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Determination of Matrix Metalloproteinases and Interleukin-8 in Bronchoalveolar Lavage Fluid in Horses with Different Lung Diseases

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This thesis dedicated to

The soul of my parents, to my wife “Heba” and my daughters “Jana and Ellen”

Content

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Abbreviations

%	Percent
↑	Increase
↓	Decrease
°C	Degree centigrade
µmol/l	Micromole per liter
$AaDo_2$	Arterial oxygen difference
APMA	4-aminophenylmercuric acetate
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BM	Basement membrane
cm H_2O	Centimeter of water
CMT-3	Chemically modified tetracyclines
COPD	Chronic obstructive pulmonary disease
ECM	Extracellular matrix
EDTA	Ethylene diamine tetracetic acid
ELISAs	Enzyme-linked immunosorbent assays
et al.	et alii "and others"
etc.	et cetera "and other things"
g	Gram
i.v.	Intravenous
IgE	Immunoglobulin E
IL	Interleukins
kDa	Kilodalton
Kg	Kilo gram
L	Liter
Max.	Maximum
Min.	Minimum
mM	Millimolar
mmHg	Millimeters of mercury

MMPs	Matrix metalloproteinases
MT-MMPs	Membrane type matrix metalloproteinases
$PaCO_2$	Partial pressure of arterial carbon dioxide
PaO_2	Partial pressure of arterial oxygen
PBS	Phosphate buffered saline
pH	Power of hydrogen
RAO	Recurrent airway obstruction
RFU	Relative fluorescence units
rpm	Rotations per minute
SD	Standard deviation
SPAOPD	Summer pasture associated obstructive pulmonary disease
TBS	Tracheobronchial secretions
TELF	Tracheal epithelial lining fluids
Th-1 and Th-2	Sub populations of T helper cells
TIMPs	Tissue inhibitors of metalloproteinases
Tris	Tris hydroxy methyl amino methan
ΔPpl_{max}	Maximal change in the interpleural pressure
$\bar{x}$	Mean

1 Introduction

Recurrent airway obstruction (RAO) is a common inflammatory/allergic condition that affects many horses worldwide, especially in the most northern countries and usually affects middle-aged to older horses. The disease is characterized by a reversible bronchoconstriction with alternation of remission and exacerbation phases. Signs during clinical exacerbation of RAO are more evident when the horse is exposed to a dusty environment and is usually accompanied by bronchospasm, mucous accumulation (Leguillette, 2003, Lowell, 1990), chronic cough, exercise intolerance, labored expiratory effort and nasal discharges (Robinson et al., 1996). Chronic interstitial pneumopathy is a chronic disease characterized by marked clinical signs including cough, weight loss, nasal discharge, exercise intolerance and severe dyspnea. It is associated with marked interstitial lesion indentified as opacity on thoracic radiographs, (Mair, 2007). Acute to subacute respiratory infection is occurring usually after bacterial or viral infection and parasitic infestation. The radiographic image showed numerous small circular opacities represent thickening of the large and medium sized airways (Ainsworth and Hackett, 2004). The basement membrane and the extracellular matrix (ECM), play a role in the maintenance of normal lung function.

In normal lung, ECM provides stability and elasticity during the inspiration and expiration processes while during a disease process the ECM is disrupted leading to aberrant remodeling of lung architecture (Stamenkovic, 2003), which is a hallmark of many lung diseases (Atkinson and Senior, 2003). The degradation of the ECM is facilitated by matrix metalloproteinases (MMPs) and is controlled by the tissue inhibitors of metalloproteinases (TIMPs), so that an imbalance between MMPs and TIMPs results in pathological consequences (Clutterbuck et al., 2010).

Matrix metalloproteinases have the capacity to cleave the structural proteins of the ECM such as collagens and elastin. They are secreted by inflammatory cells and have the enzymatic capacity to cause morphological changes in the lungs (Srivastava et al., 2007). The catalytic activity of MMPs participates in the lung homeostasis and repair (Parks and Shapiro, 2001). However, overexpression of MMPs may cause tissue damage associated with lung inflammation and

disease (Parks and Shapiro, 2001). Additionally, the role of TIMPs in parallel with the MMPs should not be neglected.

Subsequently, the evaluation of MMPs in the respiratory secretions of the equine lung is of great diagnostic and therapeutic value, particularly in RAO-affected horses in remission. The increased concentration of MMPs have been found in bronchoalveolar lavage fluid (BALF) samples of human patients with chronic obstructive pulmonary disease (COPD) compared to controls (Srivastava et al., 2007). Furthermore, understanding the role of inflammatory markers in the inflammation cascade might allow the development of novel pharmacological agents for the treatment of inflammatory lung diseases. In addition, interleukins (IL), especially IL-8, play a major role in the pathophysiology of RAO as they promote neutrophils recruitment and development of airway remodeling (Franchini et al., 1998, Lasky and Brody, 1997, Franchini et al., 2000).

The main objectives of the present study were:

1. To compare the results of clinical examination, endoscopy, radiography, BALF and tracheobronchial secretions (TBS) cytology, lung function, concentration and activity of MMP-2, MMP-8, MMP-9 and IL-8 in BALF of healthy horses and horses with RAO either in exacerbation or remission, chronic interstitial pneumopathy and acute to subacute respiratory infection.
2. To find out the correlation of the clinical examination score with MMP-2, MMP-9 concentration and percentage of neutrophils in healthy horses and diseased horses, with reference to other correlations found between the other examined parameters.
3. To validate a new quantitative method for measuring the concentration of MMP-2 and MMP-9 in healthy horses and those with lung diseases using enzyme linked immunosorbent assays (ELISAs).
4. To validate a new quantitative method for measuring the MMP-8 collagenolytic activity in healthy horses and those with lung diseases using a flourimetric assay.
5. To measure the gelatinolytic activity of MMP-2 and MMP-9 in healthy horses and diseased horses using a semi-quantitative densitometry method after gelatin zymography.

2 Review of literatures

2.1 Recurrent airway obstruction

2.1.1 General aspects and synonyms of RAO

Recurrent Airway Obstruction is defined as a reversible and an exhausting respiratory disease of horses (Moran and Folch, 2011), which markedly contributes to loss of performance in racehorses (Pearson et al., 2007). It is commonly seen in stabled horses fed hay where it is improved by housing affected horses in a dust-free stable (Lavoie, 2007). It is characterized by small airway inflammation, airway wall thickening, bronchospasm, airway neutrophilia and mucus overproduction (Tahon et al., 2009, Robinson et al., 1996).

Numerous terminologies were described by Lavoie (2007) according to clinical presentation, for example “heaves, broken-wind, chronic obstructive pulmonary disease (COPD) and RAO”, or suggested etiologies like “allergic airway diseases and hay sickness” and lung pathology like “emphysema, chronic bronchiolitis and small airway disease”. The term COPD was introduced by Sasse (1971) to describe airway inflammation and obstruction, neutrophils and mucus accumulation in the airways of mature horses with the absence of active infection.

Equine disease and human COPD are quite different. Human COPD is a progressive non-reversible disease related to smoking. In contrast, the equine disease is characterized by reversible airway narrowing due to bronchospasm and therefore it is more closely resembles human asthma, in which phases of exacerbation and remission are common. The international workshop on equine chronic airway disease, Michigan State University reported that it is no longer appropriate to use the term COPD to describe horses with airway inflammation (Robinson, 2001). Thus, heaves or recurrent airway obstruction was used to describe mature horse with airway obstruction that is reversed by a change in the environment or use of bronchodilators.

A similar clinical presentation is observed in some horses when pastured; the condition is reversible by housing affected horses in a dust-free stable. This syndrome is called summer pasture associated obstructive pulmonary disease (SPAOPD) and occurs mainly in Northern

America and shares many clinical and pathological similarities with RAO (Bowles et al., 2002, Mair, 1996).

2.1.2 Incidence

RAO is a frequent disease in stabled horses and mostly in show and jumping horses (Cunningham and Dunkel, 2008, McPherson et al., 1979b). It is commonly affects middle-aged and older horses, the older a horse, the more likely it is to become affected (Allen and Franklin, 2007, Davis and Rush, 2002, Horohov et al., 2005). It is uncommon in young horses and there is no particular breed or sex predisposition. It is reported to be more common in the most northern regions, where horses are fed hay produced during humid summer weather, in Switzerland, the RAO incidence was 54% (Bracher et al., 1991). Exposure to hay and respiratory infection in early life may also be involved in the development of this disease (Hotchkiss et al., 2007).

2.1.3 Etiology

Numerous factors influence the occurrence of the disease and lead to comparable symptoms. Exposure to hay and straw is the most common cause of RAO especially when horses are stabled during the winter season (Mair and Derksen, 2000). Over 50 species of moulds and different potential allergens have been identified in stable air that was primarily originated from hay and straw (Woods et al., 1993). *Aspergillus fumigatus, Faeniarectivirgula* and *Thermoactinomyces vulgaris* are potentially important allergens involved in the pathogenesis of RAO (McPherson et al., 1979a, McGorum et al., 1993b).

The hypothesis for the development of RAO is based commonly on an allergic reaction to inhaled moulds (Bowles et al., 2002), where predisposed horses mount an antigen-specific inflammatory response (hypersensitivity reaction I and III) to components of environmental dust and a non-specific inflammatory response to inhaled pro-inflammatory agents present in the breathing zone of stabled horses including moulds, endotoxins, particulates and noxious gases (Lavoie, 2007). Additionally, climatic changes influence the symptoms and the course of chronic pulmonary diseases. Viral influenza combined with bad management resulted in the development of RAO (Gerber, 1973).

2.1.4 Pathogenesis and the immunological basis of RAO

Airway obstruction, inflammation, mucous accumulation and tissue remodeling are the main functional changes occurring in RAO-affected horses (Lavoie, 2007). The immunological mechanisms of pulmonary inflammation in RAO are not fully understood. A hypersensitivity component is involved in the pathogenesis and development of RAO (Pirie, 2014, Robinson et al., 1996). Hypersensitivity reactions include immediate type "type I hypersensitivity", immune complex-mediated late type reactions "type III hypersensitivity reactions" and cell-mediated delayed type reaction "type IV hypersensitivity reactions" (Tahon et al., 2009, Robinson, 2001).

Type-I hypersensitivity reaction is an immediate type reaction (Tahon et al., 2009) which is initiated within minutes of inhalation of allergens that resulted in airway inflammation where the immune response starts with the activation of pulmonary mast cells resulted in inflammatory cells recruitment with an increased expression of T helper cells (Th), cytokines and chemokines (Moran and Folch, 2011, Lavoie, 2007, Woods et al., 1993).

Type-III hypersensitivity reaction is an immune complex-mediated late type reactions to inhaled allergens resulted from local pulmonary deposition of immune complexes and the activation of the complement system. This reaction requires pre-sensitization by the presence of precipitating antibodies against antigen in the serum of the affected horses (Tahon et al., 2009, Moran and Folch, 2011, Derksen et al., 1988). The late-phase response occurs after 6-9 h due to the release of inflammatory mediators including IgE (Horohov et al., 2005), cytokines and chemokines from Th-2 cells (Lavoie, 2007). Neutrophils are recruited to the lungs and accumulate in the airway lumen within 4 hours of exposure to irritants resulting in bronchospasm (Horohov et al., 2005, Fairbairn et al., 1993). Increased number of cells expressing mRNA for IL-4 and IL-5 are consistent with a predominant Th-2 cell response (Lavoie et al., 2001).

Type-4 hypersensitivity reaction is a cell-mediated delayed type reaction occur after 24 hours, where cellular elements predominate including Th-1 and Th-2 (Pirie, 2014). The Th-l cells synthesize IL-2 and interferon gamma, whereas the Th-2 lymphocytes are responsible for hypersensitivity reactions and synthesis of IL-4, IL-5 and IL-10 (Lasky and Brody, 1997).

Generally, the inflammatory changes in the wall of airways include development of edema and airway remodeling which involves mucus metaplasia, smooth muscle hypertrophy and fibrosis (Allen and Franklin, 2007), in addition to increased levels of histamine in BALF (Robinson et al., 1996). Removal of hay and straw resulted in switching to disease remission (Pirie, 2014).

2.1.5 Pathophysiology of RAO in horses

2.1.5.1 Airway inflammation and mucus secretion

Mucus accumulation and neutrophilic airway inflammation are characteristics for RAO in horses (Robinson et al., 1996, Robinson et al., 2003). Airway inflammation results in mucus hypersecretion, ciliary dysfunction and changes in the composition and properties of airway secretions (Robinson, 2001). In RAO-affected horses in remission, mucus accumulated in the airways resulting in lung function defects (Gerber et al., 2004), with goblet cell hyperplasia and submucosal gland hypertrophy (Marchette et al., 1985, Davis and Rush, 2002).

