1.99 [ab] 7.30 - 14.74
ET (sec)	0.662 ± 0.068 [a] 0.547 - 0.827	0.620 ± 0.065 [b] 0.492 – 0.766	0.565 ± 0.065 [ab] 0.477 - 0.723
AT/ET	0.39 ± 0.05 [a] 0.33 - 0.50	0.36 ± 0.03 [b] 0.31 - 0.42	0.32 ± 0.05 [ab] 0.23 - 0.39
PAD (mm)	43.2 ± 5.6 [a] 34.1 - 53.0	44.2 ± 3.4 [b] 37.8 - 50.4	52.3 ± 8.1 [ab] 41.8 - 66.2
AOD (mm)	57.7 ± 5.3 50.5 - 67.0	59.9 ± 6.9 49.7 - 72.7	54.82 ± 7.1 42.5 - 67.6
PAD/AOD	0.75 ± 0.07 [a] 0.61 - 0.85	0.74 ± 0.07 [b] 0.65 - 0.87	0.96 ± 0.10 [ab] 0.77 - 1.13
HR (bpm)	36 ± 5 [a] 28 - 44	36 ± 5 [b] 27 - 46	43 ± 7 [ab] 30 - 57

MFS: maximal frequency shift; AT: acceleration time; PAS: pulmonary artery stiffness; ET: ejection time; AT/ET: ratio of acceleration time to ejection time; PAD: pulmonary artery diameter; AOD: aorta diameter at sinotubular junction; PAD/AOD: ratio of pulmonary artery diameter to aorta diameter; HR: heart rate; sd: standard deviation; min: minimum value; max: maximum value. Different superscripts show significantly different mean values.

Table 7.3 - Echocardiographic parameters adjusted for heart rate in healthy horses, mild/moderate equine asthma (MEA) affected horses and severe equine asthma (SEA) affected horses

	HEALTHY mean ± sd/median (IQR) min -max	**MEA** mean ± sd min -max	**SEA** mean ± sd/median (IQR) min -max
AT_{HR}	0.006 ± 0.001 [a] 0.004 - 0.008	0.005 ± 0.001 [b] 0.004 - 0.006	0.004 ± 0.001 [ab] 0.003 - 0.007
PAS_{HR}	0.169 ± 0.023 [a] 0.124 - 0.198	0.179 ± 0.031 [b] 0.127 - 0.235	0.276 ± 0.067 [ab] 0.196 - 0.421
ET_{HR}	0.016 ± 0.002 0.013 - 0.020	0.015 ± 0.002 0.012 - 0.019	0.014 (0.006) 0.013 - 0.020
AT/ET_{HR}	0.009 (0.002) 0.007 - 0.012	0.009 ± 0.001 0.007 – 0.011	0.008 ± 0.002 0.005 -0.011

AT_{HR}: acceleration time adjusted for heart rate; PAS_{HR}: pulmonary artery stiffness adjusted for heart rate; ET_{HR}: ejection time adjusted for heart rate; AT/ET_{HR}: ratio of acceleration time to ejection time adjusted for heart rate. Normally distributed data are reported as mean ± standard deviation and range (minimum-maximum). Non-normally distributed data are reported as median (interquartile range) and range (minimum-maximum). Different superscripts show significantly different mean values.

7.3 Statistical analyses

Feasibility of PAS measurement

Regarding intra-observer variability, bias between the repeated measurements of MFS performed by the first operator was < 0.01 kHz (95% confidence interval, CI: -0.05 kHz to 0.05 kHz). The repeated measurements of AT had a bias of 0.003 seconds (95% CI: -0.018 sec to 0.025 sec). The calculated PAS values showed a bias of 0.04 kHz/sec (95% CI: -0.41 to 0.49 kHz/sec). All the parameters showed a very strong correlation between the repeated measurements (MFS: $r = 0.99$, $p < 0.0001$; AT: $r = 0.97$, $p < 0.0001$; PAS: $r = 0.97$, $p < 0.0001$). The intra-observer variability resulted low for all the parameters (MFS: CV = 5%; AT: CV = 8%; PAS: CV = 7%).

Regarding the inter-observer variability, MFS measured by the two operators had a bias < -0.01 kHz (95% CI: -0.11 kHz to 0.09 kHz). AT measured by the two operators showed a bias of < 0.001 seconds (95% CI: -0.028 sec to 0.029 sec). Bias between PAS values calculated by the two operators was of 0.03 kHz/sec (95% CI: -0.70 kHz/sec to 0.76 kHz/sec). All the parameters showed a very strong correlation between the two operators (MFS: $r = 0.97$, $p < 0.0001$; AT: $r = 0.96$, $p < 0.0001$; PAS: $r = 0.93$, $p < 0.0001$). The inter-observer variability resulted low for all the parameters (MFS: CV = 5%; AT: CV = 8%; PAS: CV = 9%).

Results of simple linear regression analyses for the evaluation of intra-observer variability and inter-observer variability are illustrated in Figure 7.1.