The cells present within the lung in the respiratory secretions include inflammatory cells (neutrophils, lymphocytes, macrophages and mast cells), epithelial cells and mucus-producing cells (Lavoie, 2007). An increased amount of stored mucins contributes to the modulation of inflammatory lung disease (Lugo et al., 2006).

2.1.5.2 Airway obstruction

Bronchospasm is a key feature in RAO horses where numerous inflammatory mediators have been found in the BALF of affected horses including histamine, serotonin, leukotrienes and thromboxane are responsible for the bronchospasm (Franchini et al., 1998, McGorum et al., 1993a). During acute exacerbation of RAO, airways become obstructed as a result of bronchospasm and mucus accumulation resulting in a breathing pattern characterized by a high peak breaths at the start of inspiration rapidly diminished later on (Robinson et al., 1996).

2.1.6 Diagnosis of RAO

2.1.6.1 Clinical signs

Clinical signs of RAO vary from exercise intolerance, coughing to dyspnea at rest (Davis and Rush, 2002). Features of chronic respiratory disease may be obvious including lower airway inflammation, bronchoconstriction, mucus accumulation (Leguillette, 2003), airway neutrophilia and airway obstruction (Pirie et al., 2002). The severity of the clinical disease worsens with time unless prevented by treatment and/or disease management, additionally the clinical signs are more obvious during stabling particularly when new batches of hay or straw are introduced (Mair and Derksen, 2000). Based on this, RAO-horses show alternations of signs of remission and exacerbation as follows:

2.1.6.1.1 Remission phase of RAO

RAO-affected horses in remission are clinically normal at rest with normal breathing pattern and may show few or no apparent clinical signs, but develop increasing expiratory dyspnea within several hours of exposure to allergens (McGorum and Pirie, 2008). Such cases may demonstrate mild clinical signs of coughing and nasal discharge at exercise, with exercise intolerance in racing horses (Gerber, 1973). There is an increased amount of tracheal mucus and an increased percentage of neutrophils in the TBS (Laumen et al., 2010).

2.1.6.1.2 Exacerbation phase of RAO

RAO-horses in clinical exacerbation show signs including non-productive coughing, serous or mucopurulent nasal discharge and labored expiratory effort (McGorum and Pirie, 2008, Laumen et al., 2010, Horohov et al., 2005). Exercise intolerance and flaring of nostrils may be evident (Moran and Folch, 2011). Contraction of the abdominal muscles at the end of exhalation may be present (Robinson et al., 2000). Acute severe bouts of disease occur, resulting in severe dyspnea, tachypnea, protrusion of the anus with each expiratory effort, an anxious facial expression and paroxysmal coughing (Mair and Derksen, 2000).

Auscultation of the lungs of the affected horses often reveals abnormal respiratory sounds, described as crackles and wheezes, throughout the area of the lung field or distal cervical trachea (McGorum and Pirie, 2008), abnormal breathing pattern (Laumen et al., 2010). These clinical signs are secondary to an inflammatory response that results in bronchospasm, excessive mucus production and airway obstruction. The inflammatory response is characterized by the presence of excessive mucus and inflammatory cells, primarily neutrophils, in the small airways (Horohov et al., 2005). Inflammation is accompanied by airway obstruction, which resulted from bronchospasm and obstruction of airways by mucoid exudates (Robinson et al., 1996) and reversible bronchoconstriction may occur (Moran et al., 2011). Bronchoalveolar lavage (BAL) cytology is characterized by neutrophilia (15%-85%) (Davis and Rush, 2002) that develops within a few hours after exposure to hay dusts (Fairbairn et al., 1993).

Horses with severe RAO show clinical signs even at rest, a frequent, deep cough which was explosive and paroxysmal was observed. Persistent or intermittent bilateral nasal discharge varies in nature from mucoid, mucopurulent to purulent. Obstruction of the small airways results in expiratory dyspnea and increased expiratory effort. Biphasic expiratory effort results in a double expiratory sounds, which in long-standing and severe cases can cause hypertrophy of the abdominal musculature and the development of a heave line (McPherson et al., 1978). Endoscopy reveals evidence of lower airway inflammation and cytological examination of TBS and BALF reveals severe neutrophilia (Mair, 1996).

2.1.6.2 Endoscopy

Endoscopic examination of the respiratory tract allows for direct visualization of the nasal passages, pharynx, larynx, guttural pouches, trachea and bronchi (Roy and Lavoie, 2003). It was used for grading of RAO using endoscopy score where the healthy horses had no tracheal mucus accumulation in endoscopy (Gehlen et al., 2008). Additionally, it reflects the lower airway inflammation measured by BALF cytology (Koblinger et al., 2013, Koblinger et al., 2011), mucus accumulation scoring which is a reliable clinical tool for diagnosing respiratory diseases of the horse as it was strongly correlated with neutrophilic airway inflammation evidenced by the percentage of neutrophils in the tracheal wash (Gerber et al., 2004, Laus et al., 2009). The evidence of lower airway inflammation was characterized by excessive respiratory secretions in

the distal trachea, congestion of the tracheal and bronchial mucosa and finally the tracheal bifurcation showed swelling and blunting (Mair, 1996).

Gerber et al. (2004) scored the mucus in the lower airways of horses according to mucus accumulation (clean, multiple small blobs, larger blobs, confluent stream-forming, pool-forming and extreme profuse amounts), localization (ventral, lateral, dorsal and threading), viscosity (very fluid, fluid, intermediate, viscous and very viscous) and color (yellow, white and colorless).

2.1.6.3 Pulmonary function testing

Pulmonary function tests in horses are helpful in the diagnosis of early stages of RAO development and are valuable methods for detection of respiratory insufficiency. Blood gas analysis includes arterial oxygen partial pressure (P_aO_2), arterial carbon dioxide partial pressure (P_aCO_2) and arterial oxygen difference ($AaDO_2$), while, the maximal change in interpleural pressure (ΔPpl_{max}) could be used to a greater extent under field conditions (Willoughby and McDonell, 1979, Herholz et al., 2002).

The interpleural pressure is one of the most important parameters of lung function tests in horses (Sasse, 1971). In SPAOPD, the evaluation of clinical score and ΔPp_{lmax} provided a valid estimate of severity of the disease and serves as an indicator of response to treatment (Costa et al., 2000). Horses with RAO have an increased resistance and dynamic elastance which resulted in an increase in the ΔPpl_{max} more than 15mmHg (Robinson et al., 2000, Nyman et al., 1991, Robinson et al., 2003).

2.1.6.4 Cytology of the respiratory secretions in RAO horses

The cytological examination of respiratory secretions is very important and commonly used in the diagnosis of respiratory disease affecting performance (Richard et al., 2010). It is indicated when the clinical signs of pulmonary disease are unclear to confirm the presence of the pulmonary disease and to detect the type of disease present either subclinical pulmonary disease or chronic pulmonary disease particularly RAO and lungworm infection (Dixon, 1995, Beech, 1975, Moran and Folch, 2011). The respiratory secretions include TBS and BALF.

TBS cytology does not correlate as well with pulmonary pathology as does BALF cytology (Dixon, 1995). Since RAO is associated with diffuse lung pathology, the cytological findings of BALF samples from different lung segments show good correlation (McGorum et al., 1993b). Therefore, BALF cytology is recommended for the diagnosis of RAO (Robinson, 2001, McKane et al., 1993).

In normal horses, the most predominant cell populations in TBS and BALF are macrophages and lymphocytes, while the proportions of other cell types are negligible. Macrophage percentage in TBS is about 45% and that in BALF is about 60%. Lymphocyte percentage in TBS is about 5% and that in BALF is about 35%. Neutrophils percentage in TBS is <20% and that in BALF is <5% (Allen and Franklin, 2007).

Cytology of RAO-affected horses in exacerbation is characterized by airway neutrophilia (more than 25% neutrophils in BALF) (Robinson, 2001), in addition to large amounts of mucus and increased numbers of exfoliated epithelial cells (Lavoie, 2007). Neutrophil counts in TBS poorly correlates with that of BALF in RAO (Derksen et al., 1989).

Curschmann's spirals, which are coils of inspissated mucus fibrin, may be observed in TBS due to the presence of fungal elements thus suggesting impaired mucociliary clearance and exposure to high levels of airborne organic dust (Tanner et al., 1998). RAO-affected horses rarely have increased numbers of eosinophils in their airway secretions (Vrins et al., 1991). The presence of bacteria in TBS and/or BALF from RAO-affected horses in the absence of clinical signs of bacterial infection (fever, anorexia, depression) suggests secondary colonization of the large airways and reduced mucociliary clearance, rather than primary bacterial infection (Darien et al., 1990).

A higher proportion of non-degenerated segmented neutrophils (more than 20% and in severe cases 60-85%) was seen in RAO-affected horses compared to that obtained from healthy horses (Hoffman, 2008). A higher frequency of lymphocyte apoptosis is a characteristic feature in RAO-remission horses once the allergen source is removed (Moran et al., 2011).

2.1.6.5 Thoracic radiography

Radiography is a helpful diagnostic tool for equine respiratory diseases (O'Brien and Biller, 1997). It may be useful to help rule out other types of lung disease (Leguillette, 2003, Robinson, 2001). There is a correlation between radiographic alterations and the degree, severity and duration of the clinical signs of RAO (Farrow, 1981, Bakos, 2008). The radiographic findings in RAO-affected horses ranged from interstitial pattern with equal distribution in the entire lung; bronchial radiopacity and the absence of hilar opacity as occurs with lymphadenopathy and acute pneumonia; bronchial and tracheal thickening. In addition, the loss of thoracic detail and the degree of opacification of the lung field was considered an indication of the severity of the disease (Sande and Tucker, 2004, Tilley et al., 2012).

2.2 Other respiratory diseases of horses included during the study

2.2.1 Chronic interstitial pneumopathy

It is usually diagnosed as chronic pulmonary disease and is associated with marked interstitial opacity on thoracic radiographs, characterized by a marked clinical signs of respiratory disease for a long period. It is occurring even in foals less than 6 months and improved after corticosteroid therapy (Nout et al., 2002) and may be a sporadic disease of adult horses (Buergelt et al., 1986).

This condition may show signs similar to RAO (Donaldson et al., 1998) and defines a number of diseases that are chronic and progress into pulmonary fibrosis (Wilkins, 2003a). Multiple agents may be implicated in causing chronic interstitial pneumonia including hypersensitivity pneumonitis and viral infections (McChesney, 1975, Winder et al., 1988).

Horses affected with chronic interstitial pneumonia frequently present with cough, weight loss, nasal discharge, exercise intolerance and severe dyspnea. A heave line is frequently present, nostril flare and anxious expression is usual. BALF is characterized by high cellularity (Derksen et al., 1982). Typically, thoracic radiographs reveal extensive interstitial and bronchial patterns (Wilkins, 2003b, Turk et al., 1981), interstitial infiltration with discrete and diffuse nodularity

(Wilkins, 2003a, Buergelt et al., 1986). Treatment with corticosteroids and non-steroidal anti-inflammatory drugs should be attempted in horses with acute or subacute interstitial pneumonia based on lung biopsy (Mair, 2007).

2.2.2 Acute to subacute respiratory infection

Acute to subacute respiratory infection is occurring usually after bacterial or viral infection and parasitic infestation. Horses may be affected by a variety of respiratory viruses and bacteria which were considered as a cause of airway inflammation (Leguillette, 2003). Severe viral infections are accompanied by secondary bacterial infection. TBS and BALF findings are not significantly different in secondary bacterial pneumonia and primary bacterial pneumonia, which consists of increased numbers of neutrophils (Gross et al., 1998). Bacteria may be either pathogens or a contaminant from the upper airways or the environment, it may be found intracellularly and extracellularly (Laus et al., 2009). On the other hand, eosinophilic bronchitis was seen in horses due to the infestation with *Dictyocaulus arnfieldi,* where the clinical signs observed in this condition were moist cough, large numbers of eosinophils in tracheal secretion (MacKay and Urquhart, 1979). The radiographic image of equine bronchitis and bronchiolitis showed numerous small circular opacities represent thickening of the large and medium sized airways (Ainsworth and Hackett, 2004).

2.3 Extracellular matrix

The extracellular matrix represents the scaffold that supports the alveolar wall and has a major impact on lung architecture, homeostasis and function, where it surrounds the conducting airways, alveolar cells and the vascular system and holds cells together. It also has a major function as a reservoir for cytokines and growth factors (Churg et al., 2012), fundamental to cell-cell signaling (Tayebjee and Lip, 2007).

The pulmonary ECM is subjected to a continuous turnover. Thus a dynamic equilibrium between synthesis and degradation of the ECM is maintained for physiological balance. This balance is controlled by synthesis and deposition of ECM components especially collagen, proteolytic degradation of ECM by matrix metalloproteinases (MMPs) and inhibition of MMP activity by

specific tissue inhibitors of matrix metalloproteinases (TIMPs) (Clutterbuck et al., 2010, Lu et al., 2011, Tayebjee and Lip, 2007). Disruption of the normal ECM during a disease process can lead to an inflammatory response that exacerbates aberrant lung remodeling (Frantz et al., 2010, Chapman, 2004).

2.3.1 The ECM composition

The ECM is composed of a fiber net filled with different macromolecules and their regulatory factors (Pelosi et al., 2007, Godfrey, 2009). For proper gas exchange, the components of the extracellular matrix are distributed in a fashion to reduce the boundary between erythrocytes and oxygen (Clark et al., 1983).

Two main classes of macromolecules found in the ECM are proteoglycans and fibrous proteins (Schaefer and Schaefer, 2010). The main ECM fibrous proteins are collagens, elastins, fibronectins and laminins (Godfrey, 2009). Proteoglycans fill the majority of the extracellular interstitial space in the form of a hydrated polysaccharide gel (Holgate, 2009, Jarvelainen et al., 2009). Within this complex network are signal receptor proteins and enzymes that control the turnover of this highly complex system (Tayebjee and Lip, 2007).

2.3.1.1 Collagen

Collagen is the most abundant fibrous protein within the ECM and constitutes up to 30% of the total protein mass it provides the tensile strength of the alveolar tissue. There are more than 20 different collagen types (Godfrey, 2009). It consists of three α-chains, which form a rope-like triple helical structure (Ricard-Blum and Ruggiero, 2005). The main collagen fibers are fibrillar (I, II and III) and non fibrillar (IV, V and VI) (Pelosi et al., 2007).

The most dominant types of collagen in the lung are collagen I and III, which provide the structural framework for the alveolar wall. Type IV collagen is the most important component of the basement membrane (BM), where it serves as a scaffold for binding laminin and proteoglycans (Leblond and Inoue, 1989). Collagen type I and III are located in the alveolar, bronchial and vascular walls; type II in bronchial and tracheal cartilage; type IV and V in the

basement membrane (Godfrey, 2009). The interstitium of the lung parenchyma contains mostly types I and III collagens which provide the structural framework for the alveolar wall (Suki and Bates, 2008).

2.3.1.2 Elastin

Elastic fibers represent another constituent of the ECM and contain elastin and microfibrils (Suki and Bates, 2008). Elastin is an insoluble molecule highly resistant to proteolysis. Its ability to recoil after each cycle of expansion and contraction restores the parenchyma to its previous configuration after the stimulus for inspiration has ceased (Pelosi et al., 2007). It is localized in the alveolar septa, pleura, conducting airways and vascular tissues (Godfrey, 2009). Degradation and improper repair of elastin are critical steps in the development of pulmonary emphysema and lung dysfunction (Janoff, 1985). Among matrix metalloproteinases, MMP-2, MMP-9 (Senior et al., 1991), macrophage metalloelastase (MMP-12) (Senior et al., 1989), MMP-3, MMP-10 (Murphy et al., 1991) and MMP-7 have the ability to degrade elastin.

2.3.1.3 Glycosaminoglycans and proteoglycans

Proteoglycans form a gelatinous and hydrated substance embedding the fibrous proteins (collagen and elastin); it consists of a central protein bound to one or more polysaccharides denominated glycosaminoglycans. They are located mainly in the alveolar walls and basement membrane (Godfrey, 2009).

2.4 Airway remodeling

Airway remodeling is the process that causes the patient to become largely resistant to medication and is an important factor in the development of irreversible airway conditions (Wegmann, 2008, Jeffery et al., 2000). As it results in continued disruption and modification of structural cells and tissues leading to the development of a new airway wall structure and subsequently new function (Holgate, 2009). In RAO-affected horses, remodeling results in decreasing airway lumen, increased smooth muscle mass, peribronchial fibrosis, epithelial cell hyperplasia and impaired airway function (Lavoie, 2007, Lugo et al., 2006). Regulation of

remodeling is essential for developing new therapeutics and disease management (Lu et al., 2011).

The balance between the rate of formation and breakdown of ECM is regulated by MMPs that can digest all ECM components (Tayebjee and Lip, 2007, O'Connor and FitzGerald, 1994, Stamenkovic, 2003), where it allows for normal tissue repair or contribute to pathological tissue destruction when expressed in excess (McCawley and Matrisian, 2001). Under pathological conditions, a high level of degradation may take place leading to a fibrosis and thus accelerating the remodeling process (Everts and Buttle, 2008).

2.5 Matrix metalloproteinases

Matrix metalloproteinases were first described by Gross and Lapiere (1962), who studied the degradation of triple-helical collagen during metamorphosis of a tadpole-tail. MMPs are a growing family of calcium- and zinc-dependent endopeptidases excreted by many cell types. They are biomarkers for physiological and pathological tissue remodeling in the lung, where they are responsible for the degradation of the ECM components including basement membrane collagen, interstitial collagen, fibronectin and various proteoglycans in physiological and pathological processes (Asawakarn and Asawakarn, 2012, Kupai et al., 2010, Ohbayashi, 2002, Stamenkovic, 2003). It has been suggested that MMPs can either protect against or contribute to pathology in inflammatory processes of the lung (Manicone and McGuire, 2008). Most MMPs are secreted as latent pro-enzymes and need to be activated by proteolytic conversion (Gueders et al., 2006).

2.5.1 Physiological and pathological importance of MMPs

2.5.1.1 Physiological importance

MMPs contribute to the homeostasis of many tissues due to the broad spectrum of their substrate specificity (Löffek et al., 2011). They are associated with various physiological processes such as morphogenesis, angiogenesis and tissue repair (Kupai et al., 2010). They act as regulators of the ECM composition and facilitate cell migration by removing barriers such as collagen. Also, they

are implicated in functional regulation of non-ECM molecules that include growth factors and their receptors, cytokines and chemokines, cell surface proteoglycans and variety of enzymes. It also plays an important role in physiological conditions such as normal development (Stamenkovic, 2003).

2.5.1.2 Pathological importance

In human medicine, excessive expression of MMPs may contribute to the pathogenesis of lung diseases such as lung cancer, bronchial asthma, chronic obstructive pulmonary disease, acute lung injury, pulmonary hypertension and interstitial lung disease (Ohbayashi, 2002). Increased expression of MMPs is seen in almost every tissue in which inflammation is present, regulating barrier function, inflammatory cytokine and chemokine activity (Manicone and McGuire, 2008).

Degradation of BM and ECM by MMPs results in destruction of interstitial collagen and release of degraded collagen fragments into the lower respiratory tract, which results in neutrophils influx with the production of chemoattractants by alveolar macrophages (Gaggar et al., 2008). Collagen peptides may stimulate alveolar macrophages to produce chemotactic factors for neutrophils and this mechanism may play a role in the accumulation of phagocytic cells in the lung following injury (Riley et al., 1988).

Moreover, due to the non-matrix related intra- and extracellular targets of MMPs, dysregulation of MMP activity has been implicated in a number of acute and chronic pathological processes such as arthritis, acute myocardial infarction, chronic heart failure, chronic obstructive pulmonary disease and inflammation (Kupai et al., 2010).

Over production of MMPs, especially MMP-2 and MMP-9, is associated with tissue destruction and erosion of cartilage in equine osteoarthritis (Asawakarn and Asawakarn, 2012). There is a close association of lung disease with the destructive effects of MMP-2 and MMP-9 on the basement membrane in early alveolar remodeling. The interaction of MMPs with chemical mediators and inflammatory cytokines has also been reported (Ohbayashi, 2002).

2.5.2 Structure and subfamilies of MMPs

More than 20 MMPs were identified in the degradation of the ECM. They have been classified according to their substrate specificity into collagenases (MMP-1, MMP-8 and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10 and MMP-11), matrilysin (MMP-7), macrophage metalloelastase (MMP-12) and membrane type MMP (MMP-14, MMP-15, MMP-16 and MMP-17) (McCawley and Matrisian, 2001, Srivastava et al., 2007) and other MMPs.

The determination of MMP structure is of critical importance in order to understand their substrate preferences, their association with matrix components and inhibitors. The enzyme itself is divided into several domains including N-terminal propeptide, catalytic domain, hinge region and C-terminal hemopexin domain (Liacini et al., 2002). The pro-domain of a typical MMP is about 80 amino acids, the catalytic domain contains an active site (Zn^{+2}) that binds three conserved histidines containing conserved methionine to the carboxy side of the zinc binding site. In the inactive state, the conserved cysteine in the pro-domain binds to the catalytic zinc ion and disruption of this bond leads to enzyme activation (Parks and Shapiro, 2001).

2.5.2.1 Collagenases

Collagenase activity has been detected in lung tissue affected by non-infectious diseases such as fibrosis, allergic alveolitis and chronic lung inflammation. Most of the collagenase activity in pulmonary destructive diseases is of the neutrophil type (MMP-8) (Sepper et al., 1995) but some is derived from fibroblasts, monocytes and macrophages (MMP-1) (Koivunen et al., 1997a). The increased collagenase activity in BALF and tracheal epithelial lining fluid (TELF) indicates active ongoing disease and may reflect lung tissue changes in RAO-affected horses (Raulo et al., 2001b).

2.5.2.1.1 Matrix metalloproteinase-1

It is described as an interstitial collagenase, which has a vital role in the ECM remodeling during inflammatory, and angiogenic processes. Intracellular MMP-1 accumulates during the mitotic

phase of the cell cycle and this suggests that MMP-1 have a role in cell growth (Limb et al., 2005). It is able to cleave the fibrillar collagens (types I and III) and the ECM molecules accumulated in pulmonary fibrosis (Pardo et al., 2008). Moreover, it is implicated in diseases that are characterized by excessive ECM degradation such as lung emphysema (Imai et al., 2001). MMP-1 was increased in TELF of horses with RAO with subsequent increase in the degradation of type-I collagen (Koivunen et al., 1997a). The latent form of MMP-1 is expressed in a 52-kDa and converted to active form of 42-kDa (Kim et al., 2004).

2.5.2.1.2 Matrix metalloproteinase-8

It represents the second collagenase that is synthesized in the polymorphonuclear neutrophils, so it is called a neutrophil derived collagenase. Its expression has been detected in ciliated bronchial epithelial cells, glandular cells and monocyte-macrophage-like cells in human lung tissue (Prikk et al., 2001). MMP-8 was increased in TELF of horses with RAO-affected horses (Koivunen et al., 1997a). Immunoreactivity of MMP-8 was significantly increased in TELF of horses with COPD, compared to healthy horses and was positively correlated with the amount of degradation of type-I collagen (Raulo et al., 2001b). Pro-MMP-8 PMN-type was present as bands corresponding to 75-kDa and active form corresponding to 65-kDa, and pro-MMP-8 non-PMN-type as 55-kDa and the active form as 45-kDa (Kivela-Rajamaki et al., 2003). Collagenase activity especially MMP-8 and MMP-13 was approximately 7 times higher in BALF and TELF samples obtained from RAO-horses compared to control horses and also during stabling compared to horses being maintained on summer pasture (Raulo et al., 2001b).

2.5.2.1.3 Matrix metalloproteinase-13

Like MMP-8, immunoreactivity of MMP-13 was significantly increased in TELF of horses with RAO, compared to healthy horses and a positive correlation with the amount of type-I collagen degradation was detected. Macrophages and epithelial cells were the major cellular sources of MMP-13 (Raulo et al., 2001b). MMP-13 is secreted in a latent form (60-kDa) (Freije et al., 1994) and the active form have a molecular weight of 50-kDa and 48-kDa (Knauper et al., 1996). It is able to hydrolyze fibronectin, recombinant fibronectin fragments and type IV, IX, X, and XIV

collagens, also it has a powerful collagenolytic activity, while it is five or six times less efficient to hydrolyze type I or III collagen (Knauper et al., 1997).