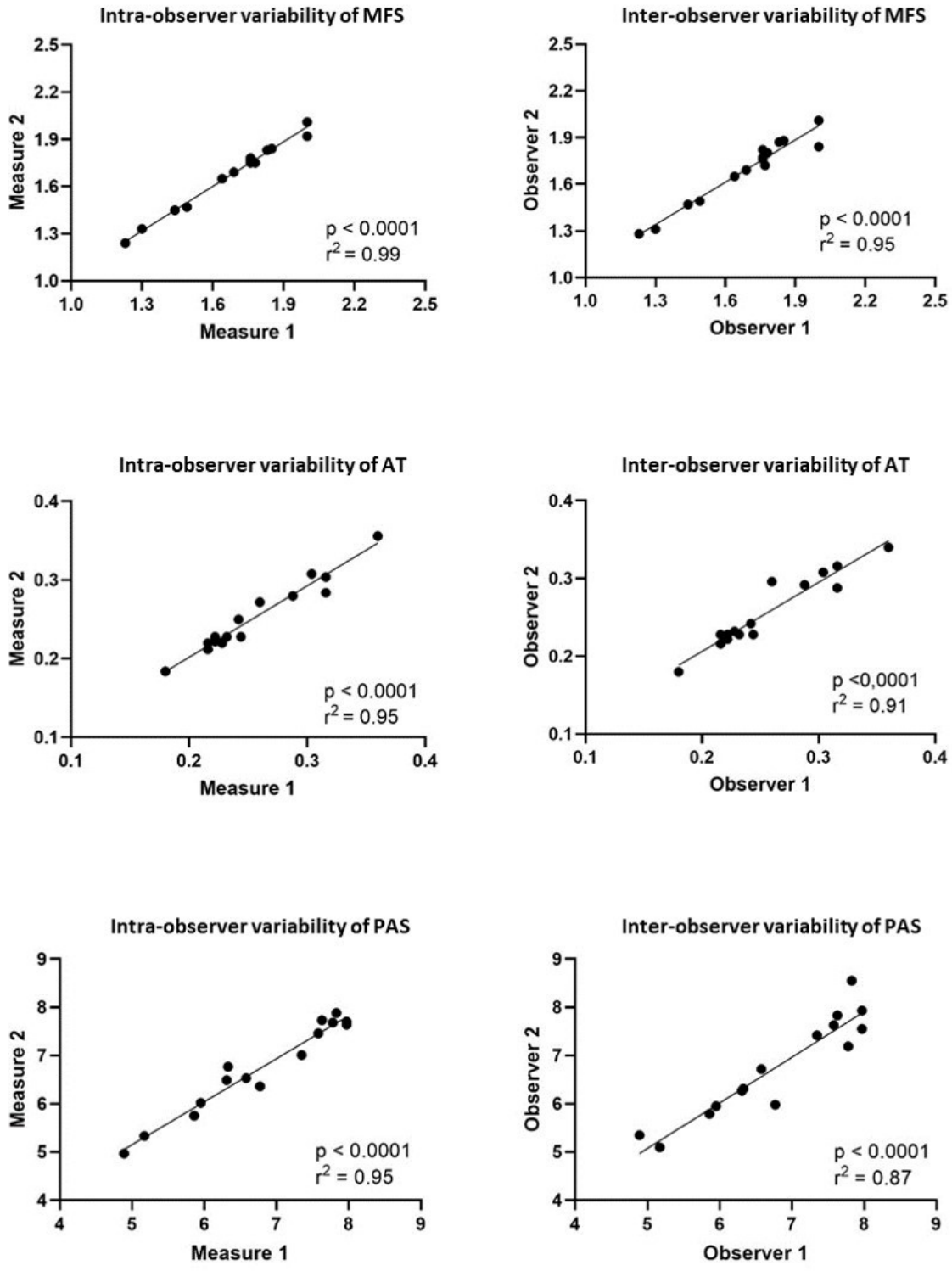

Figure 7.1 - Results of simple linear regression analyses performed for intra-observer variability and inter-observer variability of maximal frequency shift (MFS), acceleration time (AT) and pulmonary artery stiffness (PAS)

Concerning the day-to-day variability, MFS, AT and PAS showed a bias of < -0.01 kHz (95% CI: -0.19 kHz to 0.18 kHz), -0.001 sec (95% CI: -0.030 sec to 0.028 sec) and 0.08 kHz/sec (95% CI: -0.13 kHz/sec to 0.30 kHz/sec) respectively. The relationship between the measurements performed

in different echocardiographic sessions was good for all the parameters (MFS: p = 0.0416, r2 = 0.92; AT: p = 0.0026, r2 =0.99; PAS: p = 0.0071, r2 = 0.98). All the parameters showed a very strong correlation (MFS: *r* = 0.96, p = 0.0416; AT: *r* = 0.99, p = 0.0026; PAS: *r* = 0.99, p = 0.0071). The day-to-day variability resulted low for all the parameters (MFS: CV = 6%; AT: CV = 12%; PAS: CV = 10%).

About the inter-observer variability for acquisition of the image, the bias between the two operators for the measurements of MFS, AT and PAS were -0.01 kHz (95% CI: -0.18 kHz to 0.15 kHz), 0.004 sec (95% CI: -0.017 sec to 0.025 sec) and -0.01 kHz/sec (95% CI: -0.20 kHz/sec to 0.17 kHz/sec) respectively. Simple linear regression analyses showed a good relationship between the two operators for all the parameters (MFS: p = 0.0249, r2 = 0.95; AT: p = 0.0091, r2 =0.98; PAS: p = 0.0049, r2 = 0.99). All the parameters showed a very strong correlation between the two operators (MFS: *r* = 0.97, p = 0.0249; AT: *r* = 0.99, p = 0.0091; PAS: *r* = 0.99, p = 0.0049). The inter-observer variability for image acquisition resulted low for all the parameters (MFS: CV = 5%; AT: CV = 10%; PAS: CV = 9%).

Influence of age, sex, bodyweight and heart rate

No significant relationships between heart rate, age or bodyweight and MFS (HR: p = 0.471; age: p = 0.954; bodyweight: p = 0.249), AT (HR: p = 0.370; age: p = 0.358; bodyweight: p = 0.952;), PAS (HR: p = 0.735; age: p = 0.592; bodyweight: p = 0.294), ET (HR: p = 0.855; age: p = 0.545; bodyweight: p = 0.609) and AT/ET (HR: p = 0.405; age: p = 0.112; bodyweight: p = 0.557) were detected. Moreover, no differences between females and male/geldings regarding MFS (p = 0.531), AT (p = 0.521) and PAS (p = 0.737), ET (p = 0.498) and AT/ET (p = 0.385) were found.

Differences between groups

Pulmonary artery stiffness, RVSTIs, PAD, PAD/AOD, heart rate, PAS_{HR} and AT_{HR} resulted significantly different between groups (Figure 7.2). Conversely, no differences were found between groups regarding MFS (p = 0.17), AOD (p = 0.11), ET_{HR} (p = 0.19) and AT/ET_{HR} (p = 0.38). Severe equine asthma affected horses had a significant higher PAS than healthy horses (p < 0.0001) and MEA affected horses (p < 0.0001); instead, no difference was found between healthy horses and MEA group regarding PAS (p = 0.474).

Acceleration time was significantly lower in SEA affected horses compared to healthy horses (p < 0.0001) and MEA affected horses (p = 0.0003); moreover, MEA horses had a significant lower AT than healthy horses (p = 0.013).

Ejection time and AT/ET were significantly lower in SEA group compared to healthy horses (ET: $p = 0.0005$; AT/ET: $p = 0.0003$) and MEA group (ET: $p = 0.039$; AT/ET: $p = 0.006$); instead, no differences were found between healthy horses and MEA affected horses regarding ET ($p = 0.142$) and AT/ET ($p = 0.329$).

Pulmonary artery diameter and PAD/AOD were significantly higher in SEA group compared to healthy horses (PAD: $p = 0.0015$; PAD/AOD: $p < 0.0001$) and MEA affected horses (PAD: $p = 0.0010$; PAD/AOD: $p < 0.0001$); instead, no differences were found between healthy horses and MEA affected horses regarding PAD ($p = 0.88$) and PAD/AOD ($p = 0.99$).

Heart rate was significantly higher in SEA affected horses compared to healthy horses ($p = 0.0058$) and MEA affected horses ($p = 0.0022$); instead, no difference was found between healthy horses and MEA group regarding heart rate ($p = 0.99$).

Severe equine asthma affected horses had a significant higher PAS_{HR} than healthy horses ($p < 0.0001$) and MEA affected horses ($p < 0.0001$); instead, no difference was found between healthy and MEA horses regarding PAS_{HR} ($p = 0.78$).

Acceleration time adjusted for HR was significantly lower in SEA affected horses compared to healthy horses ($p = 0.0005$) and MEA affected horses ($p = 0.035$); instead, no difference was found between healthy and MEA horses regarding AT_{HR} ($p = 0.13$).

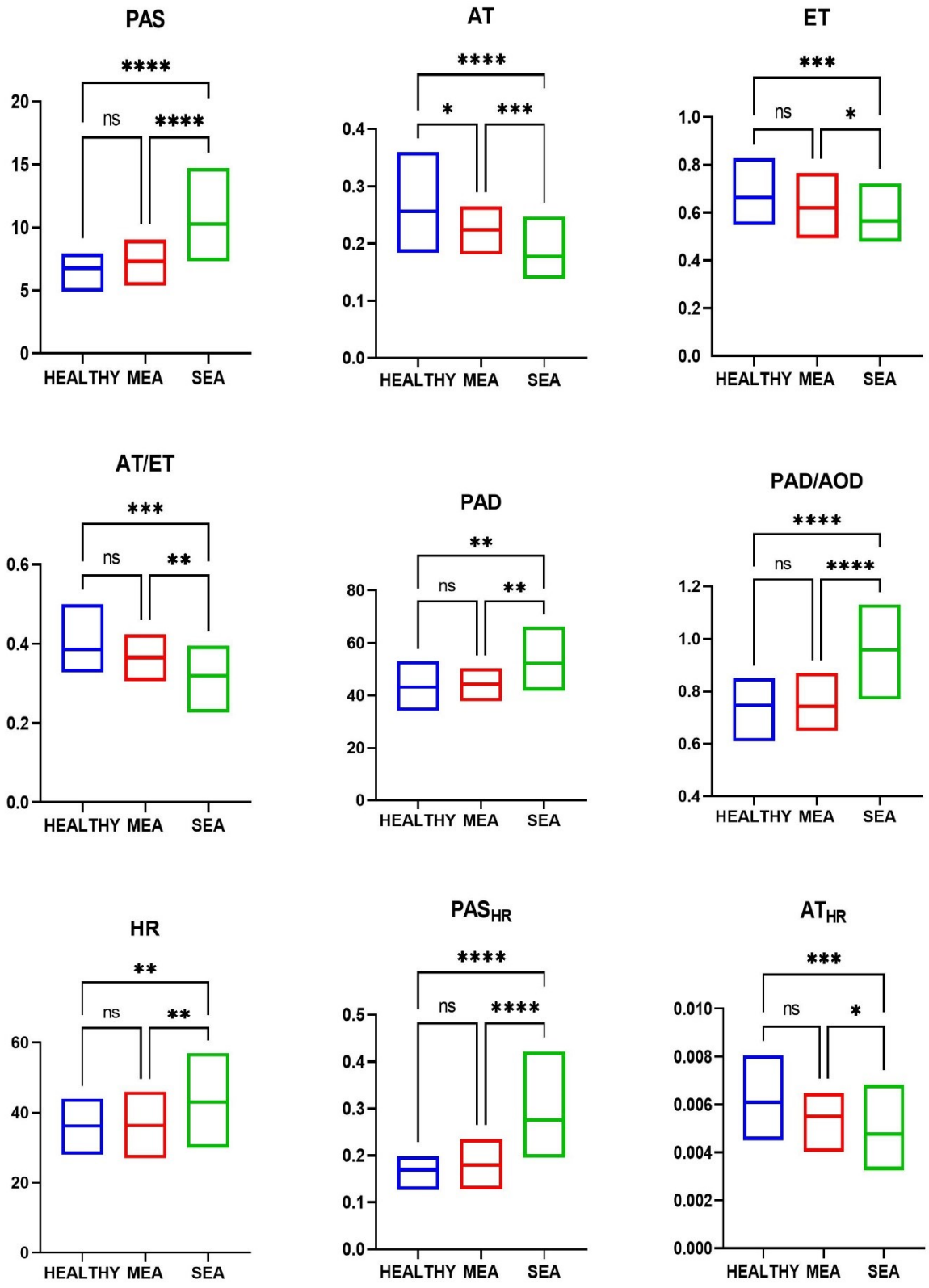

*Figure 7.2 – Graphical representations of the differences between healthy horses, mild/moderate equine asthma (MEA) affected horses and severe equine asthma (SEA) affected horses regarding pulmonary artery stiffness (PAS), acceleration time (AT), ejection time (ET), acceleration time to ejection time ratio (AT/ET), pulmonary artery diameter (PAD), ratio of pulmonary artery diameter to aorta diameter (PAD/AOD), heart rate (HR), PAS adjusted for HR (PAS_{HR}) and AT adjusted for HR (AT_{HR}). ns: not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$*