2.5.2.2 Gelatinases

The gelatinolytic MMPs include gelatinase A (MMP-2) and gelatinase B (MMP-9) which are secreted as inactive forms and are capable of destroying the basement membrane and most ECM components after their activation (Sorsa et al., 1997). They are pro-inflammatory agents that may contribute to lung dysfunction and tissue destruction in RAO horses exposed to airborne organic stable dusts where hay and straw challenges increased BALF total gelatinolytic activity in heaves horses (Nevalainen et al., 2002, Raulo et al., 2001a).

In synovial fluid, gelatinolytic activity was found at approximately 45, 72, 88, 92, 120 and 250-kDa, where higher levels of MMP-2 and MMP-9 activities were detected in synovial fluid samples from septic arthritis horses compared to controls. A correlation between MMP-9 activity and leucocytes count was identified (Fietz et al., 2008). The ability of the gelatinases to degrade elastin was found to be higher than that of stromelysins (Murphy et al., 1991).

2.5.2.2.1 Matrix metalloproteinase-2

MMP-2 is a potentially elastinolytic metalloproteinase produced by fibroblasts and other connective tissue cells (Janoff et al., 1983) that is able to hydrolyze elastin in the elastic fibers. The molecular weight of pro-MMP-2 is 65 to 75-kDa and that of lower molecular weight gelatinolytic species is below 50-kDa and it can be detected by zymography (Raulo et al., 2001a). In RAO-horses, no difference in pro-MMP-2 was found compared to healthy horses and this suggests that MMP-2 represents a house-keeping proteinase involved with normal tissue remodeling (Koivunen et al., 1997b), additionally it can be regarded as a useful marker for monitoring the progression of inflammation in the equine joint (Marttinen et al., 2006).

2.5.2.2.2 Matrix metalloproteinase-9

MMP-9 represents the largest and a complex member of MMPs that is present in low quantities in the healthy adult lung, but much more abundant in several lung diseases including asthma, idiopathic pulmonary fibrosis and RAO (Atkinson and Senior, 2003). It has the ability to degrade components of the extracellular matrix and to regulate the activity of a number of soluble proteins (Van den Steen et al., 2002). In mice, MMP-9 is involved in the remodeling of the lower airways during severe asthma and COPD (Prause et al., 2004). In horses, MMP-9 is the main gelatinase elevated in RAO-affected horses as it was observed in TELF and BALF that the MMP-9 activities was represented by 5 bands: high molecular weight gelatinase complex (above 110-kDa), pro-MMP-9 (90 to 110-kDa) and active MMP-9 (75 to 85-kDa) (Raulo et al., 2001a).

In tracheal aspirates of RAO-affected horses, the activity of high molecular weight bands (210-190 and 150-kDa) was high and also the 90 to 110-kDa bands was high in symptomatic disease phases compared to healthy horses this suggest that MMP-9 has a role in the pathogenesis of equine respiratory diseases (Koivunen et al., 1997b).

BALF gelatinolytic MMP activity in RAO-affected horses is increased after 5 hours of hay and straw challenge with the majority of gelatinolytic activity comprising pro- and active MMP-9 and the BALF neutrophils count were highly significantly correlated with levels of pro MMP-9 and active MMP-9 (Maisi et al., 2001, Nevalainen et al., 2002).

Markedly increased elastinolytic activity in TELF was found in RAO patients compared to healthy horses, suggesting participation of elastases (MMP-2, -3, -7, -9, -10 and -12) in the destruction of lung tissue during RAO (Raulo et al., 2000).

2.5.2.3 Stromelysins

Stromelysins matrix metalloproteinases include MMP-3, MMP-10 and MMP-11 and have the ability to degrade elastin, laminin, and fibronectin (Murphy et al., 1991). The main substrates for MMP-3 are collagens type II, III, and IV, fibronectin, gelatin, and elastin (Quinones et al., 1994, Curry and Osteen, 2001).

2.5.2.4 Matrilysin (MMP-7)

Matrilysin MMP-7 is the smallest MMP members and its substrates include proteoglycans, aggrecan, gelatin, fibronectin and elastin as well as BM components as laminin and type IV collagen (Murphy et al., 1991). It is expressed in the epithelium of peribronchial glands and conducting airways in normal lung while other metalloproteinases (collagenase-1, stromelysin-1 and gelatinase) were not produced by normal or injured lung epithelium (Dunsmore et al., 1998). It is highly expressed in pulmonary fibrosis and other conditions associated with airway and alveolar injury (McGuire et al., 2003). Moreover, it is expressed in injured lung and in cancer but not in normal epithelia. MMP-7 may play an important role in the bronchiolization of alveoli by promoting proliferation, migration, and attenuation of apoptosis (Wang et al., 2009).

2.5.2.5 Macrophage metalloelastase (MMP-12)

MMP-12 is the only macrophage metalloproteinase that is represented by a 54-kDa proenzyme and a 45-kDa for active form (Lagente et al., 2009). It has the ability to degrade elastin, but it also has a broad substrate range which extends beyond that of elastin alone. It also degrades type IV collagen, laminin-1, fibronectin, proteoglycans and it cleaves plasmin to angiostatin (Chandler et al., 1996, Gronski et al., 1997). It is involved in the remodeling process in pulmonary fibrosis in mice (Manoury et al., 2006). In humans, MMP-12 plays a predominant role in the inflammatory process induced by cigarette smoke, and is considered an important therapeutic target for the treatment of COPD (Lagente et al., 2009).

2.5.2.6 Membrane type MMP (MT-MMP)

It constitutes a growing subclass of recently identified MMPs that are membrane associated (Sato et al., 1994). MT1-MMP (MMP-14) is the best-characterized MT-MMP which mediates the activation of pro-MMP-2 via its interaction with TIMP-2, which serves as an intermolecular bridge for pro-MMP-2 binding to MT-MMPs. It also displays intrinsic proteolytic activity towards ECM, which is independent of MMP-2 activation (Fillmore et al., 2001, Seiki, 1999). Two other MT-MMPs (MMP-15 and MMP-16) that can be found in the lung and activate pro-MMP-2 were identified (Sato et al., 1997).

2.5.2.7 Other MMPs

This includes 5 MMPs that are not classified in the above categories. MMP-19 was identified by cDNA cloning from liver (Pendas et al., 1997). MMP-20 is primarily located within newly formed tooth enamel (Li et al., 2001). MMP-22 was first cloned from chicken fibroblast (Yang and Kurkinen, 1998). MMP-23 is mainly expressed in reproductive tissues (Velasco et al., 1999). MMP-28 is the latest member of the MMP family that mainly expressed in keratinocytes. Expression patterns in intact and damaged skin suggest that MMP-28 might functions in wound repair (Pei et al., 2000, Marchenko and Strongin, 2001).

2.5.3 Regulation of MMPs activity

The activity of MMPs is regulated at several levels including enzyme activation, inhibition and complex formation. The interactions of MMPs with other proteins, proteoglycans and their glycosaminoglycan chains can regulate their activity. Complexes formed with MMP may include interactions of specific MMP inhibitors with binding sites (Hadler-Olsen et al., 2011).

MMPs may result in significant host damage when there is aberrant regulation of their activities (Van Wart and Birkedal-Hansen, 1990). Specific inhibitors of MMPs called the tissue inhibitors of metalloproteinases (TIMPs) are secreted, which bind to MMPs and thus inhibit their enzymatic activity (Brew et al., 2000). The balance of MMPs to TIMPs determines the ECM turnover (Ohbayashi, 2002, Asawakarn and Asawakarn, 2012, Punzi et al., 2005). Unregulated MMP activity leads to pathological conditions such as arthritis, inflammation and cancer, thus MMPs may be considered as promising therapeutic targets (Löffek et al., 2011). Knowledge about MMP activity regulation is essential for understanding various physiological and pathological processes as well as for the development of new MMPs targeting drugs.

2.5.3.1 Activation

MMPs is activated in vivo by proteinases or in vitro by chemical agents such as thiol-modifying agents (4-aminophenylmercuric acetate APMA, $HgCl_2$ and *N*-ethylmaleimide), oxidized glutathione and reactive oxygen (Nagase, 1997). There is a relationship between the plasmin and

the proMMPs activation where plasmin, generated from plasminogen by tissue plasminogen activator, bound to fibrin and urokinase plasminogen activator bound to a specific cell surface receptor. Both plasminogen and urokinase plasminogen activator are membrane-associated, thus creating localized proMMP activation and subsequent ECM turnover. Plasmin has been reported to activate proMMP-1, proMMP-3, proMMP-7, proMMP-9, proMMP-10 and proMMP-13 (Lijnen, 2001). ProMMP-2 is not readily activated by general proteinases. The main activation of proMMP-2 takes place on the cell surface and is mediated by MT-MMPs (MT1-MMP and MT2-MMP) (Butler et al., 1997).

MMPs have a complex relationship with cytokines and growth factors, they can both activate or deactivate these molecules and some cytokines can cause secretion of MMPs or their activation (Löffek et al., 2011). In mice, intranasal administration of IL-17 resulted in the release of proMMP-9 in BALF associated with a pronounced local accumulation of neutrophils and increase the free soluble MMP-9 concentration (Prause et al., 2004).

2.5.3.2 Inhibition

Tissue inhibitors of metalloproteinases (TIMPs) are specific inhibitors of MMPs. Four TIMPs have been identified in vertebrates including TIMP-1, TIMP-2, TIMP-3 and TIMP-4 (Brew et al., 2000, Visse and Nagase, 2003). Under pathological conditions, changes of TIMP levels are considered to be important because they directly affect the level of MMP activity. TIMPs inhibit all MMPs tested so far, except that TIMP-1 failed to inhibit MT1-MMP (Will et al., 1996).

Proteins such as plasma α-macroglobulins are general endopeptidase inhibitors that inhibit most proteinases by trapping them within the macroglobulin. MMP-1 reacts with α_2-macroglobulin more readily than with TIMP-1 in solution (Cawston and Mercer, 1986).

Tissue factor pathway inhibitor-2 is a serine protease inhibitor that inhibits MMPs. A C-terminal fragment of the procollagen C-terminal proteinase enhancer protein has been shown to inhibit MMP-2 (Mott et al., 2000). The secreted form, membrane-bound β-amyloid precursor protein, has also been reported to inhibit MMP-2 (Miyazaki et al., 1993).

Elastinolytic activity was inhibited by EDTA and chemically modified tetracyclines (CMT-3) where CMT-3 may provide an additional treatment possibility for horses with RAO (Raulo et al., 2000), where it acts as a tissue protective drug in equine RAO (Maisi et al., 1999).

Chlorhexidine is thought to inhibit the activities of both MMP-2 and MMP-9, but MMP-2 appeared to be more sensitive than MMP-9. MMP-8 without APMA activation was also inhibited by chlorhexidine (Gendron et al., 1999).

2.5.4 MMPs as targets for therapy

MMPs appear to be ideal drug target as they are disease-associated (Fingleton, 2008). In cancer patients, targeting the invasion of the cancer is very important method for therapy which can be achieved by inhibition of MMPs. Although MMP inhibitors have failed in clinical trials (Vihinen et al., 2005), recent data indicates that the use of selective inhibitors might lead to new therapies for acute and chronic inflammatory diseases (Hu et al., 2007).

Developing rational therapies requires further identification of specific MMP substrates and the proteolytic activity consequences (Manicone and McGuire, 2008). In lung diseases, MMPs may be considered as a drug target (Kupai et al., 2010). Several promising therapeutic approaches to inhibit MMPs have been started in the field of oncology, it is still a challenge to find specific MMP inhibitors that may be effective in lung diseases (Ohbayashi, 2002).

2.6 Interleukins

Interleukins (IL) are potent chemo-attractants for neutrophils that are secreted after activation of alveolar macrophages by dust components especially fungal antigens (Franchini et al., 1998). They play a major role in the pathophysiology of RAO as they promote neutrophils recruitment and development of airway remodeling (Lasky and Brody, 1997, Franchini et al., 2000, Franchini et al., 1998). In human medicine, the interleukins implicated in chronic airway inflammation include IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-11, IL-12 and IL-13 (Lasky and Brody, 1997). In the present study, we will focus mainly on IL-8.

2.6.1 Interleukin-8

IL-8 is one of the chemotactic cytokines, which is important in the recruitment of neutrophils into the airways in RAO, and has been the focus of several studies. It is synthesized by many cells in the lung including neutrophils, macrophages, epithelial cells, fibroblasts and smooth muscle cells. The alveolar macrophage plays a central role by producing interleukin-1 and tumor-necrosis factor, which are potent stimuli for the induction of IL-8 (Franchini et al., 2000, Kunkel et al., 1991). IL-8 is considered a regulator of type-1 hypersensitivity reactions, thus, it represents an important substance in the pathogenesis of RAO independently of the immunologic mechanisms that enhance its secretion (Franchini et al., 2000).