Correlation between PAS or RVSTIs and PAD/AOD

Considering the entire studied population PAS and PAD/AOD were not-normally distributed; instead, AT, ET and AT/ET were normally distributed. The median PAS and median PAD/AOD of all horses were 7.67 kHz/sec (IQR: 1.98; range: 4.89 - 14.74 kHz/sec) and 0.77 (IQR: 0.15; range: 0.61 - 1.13) respectively. The mean AT, ET and AT/ET were 0.220 ± 0.043 seconds (range: 0.138 - 0.360 seconds), 0.616 ± 0.075 seconds (range: 0.477 - 0.827 seconds) and 0.36 ± 0.05 (range: 0.23 - 0.50) respectively. A significant positive correlation ($p = 0.0001$; $r = 0.563$) between PAS and PAD/AOD was detected; PAS increased with the increasing of PAD/AOD. Moreover, a significant negative correlation was detected between AT ($p < 0.0001$; $r = - 0.574$) and AT/ET ($p = 0.0002$; $r = - 0.543$) and PAD/AOD; AT and AT/ET decreased with the increasing of PAD/AOD. No correlation was found between ET and PAD/AOD ($p = 0.0924$; $r = 0.263$).

Sensitivity and specificity of PAS and AT

Fifteen horses were affected by SEA and 38 horses were not affected by SEA (healthy and MEA horses).

Forty-eight values of PAS ranging from 5.10 kHz/sec to 13.68 kHz/sec were evaluated as cut-off for the diagnosis of SEA. Sensitivity ranged from 100% to 6.67%, whereas specificity ranged from 2.63% to 100%. A cut-off value of 7.28 kHz/sec had 100% sensitivity but a specificity of 50% whereas a cut-off value of 9.12 kHz/sec had 100%specificity but a sensitivity of 66.67%. The cut-off value of 8.18 kHz/sec showed the best compromise between sensitivity (93.33%) and specificity (86.84%). The ROC curve is illustrated in Figure 7.3. The area under the ROC curve was 0.94 (95% CI: 0.88 -1.00; $p < 0.0001$).

Forty-five values of AT ranging from 0.138 sec to 0.335 sec were evaluated as cut-off for the diagnosis of SEA. Sensitivity ranged from 100% to 6.67%, whereas specificity ranged from 2.63% to 100%. A cut-off value of 0.249 sec had 100% sensitivity but a specificity of 21.05% whereas a cut-off value of 0.173 sec had 100% specificity but a sensitivity of 46.67%. The cut-off value of 0.202 sec showed the best compromise between sensitivity (86.67%) and specificity (89.47%). The ROC curve is illustrated in Figure 7.3. The area under the ROC curve was 0.89 (95% CI: 0.76 - 1.00; $p < 0.0001$).

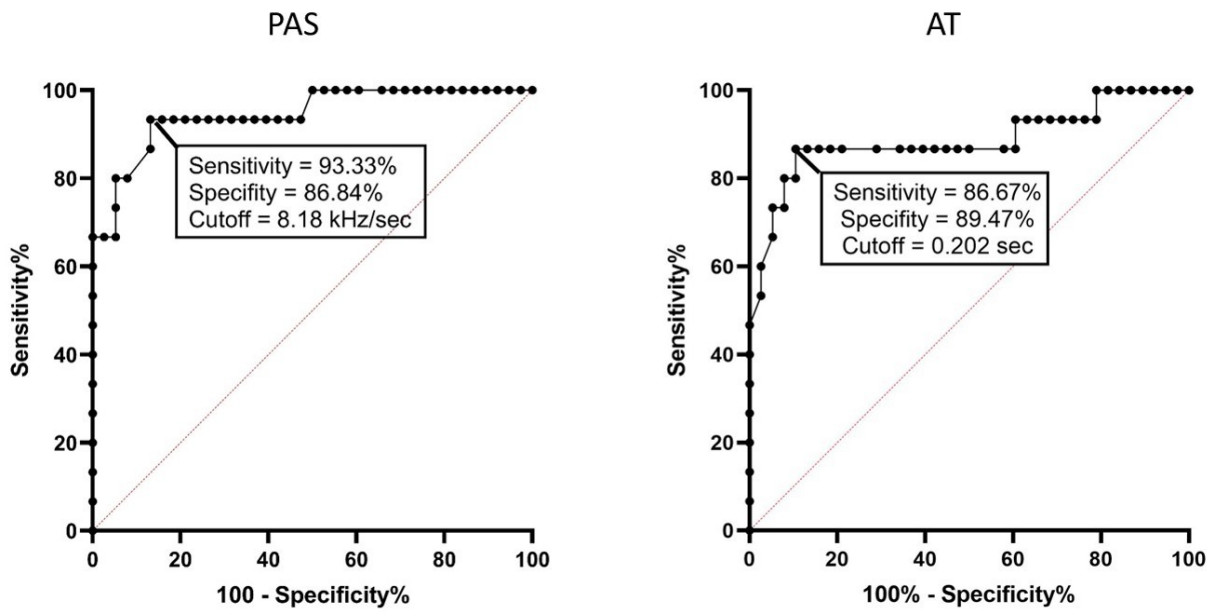

Figure 7.3 – Receiving Operator Characteristics (ROC) curve for pulmonary artery stiffness (PAS) and acceleration time (AT). The PAS cut-off value of 8.18 kHz/sec showed the best compromise between sensitivity (93.33%) and specificity (86.84%). The AT cut-off value of 0.202 sec showed the best compromise between sensitivity (86.67%) and specificity (89.47%).

Chapter 8

DISCUSSION

Pulmonary Artery Stiffness is a PW Doppler echocardiographic parameter used in human medicine to early detect an increase in pulmonary artery stiffness due to remodeling of the pulmonary vessel wall (Görgulü *et al.*, 2003; Altiparmak *et al.*, 2016; Baysal and Has, 2019). In human medicine, it has been reported an increase of PAS value in people with chronic respiratory diseases, such as asthma and OSAS; moreover, in these patients, PAS seems to increase with the severity of the disease (Weir-Mccall *et al.*, 2015; Altiparmak *et al.*, 2016).

Other PW Doppler parameters useful for the evaluation of pulmonary vascular bed are the right ventricular systolic time intervals, such as acceleration time, right ventricular ejection time and acceleration time index (Celermajer and Playford, 2017; Grapsa and Tzemos, 2017). It is known that a decreased AT and a reduced AT/ET are indicative of high vascular resistance and pulmonary hypertension in humans (Görgulü *et al.*, 2003; Celermajer and Playford, 2017; Grapsa and Tzemos, 2017) and dogs (Schober and Baade, 2006; Serres *et al.*, 2017; Reinero *et al.*, 2020).

The pulmonary vascular bed of patients with chronic respiratory diseases can undergo structural changes caused by recurrent episodes of hypoxemia and hypercapnia, associated with inflammatory mediators and cytokines released due to chronic inflammation (Görgulü *et al.*, 2003; Altiparmak *et al.*, 2016). These modifications consist in an increase in smooth muscle mass of the pulmonary arterioles and excessive deposition of matrix proteins in the wall of large pulmonary arteries leading to a thickening of the pulmonary artery wall. These structural modifications compromise the elastic properties of pulmonary arteries leading to a loss of compliance and this may be the reason for a high PAS value. Moreover, the loss of compliance leads to an increase in pulmonary vascular resistance and contributes to the elevated oscillatory load that increases right ventricular systolic pressure. The stiffness itself causes further damages to the pulmonary vascular bed over time, ultimately leading to pulmonary hypertension (Görgulü *et al.*, 2003; Altiparmak *et al.*, 2016; Baysal and Has, 2019).

It is demonstrated that the loss of compliance of pulmonary artery shortens the duration of right ventricular systolic ejection time primarily due to a decrease in the acceleration time of the pulmonary flow trace. Since in a stiff pulmonary artery AT decreases, the velocity and MFS increases in order to maintain the flow constant. Therefore, PAS has been developed as an

echocardiographic parameter calculated with a formula associated with AT and MFS (Görgulü *et al.*, 2003; Altiparmak *et al.*, 2016).

As human asthma, equine asthma causes thickening of the pulmonary vessel wall over time, leading to progressive increase of pulmonary vascular resistance and consequently PHT (Decloedt *et al.*, 2017a; Ceriotti *et al.*, 2020). The hypothesis is that evaluation of PAS and RVSTIs in asthmatic subjects could be useful also in horses. However, no studies are available on PAS in the veterinary medicine literature and only two studies reported RVSTIs measured in horses with increased pulmonary pressure (Lightowler *et al.*, 2009; Decloedt *et al.*, 2017a).

This is the first study that evaluates PAS in veterinary medicine and in particular on equine patients. This study assessed the measurement of PAS by PW Doppler of pulmonary outflow acquired from the right parasternal short-axis view at the level of the pulmonary artery as described by Blissitt and Bonagura (Blissitt and Bonagura, 1995). The image was easily obtained in all patients; the pulmonary flow trace was acquired positioning the sample volume on the arterial side of the pulmonary valve and paying attention to maintain the sample volume in the centre of artery during systole. No correction of the sampling angle was made. A satisfactory alignment between blood flow and ultrasound beam was assumed when the audible signal was clear and the envelope of the waveform was complete.

First of all, the feasibility of PAS was assessed in healthy horses evaluating its intra-observer variability, inter-observer variability, inter-observer variability of image acquisition and day-to-day variability. The analyses showed a low variability between the repeated measurement performed by the same operator or by different operators. Moreover, also the inter-observer variability for image acquisition and the variability between different echocardiographic sessions resulted low. These results demonstrated that PAS can be measured consistently in horses by PW Doppler echocardiography across the pulmonary valve.

This technique resulted quite easy and well tolerated by patients; therefore, the evaluation of PAS could be easily included in the standard echocardiographic examination of horses. However, the measurement of PAS was attempted only from the right parasternal short axis view, and the feasibility of PAS measured by PW Doppler acquired from other nonstandard view, such as the left parasternal view (Decloedt *et al.*, 2017b), has yet to be assessed. Moreover, it would be useful to evaluate possible differences in the PAS values obtained from the different views.

Regarding the value of PAS in healthy horses, this study detected a minimum value of 4.89 kHz/sec and a maximum value of 7.94 kHz/sec with a mean value of 6.76 ± 0.97 kHz/sec. This study did not determine PAS reference intervals due to the limited number of healthy horses included; therefore, a further study on a large number of healthy subjects is necessary to establish the normal reference range of PAS in healthy horses. In human medicine, despite the use of PAS as early predictor of PHT and numerous studies regarding its measurement in pathologic conditions, there is no agreement concerning echocardiographic PAS reference range in healthy people (Yildirim *et al.*, 2017). In fact, wide differences in values of PAS measured in healthy people are reported by different studies. A mean PAS of 7.41 ± 1.32 kHz/sec was reported in healthy people used as controls in a study that investigated PAS in patients with heart failure with reduced ejection fraction (Yildirim *et al.*, 2017). Another study, evaluating differences between recently diagnosed asthmatic patients and healthy people, reported a mean PAS of 22.4 ± 4.1 kHz/sec in the latter (Baysal and Has, 2019). Finally, other two studies assessed PAS in patients with OSAS, indicating for the control group a mean PAS value of 18.0 ± 3.5 kHz/sec (Altiparmak et al., 2016) and of 18.6 ± 6.3 kHz/sec (Ozkececi et al., 2016).