The expression levels of IL-8 in BALF cells in control and RAO-susceptible horses was measured during remission and at 24 hours and 5 weeks post-stabling and hay exposure. During the chronic phase, IL-8 expression level was 3-fold greater in RAO-affected horses compared to healthy horses (Ainsworth et al., 2003).

The concentrations and activity of IL-8 in BALF were greater in horses with RAO compared to healthy horses and was greater in RAO-affected horses exposed to hay dust challenge compared to non exposed RAO-affected horses and mainly accompanied by an increase in neutrophils percentage in BALF (Franchini et al., 2000).

2.6.2 Other interleukins implicated in respiratory disease development

In RAO-affected horses, an increased number of cells expressing mRNA for IL-4 and IL-5 were observed. Studies on IL-10 were performed and were not found to play a role in the active inflammation of RAO (Lavoie et al., 2001). IL-1 (α and β) and IL-6 are responsible for acute lung inflammation where they mediate and modulate the healing process, but if over-expressed may exacerbate the severity of the disease condition (Thacker, 2006, Murtaugh et al., 1996). In relation to seasonal changes, there was an elevation of BALF neutrophils during winter stabling that coincided with an increase in the IL-6 mRNA expression in BALF cells 3.7-fold (Riihimaki et al., 2008b). The expression of IL-23, IL-1β and early expression of IL-17 in RAO-affected horses was increased after exposure to hay dust (Ainsworth et al., 2007, Debrue et al., 2005).

However, this increase in IL-17 is preceded for several days by an increase in IL-8 gene expression (Ainsworth et al., 2006). Some studies reported elevated levels of IL-4 and IL-13 mRNA in the airways and peripheral blood of horses with RAO (Bowles et al., 2002).

3 Materials and methods

3.1 Horses

A total of 64 Warmblood horses, which were admitted to the Equine Clinic, Freie Universität Berlin, were used in the present study. Fifteen horses owned by the clinic served as controls, which had no clinical signs or history of respiratory disease, the clinical examination and laboratory findings were normal. Forty-nine horses had been referred to the clinic for a detailed examination due to a history of respiratory tract disease. The basic data of individual horses such as breed, age, gender, weight, height and a brief description of the admission history are included in Tables 1 and 2. Clinical history, physical examination, endoscopy, radiography of the lung and neutrophils content in BALF and TBS samples were used as the basis for grouping of diseased horses. Horses showing any other health problems (e.g. Lameness, colic etc.) were not included in the present study. The present study was approved by the Institutional Animal Care and Use Committee of Berlin (LaGeSo, Landesamt für Gesundheit und Soziales) and the owners of the horses signed a consent form for further examinations approval.

The diseased group contained a total of 49 horses and was subdivided into RAO-affected horses (n=35) which were further classified into RAO in exacerbation (Group II, n=17) and RAO in remission (Group III, n=18). Other diseased horses were classified into horses with chronic interstitial pneumopathy (Group IV, n=11) and horses with acute to subacute respiratory infection (Group V, n=3).

RAO-affected horses during disease remission appear clinically healthy at rest. They become exercise intolerant with cough at the onset of exercise. During exacerbation, auscultation during forced re-breathing may reveal wheezes that may be heard throughout the lung fields and expiratory crackles at the periphery of the lungs. During periods of exacerbation, further clinical signs include nasal discharge which may be serous, seromucous or mucopurulent, exercise intolerance and labored breathing. Coughing episodes are usually common. In severe cases, an increased respiratory rate, extended neck and head, flared nostrils and double expiratory effort are evident. A heave line and anus pump may be evident. Horses affected with chronic interstitial pneumopathy were most often presented for exercise intolerance and coughing.

Table 1: List of the clinically healthy horses used in the present study with reference to the basic data and the history collected from every horse on admission to the clinic.

Horse no.	Age (year)	Sex	Weight (Kg)	History
1	9	Gelding	560	no history of respiratory disease
2	4	Mare	600	
3	5	Mare	320	
4	9	Mare	420	
5	4	Mare	500	
6	11	Gelding	650	
7	9	Mare	450	
8	10	Mare	420	
9	17	Gelding	340	
10	10	Gelding	350	
11	9	Gelding	350	
12	6	Gelding	290	
13	6	Gelding	360	
14	13	Mare	300	
15	11	Gelding	470	

Table 2: List of horses with lung diseases used in the present study with reference to the basic data and the history collected from every horse on admission to the clinic.

Horse no.	Age (year)	Sex	Weight (Kg)	History
16	16	Gelding	455	Cough, dyspnea, nasal discharge
17	23	Gelding	430	Cough
18	15	Gelding	300	Cough
19	11	Gelding	520	Cough
20	20	Gelding	500	Cough
21	23	Mare	450	Spontaneous cough

Table 2 continued:

Horse no.	Age (year)	Sex	Weight (Kg)	History
22	19	Mare	400	Known history of RAO
23	10	Gelding	450	Cough
24	16	Gelding	500	Known history of RAO, dyspnea, exercise intolerance
25	15	Mare	480	Dyspnea, dry painful attacks of cough
26	22	Gelding	450	Increased breathing effort
27	23	Mare	450	Cough
28	19	Gelding	400	Dyspnea, increased respiratory rate
29	12	Gelding	350	Cough, dyspnea, exercise intolerance
30	11	Mare	500	Cough
31	22	Mare	550	Known history of RAO
32	18	Mare	500	Known history of RAO
33	16	Mare	488	Cough and nasal discharge
34	11	Gelding	535	Unclear
35	5	Mare	450	Strong cough after change of stable with daily pasture
36	13	Gelding	600	Known history of RAO, dyspnea
37	13	Mare	500	Cough after moving to open stable with sandy ground and dry hay feeding
38	12	Gelding	620	Productive cough, exercise intolerance
39	18	Gelding	590	Cough, abnormal breathing
40	6	Mare	440	Cough at the beginning of exercise
41	12	Mare	550	Cough
42	16	Gelding	550	Known history of RAO, cough
43	11	Gelding	535	Known history of RAO, cough
44	9	Mare	450	Frequent cough
45	13	Gelding	450	Increased respiratory rate
46	8	Gelding	450	Exercise intolerance, nasal discharge, cough
47	14	Gelding	600	Intense cough
48	22	Gelding	450	Known history of RAO, dry cough, exercise intolerance

Table 2 continued:

Horse no.	Age (year)	Sex	Weight (Kg)	History
49	13	Gelding	500	Cough, exercise intolerance, open stable with dry hay
50	17	Mare	590	Acute dyspnea since long period
51	11	Gelding	596	Attacks of cough, severe dyspnea
52	14	Mare	594	Exercise intolerance, increased breathing rate
53	9	Gelding	550	Poor performance, dyspnea, increase breathing after mild exercise
54	12	Gelding	500	Cough
55	19	Gelding	500	Cough
56	9	Mare	250	known history of RAO, poor performance, cough
57	16	Gelding	550	Cough
58	16	Mare	550	Exercise intolerance, epistaxis several times
59	13	Mare	650	Prolonged recovery after exercise, cough
60	7	Mare	600	known history of RAO, exercise intolerance, increased breathing, sporadic cough
61	10	Gelding	270	Unclear
62	19	Mare	450	Cough
63	9	Mare	450	Cough, nasal discharge
64	1	Mare	400	Frequent cough

3.2 Methods

3.2.1 Physical examination

On the day of admission, all horses were kept on the same conditions, once all examinations had been performed bedding was changed to wood shavings, feeding consisted of damp hay and oats squeezed. Special clinical examination of the respiratory tract was performed in all patients.

Rectal temperature (°C), respiratory rate (breaths/min), heart rate (beats/min), capillary refill time and the color of mucous membranes were recorded.

Auscultation was performed before and during a rebreathing examination using a plastic bag. Blood gas analysis, pulmonary function testing, radiography, tracheo-bronchoscopy with TBS and BAL sampling were performed. Clinical score was determined. The protocol for the clinical examination during the 3 days examination in the Equine Clinic is shown in Table 3.

3.2.1.1 Clinical scoring

Clinical examination scores including cough induction, dyspnea at rest, lung percussion, lung auscultation, endoscopy, BAL and blood gas analysis scores were recorded according to the method modified after Ohnesorge et al. (1998) (Table 4), where the horses were grouped into clinically healthy if the clinical score ranged from 0-1, RAO in remission when it is 2-6 and RAO in exacerbation when it is 7-14 (Table 5).

3.2.1.2 Exercise tolerance test

Exercise tolerance test was done for most horses. Some horses with lameness and severe respiratory distress were discarded from the exercise tolerance test. The standardized exercise test used in the present study consisted of a 10 min trot and 5 min canter in the exercise track. Heart and respiratory rates were recorded before the test, at zero time after exercise and every 5 min until it returned to normal values measured before exercise (in health the respiratory and heart rates return to normal within 15 min). Blood samples for measurement of lactate were taken before and immediately after exercise.

Table 3: Clinical examination protocol adopted during the present study.

Days of examination	Type of procedures
1st day	- Delivery and stabling of patients - History and signalment recording - 1st clinical examination and clinical score - 1st blood gas analysis - Hematocrit and blood differential cell count
2nd day	- 2nd clinical examination and clinical score - 2nd blood gas analysis - Exercise tolerance test (trot for 10 min, gallop for 5 min) - Lactate concentration before and after exercise - Thorax radiography (caudoventral/caudodorsal) - Interpleural pressure - Tracheo-bronchoscopy with TBS collection - Collection of BALF samples
3rd day	- 3rd Clinical examination and clinical score - 3rd Blood gas analysis

3.2.1.3 Pulmonary function testing

The pulmonary function testing was performed using a Ventigraph® (model PG100/REC, Boehringer Ingelheim, Ingelheim, Germany) that measures the maximum change in the interpleural pressure (ΔPpl_{max}), which is defined as the difference in esophageal pressure from peak inspiration to peak expiration. The esophageal probe of the Ventigraph® was placed through the right nostril of the horse into the esophagus, where pressure changes correlate to those in the intrapleural spaces. The horse was given time to return to normal respiratory rhythm then the Ventigraph measurements were recorded. A blinded analyst was used to determine the respiratory rate and the ΔPpl_{max} (Ainsworth et al., 2003). Interpleural pressure differences <4 mmH_2O were considered physiological, 4-8 mmH_2O mildly increased, 8-14 mmH_2O moderately increased and ≥15 mmH_2O severely increased, characteristic for exacerbation of RAO (Pearson et al., 2007). Blood gas analysis was performed (see 3.2.2.1.2).

Table 4: Clinical examination scores during the present study modified after Ohnesorge et al. (1998).

		Score	Max. points
1. Cough induction score	No cough after manual compression of larynx	0	1
	Coughing during manual larynx compression	1	
	Very frequent coughing	1	
	Spontaneous coughing	1	
2. Dyspnea at rest score	Normal	0	3
	Prolonged expiration	1	
	Abdominal breathing	1	
	Nostril flare during inspiration and returns to normal at end inspiration	2	
	Sinking of the intercostal area	3	
	Nostril flare during inspiration and expiration	3	
	Heaves line	3	
	Anal pumping	3	
3. Lung percussion score	3 fingers	0	2
	Handbreadth	1	
	Damping	2	
4. Lung auscultation score	Normal lung sound	0	2
	Rattling	2	
	Crackle	2	
	Wheezing	2	
5. Endoscopy score	Normal endoscopy	0	2
	Significantly increased secretions with moderate viscosity	1	
	Highly increased secretions with high viscosity	2	
	Thickened tracheal bifurcation	1	
6. BAL score	Neutrophils <8%	0	3
	Neutrophils 8-15%	1	
	Neutrophils 15-25%	2	
	Neutrophils >25%	3	
7. Blood gases score	$AaDo_2$: 0-7 mmHg	0	2
	$AaDo_2$: 7-14 mmHg	1	
	$AaDo_2$: >14 mmHg	2	

Table 5: Total score, disease grade of patients in different disease groups modified after Ohnesorge et al. (1998).