The possible influence of age, bodyweight, gender and heart rate on RVSTIs and PAS was investigated in the present study.

None of these factors were found to influence AT. Similarly, a previous study found no correlation between AT and age, bodyweight or sex in horses (Blissitt and Bonagura, 1995). Moreover, it has been reported that AT in dogs is not affected by age, bodyweight, heart rate, breed and right ventricular segmental shortening fraction (Kirberger *et al.*, 1992; Schober and Baade, 2006). Also in humans, AT is not influenced by gender (Habash *et al.*, 2019). Conversely, it has been demonstrated that AT in children correlates positively to age and body surface area (BSA) and negatively to heart rate; however, these correlations become weaker with the increasing age (Habash *et al.*, 2019). The influence of heart rate on AT in adult people is controversial. In fact, a study in which patients' heart rate ranged widely (38-180 bpm) showed that the correlation between AT and mean pulmonary artery pressure improved when patients with extreme values of HR were excluded or when AT was corrected for HR (Chan *et al.*, 1987). However, other studies found that this correction was not necessary (Dabestani *et al.*, 1987; Yared *et al.*, 2011).

Results of the present research suggest that also ET is not influenced by age, bodyweight, gender and HR. Similarly, no correlation between ET and age, bodyweight or sex were reported in a previous study on healthy horses (Blissitt and Bonagura, 1995). Moreover, it has been reported

that ET is not influenced by bodyweight in dogs (Schober and Baade, 2006) and by gender in humans (Habash *et al.*, 2019). Conversely, in dogs ET is affected by age, HR and right ventricular segmental shortening fraction (Schober and Baade, 2006). Furthermore, it has been reported that in children ET is positively correlated to age and BSA while it is negatively correlated to HR; however, these correlations become weaker with the increasing age (Habash *et al.*, 2019).

In addition, none of the considered factors in this project seemed to influence AT/ET. Similarly, it has been demonstrated that AT/ET in dogs is not influenced by HR, bodyweight and right ventricular segmental shortening fraction (Schober and Baade, 2006). Moreover, in humans, AT/ET is not affected by sex (Habash *et al.*, 2019). Conversely, it has been reported that, in children, AT/ET is weakly correlated to age, BSA and HR (Habash *et al.*, 2019). Furthermore, it has been reported that also in dogs AT/ET is influenced by age; however, authors themselves stated that this influence is negligible (Schober and Baade, 2006).

In horses, PAS seemed to be not influenced by age, bodyweight, gender and heart rate. Similarly, in human medicine, it has been reported that PAS is not affected by age and sex; however, it is affected by BMI (body mass index) (Ozkececi *et al.*, 2016).

Summarizing, no factors among age, bodyweight, gender and heart rate seemed to influence RVSTIs and PAS in the healthy group of horses in our study. However, these results could be influenced by the small sample size. Moreover, it should be considered that all the subjects had a normal heart rate during the echocardiographic examination and therefore, the range of heart rate considered is quite narrow (28 - 44 bpm). Even if, usually, echocardiographic measurements should be taken only with normal heart rate, this may not always be possible, particularly in pathological conditions such as severe asthma. Since a wider range of HR was found in SEA affected horses, we used the formula for HR correction suggested in humans for AT (Chan *et al.*, 1987) to evaluate possible differences in PAS and RVSTIs between groups.

In addition, all the healthy subjects were aged between 3 and 16 years old; elderly subjects, in which may be a reduction of pulmonary artery distensibility due to age, as described in humans (Castillo *et al.*, 1967; Townsley, 2013), were not considered in the present study. Further studies considering subjects with a wider range of age, might be necessary to verify this possible influence.

Moreover, growing subjects were not included in our research. In humans, it has been reported that RVSTIs are influenced by some factors during growth and that these influences become

weaker with the increasing age (Habash *et al.*, 2019); thus, it would be interesting to evaluate these parameters also in growing horses.
Eventually, it would be interesting to investigate also the possible effect of breeds, training and athletic discipline performed by horses on PAS and RVSTIs. These last analyses were not performed in this project due to the limited number of subjects and their wide range of breeds and athletic discipline.

In the present study, PAD and PAD/AOD resulted significantly higher in SEA affected horses compared to healthy and MEA subjects. These findings are in accordance with literature that reported higher PAD in SEA affected horses during exacerbation (Johansson *et al.*, 2007). Moreover, it has been reported that a pulmonary artery dilation and a PAD/AOD greater than 1 are probably indicative of PHT (Reef *et al*., 1998; Marr, 2010).
Evaluating possible differences between healthy horses, MEA affected horses and SEA affected horses regarding RVSTIs, significant differences were found. In particular, RVSTIs were significantly lower in SEA group compared to healthy horses and MEA group; moreover, MEA affected horses had a significant lower AT than healthy horses. In literature, a study reported a case of a pony affected by SEA with PHT that presented a very decreased AT (60 msec) and ET (240 msec) compared to normal values (AT: 160 - 270 msec; ET: 450 - 580 msec); moreover, this pony had a AT/ET of 0.25 (Lightowler *et al.*, 2009). Conversely, a study that evaluated RV function in 6 horses affected by SEA, did not find differences between SEA affected horses in remission and control group regarding AT and ET (Decloedt *et al.*, 2017a); however, in our research, SEA affected horses were symptomatic and this could explain the different results. Similarly to the results of the present research, in humans it has been reported a decreased AT in asthmatic patients compared to the controls; although within normal limits, pulmonary artery pressure in these patients was higher than controls (De-Paula *et al.*, 2018; Baysal and Has, 2019) and this may be the reason of decreased AT. In fact, in human medicine, it is demonstrated that AT is inversely correlated to pulmonary pressure measured invasively by right cardiac catheterization (Kitabatake *et al.*, 1983; Görgulü *et al.*, 2003; Celermajer and Playford, 2017; Grapsa and Tzemos, 2017; De-Paula *et al.*, 2018). The same relationship has been reported also in dogs (Uehara, 1993; Schober and Baade, 2006; Visser *et al.*, 2016; Serres *et al.*, 2017; Reinero *et al.*, 2020). Under normal conditions, the peak flow velocity occurs in mid-systole because the pulmonary vascular bed has a high compliance. Conversely, with increased pulmonary pressure, there is a higher impedance to flow due to a decreased distensibility of the pulmonary vascular bed; this high

impedance is more manifested during the earliest phase of the ejection and, therefore, the acceleration time decreases (Schober and Baade, 2006). Moreover, in humans and dogs, it has been reported that also AT/ET is inversely correlated to pulmonary pressure (Kitabatake *et al.*, 1983; Schober and Baade, 2006; Celermajer and Playford, 2017; Serres *et al.*, 2017). In this study a negative correlation of AT and AT/ET with PAD/AOD was detected; since PAD/AOD is considered an indirect marker of pulmonary hypertension (Johansson *et al.*, 2007), these correlations may suggest that AT and AT/ET could be inversely correlated to pulmonary pressure also in horses. However, this hypothesis needs to be verified with further studies comparing AT and AT/ET values with pulmonary pressure measured invasively.

The present study evaluated also possible differences in PAS value between healthy horses and asthmatic horses. Results indicated that SEA affected horses had a significant higher PAS than healthy horses and MEA affected horses. Similarly, in human medicine, it is demonstrated that asthmatic patients have higher PAS values than healthy controls (Baysal and Has, 2019). An increased PAS is reported also in people with other chronic respiratory diseases, such as OSAS and chronic obstructive pulmonary disease (Weir-Mccall *et al.*, 2015; Altiparmak *et al.*, 2016; Ozkececi *et al.*, 2016). As described before (see the beginning of this chapter), this increase in PAS value is due to a loss of compliance of the pulmonary arteries caused by structural modification induced by recurrent hypoxemia and hypercapnia associated with chronic inflammation. These modifications lead to pulmonary hypertension over time; therefore, PAS is used as an early predictor of PHT in humans (Görgulü *et al.*, 2003; Altiparmak *et al.*, 2016; Baysal and Has, 2019).

Equine asthma and human asthma share numerous similarities regarding aetiology, clinical presentation, pathological changes and immunologic response (Leclere *et al.*, 2011; Bullone and Lavoie, 2015; Lange-Consiglio *et al.*, 2019; Bullone and Lavoie, 2020). Like human asthma, equine asthma induces thickening of the pulmonary vessel wall over time, leading to progressive increase of pulmonary vascular resistance and consequently pulmonary hypertension (Decloedt *et al.*, 2017a; Ceriotti *et al.*, 2020). Due to these similarities and the similar difference of PAS value in asthmatic subjects compared to healthy ones, it could be hypothesized that, even in horses, the increased PAS may be due to structural changes of the pulmonary vascular bed and that it may be associated with an increase of pulmonary pressure. This hypothesis is supported by the detection, in our research, of a correlation between PAS and PAD/AOD which is considered an indirect marker of pulmonary hypertension (Johansson *et al.*, 2007). However, further studies

are necessary to confirm this hypothesis comparing PAS values with pulmonary pressure measured invasively by right cardiac catheterization and with histology of lung biopsies. Moreover, PAS value in SEA affected horses ranged widely (7.30 - 14.74 kHz/sec); these could be due to different severity of the disease between subjects or due to a different status of the disease (acute exacerbation, low symptomatic or in remission). Therefore, it could be useful to evaluate in the future possible differences between SEA affected horses in remission and during exacerbation. Finally, since it is demonstrated that pulmonary vascular bed remodeling is reversible after 12 months of antigens avoidance (Ceriotti *et al.*, 2020), it would be interesting to evaluate PAS in SEA affected horses before and after long-term of antigens avoidance to verify if it can turn within normal limits.

Heart rate was significantly higher in horses with SEA than in healthy subjects and horses with MEA. Heart rate was above the normal range in 7 of 15 horses with severe asthma. As reported, no influence of heart rate on PAS and RVSTIs was found in healthy horses; however, as heart rate was within normal limits in these horses, we hypothesized that higher heart rate values, more commonly found in SEA horses, might affect these measurements. Therefore, possible differences between groups were evaluated also for PAS and RVSTIs adjusted for heart rate. The parameters were adjusted for heart rate by dividing them by the square root of RR intervals, as suggested for AT in humans (Chan *et al.,* 1987). A significant difference between SEA affected horses and healthy or MEA subjects was found for PAS and AT as well as for PAS_{HR} and AT_{HR}. These findings may suggest that SEA affected horses had a higher PAS and a lower AT due to the disease rather than to variations in heart rate and tachycardia. Conversely, ET and AT/ET that differed between SEA and other subjects, when adjusted for heart rate resulted not significantly different between groups. These findings suggest that these parameters could be influenced by variation in heart rate and confirm the need of investigating the influence of heart rate on ET and AT/ET in a larger population with a wider heart rate range.

A ROC curve was performed to determine the diagnostic potential of PAS and AT in the evaluation of horses with severe equine asthma. Three cut-off values for each parameter were identified: the point with 100% sensitivity, the point with 100% specificity and the point of best compromise between specificity and sensitivity.

Regarding PAS, a cut-off of 7.28 kHz/sec identified SEA affected horses with 100% sensitivity; in other words, a PAS > 7.28 kHz/sec identified all the affected subjects. However, the specificity of

this cut-off is low (50%) and therefore, it leads to high false positives. In fact, with this cut-off 19/38 of not-SEA affected subjects were erroneously classified as SEA.