Group	Diagnosis	Score
I	Clinically healthy horses	0 – 1
III	RAO-affected horses in remission	2 – 6
II	RAO-affected horses in exacerbation	7 – 14

3.2.1.4 Endoscopy

An endoscopic examination was performed for the nasopharynx, larynx and trachea to the level of tracheal bifurcation using a 160cm, flexible videoendoscope (Videomed GmbH, Munich, Germany). Horses were restrained with a nose-twitch (Laus et al., 2009) and sedated using *detomidine*® at a dosage of 0.1 - 0.2 mg/kg BDW i.v. Mucous accumulation and viscosity in the lower airways was recorded and scored according to the score suggested by Gerber et al. (2004), Ohnesorge et al. (1998). Samples of tracheal wash for cytological examination were collected.

3.2.1.5 Thoracic radiography

Caudoventral and caudodorsal latero-lateral thoracic radiographs were performed on the 2nd day of admission before and after endoscopy by the Department of Diagnostic Imaging, Equine Clinic, Freie Universität Berlin. All radiographs were interpreted by a radiologist. The lesions assessed on lung radiography include bronchial wall thickening, generalized interstitial pattern and cranial-caudal focal interstitial pattern with increased vascular and interstitial opacity in horses with chronic interstitial pneumopathy (Doucet and Viel, 2002).

3.2.2 Samples and sampling methods

3.2.2.1 Blood samples

3.2.2.1.1 Peripheral blood

Venous blood samples were collected by means of jugular venipuncture into a plain syringe then put in a 5 ml EDTA tubes (Sarstedt, Nümbrecht, Germany) and mixed gently. Hematocrits were determined using a Micro-hematocrit centrifuge (Haemofuge Heraeus Sepatech, Germany) at 12000 rpm for 3 min. Plasma total protein concentration was measured by a total solid refractometer (Reichert GmbH, Seefeld, Germany) using the sample centrifuged for the hematocrit measurement. Rest of the sample was used for differential blood cell count and other CBC parameters by automated method using pocH-100 iv Diff (Sysmex Europe GmbH, Norderstedt, Germany).

3.2.2.1.2 Arterial blood

For blood gas analysis, arterial blood was collected from the right common carotid artery puncture hand fest above the shoulder joint in the distal third of the neck between the trachea and the jugular vein using a 4.5 cm long 22 gauge needle. The sample was collected into 3 heparinized capillary tubes and capped rapidly after collection and then strong pressure with closed fist should be kept on the puncture site for 1 min. The samples were analyzed immediately using the automated blood gas analyzer Cobas b 123 POC system® (Roche, Swisslab, Germany). The result was corrected according to horse's temperature, where the temperature was inserted manually after inserting the sample into the machine and the mean of the 3 analyses was calculated (Rose and Rossdale, 1981). The results obtained from the analyzer include $PaCO_2$, PaO_2, $AaDO_2$, pH and HCO_3. Blood gas analysis was taken into account before performing endoscopy and BALF collection, where it should be within the reference range otherwise the procedures postponed.

3.2.2.2 Respiratory fluid samples

The samples collected for analysis during the present study were tracheobronchial secretions (TBS) and bronchoalveolar lavage fluid (BALF).

3.2.2.2.1 TBS collection and processing

The tracheobronchial secretions were collected while performing the endoscopic examination of the trachea, where a plastic catheter was inserted into the biopsy channel of the endoscope and the secretions present in the trachea were collected by suction with 20 ml syringe, and then the sample was submitted for cytological examination. A drop of TBS was placed on a glass slide then a thin film was made, left to dry, then stained according to May-Grünwald Giemsa staining method (Sigma-Aldrich Chemie GmbH, Germany) and examined under a light microscope with an oil immersion lens (100x).

3.2.2.2.2 BAL collection and processing

Following endoscopy and TBS collection, a plastic catheter was inserted into the biopsy channel of the endoscope and 20 ml of *2% lidocaine*® (bela-Pharm GmbH, Vechta, Germany) were infused around the tracheal bifurcation of the trachea. Then BAL was performed using a 300 cm Silicone Bronchoalveolar Lavage Catheter (Smiths Medical ASD, Inc, USA), which was passed nasally into the distal respiratory tract and wedged into the bronchus by mean of an air balloon. 500 ml of pre-warmed *Phosphate Buffered Saline*® (Lonza, Verviers, Belgium) was infused into the bronchi through BAL catheter as recommended by the International Workshop on Equine Chronic Airway disease (Robinson, 2001) and immediately aspirated.

After the sample was collected the recovered volume was recorded. The collected volume was divided into 2 portions, one for cytological examination and the second for biochemistry. For cytological examination, the samples were centrifuged at 1500 rpm for 10 min in a Universal Centrifuge Hermle Z300 (Hermle Labortechnik GmbH, Germany), then a direct smear, staining and examination was performed as described under TBS. For biochemical analysis, BALF was centrifuged in a Table Top Refrigerated Centrifuge Hermle Z326K (Hermle Labortechnik GmbH,

Germany) at 1500 rpm for 10 min and the temperature was 4^{o}C. The cell-free supernatant was collected and stored at -80^{o}C until assayed.

3.2.3 Laboratory assays

3.2.3.1 Gelatin zymography

Gelatin zymography is most often used for the detection of MMP-2 and MMP-9 activity according to the method modified after Laemmli (1970).

3.2.3.1.1 Samples dilution

BALF samples were diluted with sample buffer (Table 6) in a ratio of 1:1 and incubated at 37˚C for 30 min prior to electrophoresis.

3.2.3.1.2 Polyacrylamide Gel Electrophoresis (PAGE)

Gelatin zymogram gels (Life technologies, Van allen way carlsbad, USA) were placed into the electrophoresis chamber (XCell, Novex Experimental Technology, Japan). The smaller side of the cassette faced inward. The chamber was filled with 1X running buffer (Table 7).

3.2.3.1.3 Loading the gel

Ten µl of protein marker (Spectra™ Multicolor Broad Range Protein Ladder, Thermo Scientific, Rockford, USA) were loaded in one well and 18 µl of each sample in specified wells using special gel loading tips (Carl Roth GmbH & Co.KG, Karlsruhe, Germany). The lid was placed on the Electrophoresis cell and the electrode cords were connected correctly to an electrophoresis power supply (Novex Programmable Power Supply Model-3540, San Diego, USA).

3.2.3.1.4 Running the gel

Electrophoresis was run at 125 V constant current until the indicator dye reaches the bottom of the gel (usually after 2 hours), then the gel was removed and placed in a container with 100 ml of

2.5% renaturing buffer (Triton® X-100, Ferak Berlin GmbH, Berlin) and incubated at room temperature for 30 min with gentle agitation. The renaturing buffer was removed and 100 ml of developing buffer were added (Table 8). Incubation for 30 min at room temperature with gentle agitation followed. The developing buffer was removed and 100 ml of developing buffer were added then incubation at 37°C followed overnight. The developing buffer was removed and the gel was washed 3 times (5 min each) with deionized water at room temperature with gentle agitation.

3.2.3.1.5 Staining the gels

The gel was stained by adding 20 ml of PageBlue™ Protein Staining Solution (Fermentas Life Sciences, Vilnius, Lithuania) for 1 hour at room temperature with gentle agitation. The stain was decanted and the gel was washed with deionized water for 1 hour with gentle agitation.

3.2.3.1.6 Analyzing the gels

Gelatinolytic activity was detected as transparent bands against a dark blue background. Gels were scanned to digitalize them and analyzed by densitometry using digital image analyzing software ImageJ v1.47 (Wayne Rasband, National institutes of health, USA, http://imagej.nih.gov/ij) as described by Hu and Beeton (2010) for objective quantification of bands and the results were presented based on peak area.

Table 6: The composition of 50 ml of sample buffer used for sample dilution in gelatin zymography.

Name of ingredients	Amount	Company, Country
50 mM Tris/HCl	0.39 g	Carl Roth GmbH + Co.KG, Karlsruhe, Germany
1.5% SDS	0.75 g	Carl Roth GmbH + Co.KG, Karlsruhe, Germany
5% Glycerol	2.5 ml	Carl Roth GmbH + Co.KG, Karlsruhe, Germany
0.005% Bromophenol blue	0.0025 g	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
The pH was adjusted to 6.8		

Table 7: Composition of 1 liter of running buffer for gelatin zymography.

Name of ingredients	Amount	Company, Country
25 mM Tris/HCl	3.03 g	Carl Roth GmbH + Co.KG, Karlsruhe, Germany
0.1% SDS	1 g	Carl Roth GmbH + Co.KG, Karlsruhe, Germany
200 mM Glycin	15 g	Carl Roth GmbH + Co.KG, Karlsruhe, Germany
400 ml of deionized water were added and gently mixed with heating at 100^{o}C, the pH was adjusted to 8.3, and then the amount was completed to 1 liter.		

Table 8: Composition of the developing buffer.

Name of ingredients	Amount	Company, Country
50 mM Tris/HCl	3.94 g	Carl Roth GmbH + Co.KG, Karlsruhe, Germany
50 mM CaCl2	3.67 g	Serva, Feinbiochemica GmbH & Co., Heidelberg, Germany
10 mM NaCl	0.29 g	Carl Roth GmbH + Co.KG, Karlsruhe, Germany
0.05% Brij 35	0.26 g	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Deionized water was added and gently mixed then the pH was adjusted to 7.6, then the amount was completed to 500 ml		

3.2.3.2 Enzyme linked immunosorbent assay (ELISA)

The ELISA tests used in the present study were sandwich enzyme immunoassays for quantitative measurement of MMP-2, -9 and IL-8. Standards and samples were set up in duplicates.

Assay procedures: Standards were prepared according to the user manual. 100 µl of each dilutions of standard, blank and samples were added into the appropriate wells, covered with plate sealer and incubated at 37^{o}C for 2 hours. Then the liquid was removed. 100 µl of detection reagent "A" working solution were added to each well, covered by plate sealer and then incubated at 37^{o}C for 1 hour. The liquid was removed, and then 350 µl of washing buffer were used for washing and let to sit for 1-2 minutes. The liquid was removed from all wells completely by blotting the plate onto absorbent paper. The wells were washed 3 times. After the last wash, any remaining wash buffer was removed by decanting. 100 µl of detection reagent "B" working solution were added to each well and incubated at 37^{o}C for 30 minutes. The washing was

performed 5 times. 90 µl of TMB substrate solution was added to each well. Covered with a plate sealer and the wells were incubated for 15-25 minutes at 37°C and protected from light. The liquid turned blue by the addition of substrate solution. 50 µl of Stop Solution was added to each well, so the liquid turned yellow. The absorbance was measured with an ELISA microplate reader (BioRad Laboratories, Hercules, USA) at 450 nm immediately. Calculation of the unknown samples' concentration was made by inverted standard curves using the Excel software program.

The MMP-2 ELISA was performed using Enzyme-Linked Immunosorbent Assay Kit for Equine MMP-2 (Uscn Life Science Inc., Wuhan, China). The MMP-9 ELISA was performed using equine MMP-9 kit (Uscn Life Science Inc., Wuhan, China). The IL-8 ELISA was performed using equine IL-8 kit (Uscn Life Science Inc., Wuhan, China).

3.2.3.3 Fluorescent method for MMP-8 concentration

Sensolyte® 520 MMP-8 Assay Kit (Anaspec, Inc. Fermont, USA) is a convenient assay for high throughput screening of MMP-8; the components of the kits are illustrated in Table 9. The samples preparation and analysis procedures were followed.

3.2.3.3.1 pro-MMP-8 activation in the samples

BALF samples and recombinant human MMP-8 proenzyme were incubated with APMA (Component C) at a final concentration of 1 mmol/l for 1 hour at 37°C to activate pro-MMPs.

3.2.3.3.2 MMP-8 substrate working solution

MMP-8 substrate working solution was prepared by adding MMP-8 substrate (Component A) to assay buffer (Component D) in a ratio of 1 to 100.

3.2.3.3.3 Negative and positive controls

Negative control contained assay buffer (Component D) and the positive control contained recombinant human purified MMP-8 proenzyme. Assay buffer was used as a blank.

Table 9: Components of Sensolyte® 520 MMP-8 Assay Kit.

Component	Description	Quantity
Component A	MMP-8 substrate 5-FAM/QXL™520 FRET peptide Ex/Em=490 nm/520 nm upon cleavage	60 µl
Component B	5-FAM-Pro-Leu-OH, fluorescence reference standard Ex/Em=490 nm/520 nm	1 mmol/l, 10 µl
Component C	APMA, 4-aminophenylmercuric acetate	1 mol/l, 20 µl
Component D	Assay buffer	20 ml
Component E	Stop solution	10 ml
Human MMP-8 proenzyme	human MMP-8, concentration 10 µg/ml	100 µl

3.2.3.3.4 Standard preparation

Fluorescence reference standard (component B) was diluted from 1 mmol/l to 5 µmol/l in assay buffer (D). Double-fold serial dilutions were made to get concentrations of 2.5, 1.25, 0.625, 0.3125, 0.156 and 0.078 µmol/l.

3.2.3.3.5 Starting the enzymatic reaction

The enzymatic reaction was started by adding 50 µl of each of MMP-8 containing samples, negative control, positive control, blank and standards in specified wells in the 96-well black bottom microplate. Then 50 µl of MMP-8 substrate working solution were added to all wells. The reagents were mixed by shaking the plate gently for 30 sec. The plates were incubated at 37°C for 30 to 60 min, during which the plate was kept from direct light. Then 50 µl of stop solution (Component E) were added and the reagents were mixed. Fluorescence intensity was measured at Ex/Em=490/520 nm using FLUO Star Optima® (BMG Labtech GmbH, Ortenberg, Germany).

3.2.3.3.6 Calculations

The blank was subtracted from the readings of the other wells. The fluorescence readings were expressed in relative fluorescence units (RFU). Calculations were made by plotting data of standards as RFU versus concentration of test compounds or enzyme concentration. The obtained equation from the standard curve was used to calculate the concentration of MMP-8 in BALF samples in µmol/l (Stryer, 1978).

3.2.4 Statistical analysis

Data entry was managed by a Microsoft® Access Database 2007 file designed specifically for this purpose (Figures 18, 19, 20, 21 and 22). Data was statistically analyzed and graphically presented using SPSS Statistics® 17.0 (Version 17.0 released 2008, SPSS Inc., Chicago) and expressed as mean values ± standard deviation (SD). The data was tested for normal distribution using Shapiro Wilks W Test. As some was found normally distributed while others were found not, we preferred to do non parametric tests for the whole data.

The level of significance was set at <0.05. All data were included in a descriptive analysis including mean, standard deviation, minimum and maximum and were presented in the prospective tables. A discriminant analysis was used to proof the correctness of the classification.

Kruskal-Wallis H test was used to compare the control group and different disease groups. A p-value of <0.05 was considered significant. Pairwise comparisons using Mann-Whitney U test followed Kruskal-Wallis test for 2-group comparison, the significance was corrected using Bonferoni adjustment (0.05/10=0.005) indicating that a difference would only be considered significant if the p-value was below 0.005. The p-values are listed in Tables 27, 28, 29 and 30.

Spearman rank correlation coefficients were calculated between clinical examination scores, blood gases score and between these variables and the total examination score, BALF neutrophils percentage, TBS neutrophils percentage, MMP-2, MMP-9, IL-8 concentrations and MMP-8 activity. The spearman correlation coefficients were interpreted using the scale provided by Salkind, where the values between (0.8 and 1.0), (0.6 and 0.8), (0.4 and 0.6), (0.2 and 0.4) and

(0.0 and 0.2) were defined as very strong, strong, moderate, weak and very weak or no relationship respectively (Chung, 2007). The level of significance was set at $p < 0.05$.

Results are presented either in table form or in diagrams for the evaluation of each parameter separately. In addition some results are presented using box plots, where the interquartile range was represented by the box with 50% in the middle between the 25^{th} and 75^{th} percentile.

4 Results

4.1 History, clinical signs and basis of classifications

A total of 64 warmblood horses, which were admitted to the Equine Clinic, Freie Universität Berlin, were used in the present study. They were divided into 5 groups according to the clinical signs presented, total examination scores, BALF and TBS neutrophils percentage, MMP-2, MMP-9 and IL-8 concentrations as well as MMP-2, MMP-8 and MMP-9 activities. In addition, a discriminant analysis was used to prove the correctness of the classification on the basis of investigated parameters, where correct classification rates for different parameters are presented in Table 20 in the Appendix. A correct classification rate above 50% was observed for groups I, II, III and IV but not for group V. The different diagnosis groups are listed in Table 10.

Clinically healthy horses (Group I, n=15, 23.4%), presented in Table 1, owned by the clinic were used as controls, which showed no previous history or signs of respiratory tract disorders, clinical examination score <2, no tracheal secretions, BALF neutrophils percentage ≤8%, $AaDO_2$ ≤8 mmHg and interpleural pressure ≤4 cm H_2O.

RAO-affected horses in exacerbation (Group II, n=17, 26.6%), horses numbers 16 to 32 in Table 2, were presented with a history of dry, recurrent, painful attacks of cough that might be spontaneous, mild to severe inspiratory and expiratory dyspnea, mild serous to seromucoid nasal discharge, exercise intolerance, increased breathing effort and increased respiratory rate, BALF neutrophils percentages ≥25%, $AaDO_2$ > 8 mmHg.

RAO-affected horses in remission (Group III, n=18, 28.1%), horses numbers 33 to 50 in Table 2, showed no dyspnea at rest, but cough and exercise intolerance was a characteristic sign, mostly normal breath sounds, little amount of serous to seromucoid nasal discharge, prolonged recovery after exercise, BALF neutrophils percentage ≥8%, $AaDO_2$ > 4-8 mmHg or interpleural pressure >4 cm H_2O.

Horses with chronic interstitial pneumopathy (Group IV, n=11, 17.2%), horses numbers 51 to 61 in Table 2, were presented with sporadic cough and exercise intolerance accompanied by an

increased breathing rate during mild exercise and severe dyspnea with mild serous nasal discharge, increased interstitial opacity of thoracic radiographs and an increased percentage of macrophages in TBS and/or BALF cytology.

Horses with acute to subacute respiratory infection (Group V, n=3, 4.7%), horses numbers 62 to 64 in Table 2, were characterized by the presence of frequent cough with yellowish serous to mucopurulent nasal discharge, increased percentages of degenerated neutrophils in TBS and/or BALF cytology and evidence of toxic changes or phagocytosed bacteria.

Table 10: Diagnosis groups of horses used in the present study showed numbers and percentage of each group.

Groups	Diagnosis	n=	%
I	Clinically healthy horses	15	23.4
II	RAO-affected horses in exacerbation	17	26.6
III	RAO- affected horses in remission	18	28.1
IV	Horses with chronic interstitial pneumopathy	11	17.2
V	Horses with acute to subacute respiratory infection	3	4.7

4.2 General information on the patients

4.2.1 Gender

With reference to gender, in the whole population 35 horses were geldings (54.7%) and 29 horses were mares (45.3%). The gender of horses in subgroups is presented in Table 21 in the Appendix and are illustrated in a column chart (Figure 1).

4.2.2 Age and body weight of horses

The average age of the whole population of horses used in the present study was 12.74±5.26 years. The clinically healthy horses were presented with a mean age of 8.75±3.49 years; RAO-affected horses in exacerbation had a mean age of 17.44±4.5 years; RAO-affected horses in remission had a mean age of 12.49±4.23 years; horses with chronic interstitial pneumopathy were admitted with a mean age of 12.25±3.71 years and finally horses with acute to subacute respiratory infection had a mean age of 9.42±8.77 years (Table 22 in the Appendix, Figure 2). The average weight of the whole population of horses used in the present study was 473.79±96 kg (Table 22, Appendix).

4.3 General clinical examination

The mean values of rectal temperature, respiratory and heart rates in each group were measured and listed in Tables 11, 27 and Figure 3. Temperature showed no significant difference in all groups reflecting the nature of the chronic disease studied. Respiratory rate was significantly increased in RAO-affected horses in exacerbation (21.76±5.82 breaths/min) compared to clinically healthy horses (14.09±1.71 breaths/min), while other groups showed no significant changes. Heart rate showed no significant changes in all groups.

4.4 Clinical examination of the lung

Clinical examinations of the lower airways including both lungs were performed by auscultation as well as percussion. In clinically healthy horses, there were normal sounds on auscultation and percussion of both sides of the lung. RAO-affected horses in exacerbation showed mild to loud rattles on the trachea and all over the lung field as well as wheezes in most cases. RAO-affected horses in remission presented with mild to moderate inspiratory and expiratory noises, mild wheezes and rattles on the trachea and ventral lung field. Horses with chronic interstitial pneumopathy revealed mild to moderate inspiratory and expiratory noises and mild rattles over the lung field. Horses with acute to subacute respiratory infection were characterized by mild to moderate inspiratory noises and mild rattles over the trachea.

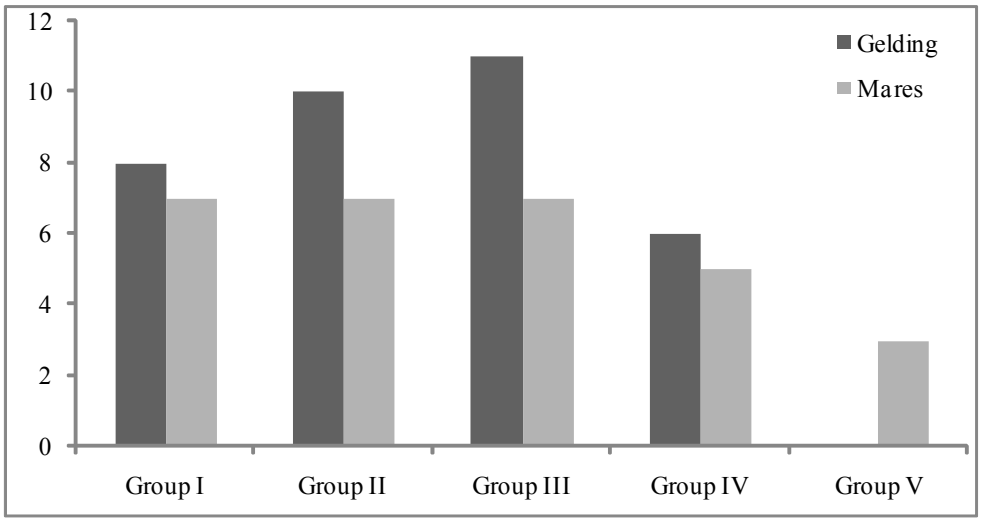

Figure 1: Gender of horses used in the present study according to their groups.

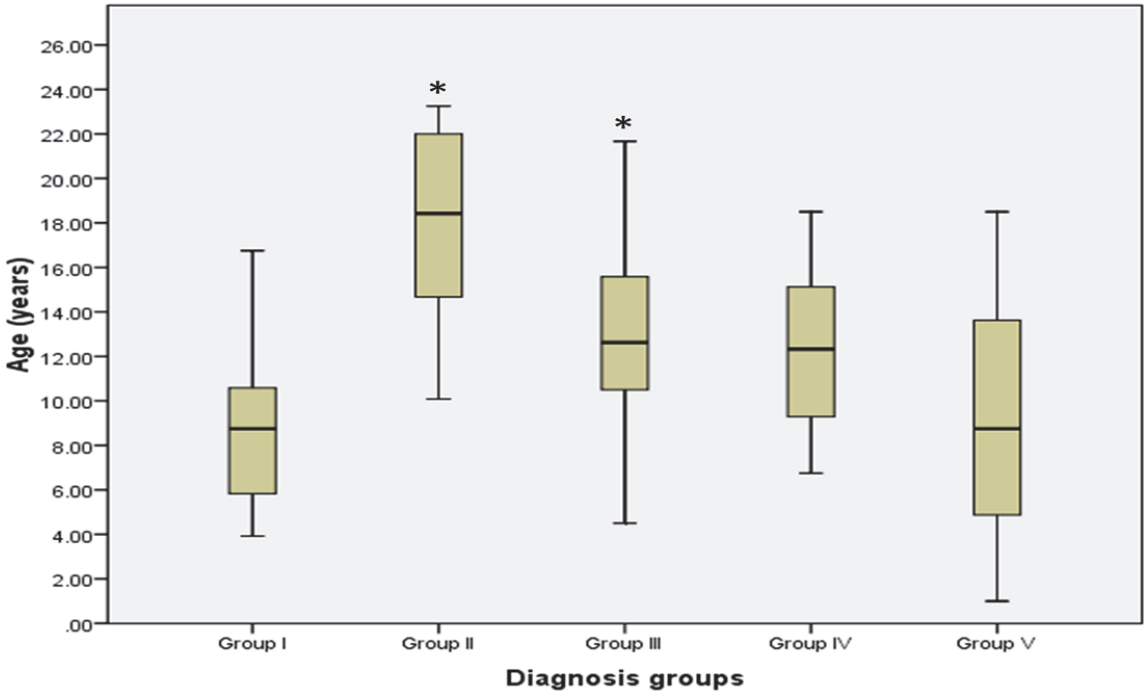

Figure 2: Box plot of age in different horses in the present study indicates that horses with RAO in remission and exacerbation have older ages.