Conversely, a cut-off of 9.12 kHz/sec identified not-SEA affected horses with 100% specificity; in other words, a PAS < 9.12 kHz/sec identified all the not severely affected subjects. However, the sensitivity of this cut-off is low (66.67%) and therefore, it has lot of false negative. With this cut-off all the not-SEA affected horses of our sample were identified but only 10/15 (66.67%) of severe asthmatic horses were correctly classified as SEA.

The best compromise between sensitivity (93.33%) and specificity (86.84%) is represented by a cut-off of 8.18 kHz/sec. In our sample, this cut-off classified correctly 14/15 (93.33%) SEA affected horses and 33/38 (86.84%) not-SEA affected horses. It must be highlighted that all the healthy horses had a PAS lower than 8.18 kHz/sec; the 5 subjects erroneously classified as SEA affected horses by this cut-off had mild/moderate equine asthma.

Regarding AT, a cut-off of 0.249 sec identified SEA affected horses with 100% sensitivity; in other words, a AT < 0.249 sec identified all the affected subjects. However, the specificity of this cut-off is very low (21.09%) and therefore, it leads to very high false positives. In fact, with this cut-off 30/38 (78,94%) of not-SEA affected subjects were erroneously classified as SEA.

Conversely, a cut-off of 0.173 sec identified not-SEA affected horses with 100% specificity; in other words, a AT > 0.173 sec identified all the not severely affected subjects. However, the sensitivity of this cut-off is low (46.67%) and therefore, it has lot of false negative. With this cut-off all the not-SEA affected horses of our sample were identified but only 7/15 (46.67%) of severe asthmatic horses were correctly classified as SEA.

The best compromise between sensitivity (86.67%) and specificity (89.47%) is represented by a cut-off of 0.202 sec. In our sample, this cut-off classified correctly 13/15 (86.67%) SEA affected horses and 34/38 (89.47%) not-SEA affected horses. Conversely to the PAS cut-off value, not all the healthy horses had a AT greater than 0.202 sec; in fact, one healthy subject and three horses with mild/moderate equine asthma were erroneously identified as SEA affected horses.

Comparing these results, it seems that PAS is a more sensitive and specific parameter than AT for the diagnosis of SEA.

Additionally, considering together the cut-off of both PAS > 8.18 kHz/sec and AT < 0.202 sec, the specificity increases to 97.37%. In fact, a PAS lower than 8.18 kHz/sec associated to a AT greater than 0.202 sec identified correctly 37/38 of not-SEA affected horses. The only subjects erroneously classified as SEA affected horse had mild/moderate equine asthma. However, the

sensitivity (86.67%) of this association is slightly lower than that of PAS alone (93.33%); in fact, a PAS greater than 8.18 kHz/sec associated to a AT lower than 0.202 sec classified correctly 13/15 SEA affected horses.

Finally, as in humans, PAS could increase also due to other chronic diseases. Therefore, further studies are required to evaluate PAS in horses affected by other diseases, such as left-side heart diseases, pulmonary fibrosis or pneumonia. Furthermore, it would be very interesting to verify the association between PAS and pulmonary pressure comparing PAS values with invasive measurements of pulmonary pressure and, in case, to determine a PAS cut-off for the diagnosis of pulmonary hypertension due to chronic diseases.

Chapter 9

CONCLUSIONS

First of all, this research permitted to introduce a new echocardiographic parameter in the echocardiographic examination of horses, evaluating for the first time PAS in veterinary medicine. In fact, results suggested that PAS can be measured consistently by pulsed-wave Doppler echocardiography across the pulmonary valve from the right parasternal short axis view in horses. Moreover, the technique is quite easy and well tolerated by subjects and can add useful information to the right ventricular systolic time intervals in the evaluation of horses with chronic pulmonary disease.

PAS and RVSTIs were not found to be influenced by age, bodyweight, gender and heart rate in healthy horses.

Moreover, this research detected a significant higher PAS and lower RVSTIs in SEA affected horses compared to healthy subjects and MEA affected ones. In addition, a positive correlation between PAS and PAD/AOD and a negative correlation between AT or AT/ET and PAD/AOD were found. These findings, in association with several similarities between equine asthma and human asthma, suggest that these parameters could be correlated to pulmonary pressure even in horses.

In addition, the study demonstrated that PAS and AT are not influenced by tachycardia in SEA affected horses.

Finally, this study determined that a PAS value of 8.18 kHz/sec and a AT value of 0.202 sec are the best cut-off values, with a very high sensitivity (PAS: 93.33%; AT: 86.67%) and specificity (PAS: 86.84%; AT: 89.47%), for the diagnosis of SEA. The association of the two cut-off has a sensitivity of 86.67% and a specificity of 97.37%.

The present research, in addition to having brought several novelties, opens the door to further studies. In fact, several ideas emerged from this project:

- the correlation of PAS values with histology of lung biopsies findings and with pulmonary pressure measured invasively by right cardiac catheterization could be useful to verify the hypothesis that increased PAS may be due to structural changes of the pulmonary vascular bed and that it may be associated with an increase in pulmonary pressure. In

case these hypotheses will be confirmed, it could be interesting to determine a PAS cut-off for the diagnosis of pulmonary hypertension

- to correlate RVSTIs values with invasively measured pulmonary pressure by cardiac catheterization in order to verify the hypothesis that AT and AT/ET could be inversely correlated to pulmonary pressure in horses
- to determine reference intervals of PAS in healthy horses
- to verify the feasibility of PAS measured by PW Doppler acquired from other nonstandard views and to evaluate possible differences in PAS values obtained from the different views
- to investigate the influence of a wider range of age on PAS and RVSTIs, including elderly subjects and to evaluate these parameters in growing horses
- to evaluate the influence of a wider range of heart rate on PAS and RVSTIs in order to verify if bradycardia and tachycardia can affect PAS value
- to verify the possible effect of breeds, training and athletic discipline performed by horses on PAS and RVSTIs
- to investigate possible differences in PAS values between SEA affected horses in remission and during exacerbation or before and after long-term of antigen avoidance
- to evaluate PAS in horses affected by other chronic diseases, such as left-side heart diseases and pulmonary fibrosis.

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