Table 11: Mean rectal temperature, respiratory and heart rates in different disease groups, the results are expressed as mean values ± SD. The * indicates significance. The detailed significances for the inter-groups comparison are listed in Table 27 in the Appendix.

	Temperature (^{o}C)	Respiratory rate (breaths/min)	Heart rate (beats/min)
Group I (n=15)	37.4±0.3	14.09±1.71	37.47±4.51
Group II (n=17)	37.4±0.3	21.76±5.82 *↑	38.71±2.5
Group III (n=18)	37.54±0.18	17.46±4.35	40.28±3.78
Group IV (n=11)	37.52±0.25	16.18±3.88	36.61±3.44
Group V (n=3)	37.6±0.38	13.11±0.38	36.44±3.36

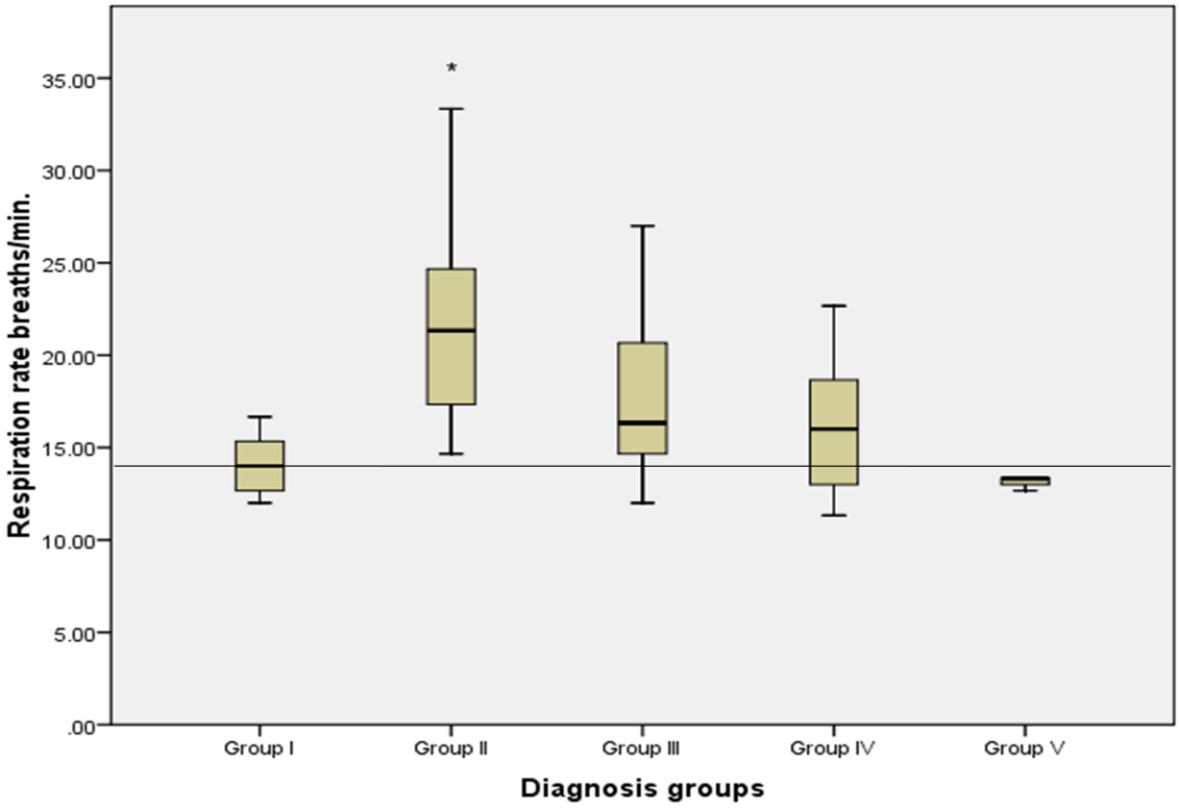

Figure 3: Box plot for respiratory rate in different groups of horses used in the present study. The * in the graph indicates significance compared to clinically healthy horses.

4.5 Clinical examination score system

Clinical examinations were performed according to a score system modified after Ohnesorge et al. (1998) (Table 4); the results of clinical scores are presented in Tables 23 and 28 in the Appendix. There were significant differences in cough induction score, dyspnea at rest score, endoscopy score, BAL score and blood gas analysis score between different groups (Figures 4 and 5). The frequencies of clinical score in different groups are listed in Table 24 in the Appendix. Lung percussion and auscultation scores showed no difference in all groups compared to controls and there were also no inter-group differences.

Cough induction score showed a significant increase in RAO-affected horses in exacerbation (0.47±0.5), remission (0.44±0.51) and horses with acute to subacute respiratory infection (0.67±0.58) compared to clinically healthy horses (0.07±0.26). Dyspnea at rest score showed a significant increase in RAO-affected horses in exacerbation (1.65±1.1) compared to clinically healthy horses (0±0). Also, RAO-affected horses in exacerbation were significantly increased compared to RAO-affected horses in remission (0.33±0.76) and horses with chronic interstitial pneumopathy (0.27±0.9).

Endoscopy score showed a significant increase in RAO-affected horses in exacerbation (1.82±0.39), RAO-affected horses in remission (1.17±0.71), and horses with chronic interstitial pneumopathy (0.91±0.7) and in horses with acute to subacute respiratory infection (2±0) compared to clinically healthy horses (0±0). Also, endoscopy score in RAO-affected horses in exacerbation was significantly increased compared to RAO-affected horses in remission and horses with chronic interstitial pneumopathy.

Bronchoalveolar lavage score showed a significant increase in RAO-affected horses in exacerbation (3±0), RAO-affected horses in remission (1.39±1.04) and horses with acute to subacute respiratory infection (1.33±1.53) compared to clinically healthy horses (0±0). Also, BAL score in RAO-affected horses in exacerbation was significantly increased compared to RAO-affected horses in remission, horses with chronic interstitial pneumopathy (0.45±0.69) and horses with acute to subacute respiratory infection.

Blood gas score showed a significant increase in RAO-affected horses in exacerbation (0.76±0.75) compared to clinically healthy horses (0.2±0.4). Also, blood gas score in RAO-affected horses in exacerbation showed a significant increase compared to RAO-affected horses in remission (0.17±0.38).

Finally, the total examination score showed a significant increase in RAO-affected horses in exacerbation (8.12±2.23), RAO-affected horses in remission (3.88±1.41), horses with chronic interstitial pneumopathy (2.36±2.66) and horses with acute to subacute respiratory infection (4.33±2.52) compared to clinically healthy horses (0.27±0.46). Also, the total examination score in RAO-affected horses in exacerbation was significantly higher than in RAO-affected horses in remission and horses with chronic interstitial pneumopathy.

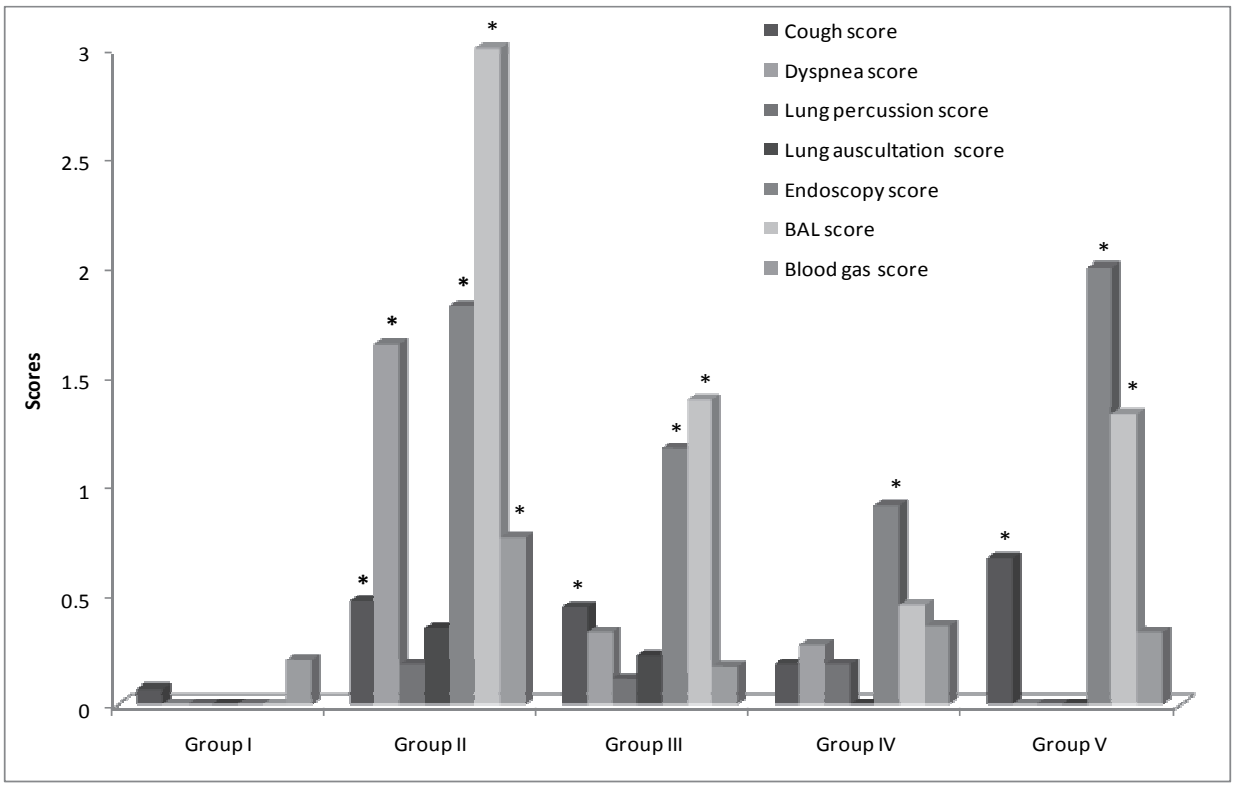

Figure 4: Clinical examinations score in different disease groups. The * indicates significance compared to clinically healthy horses.

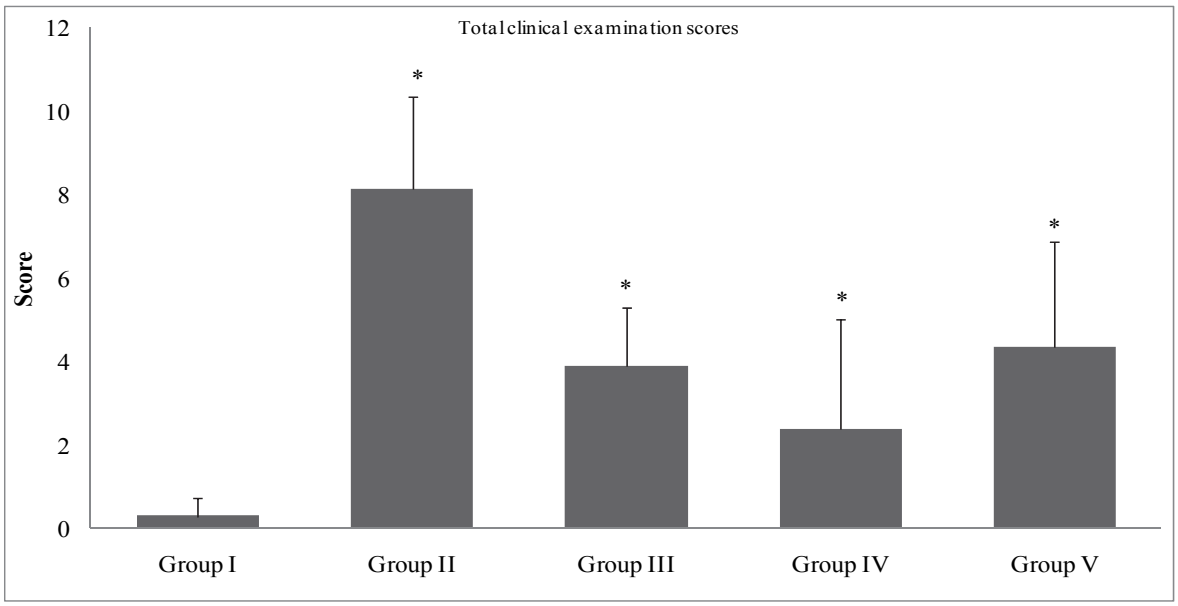

Figure 5: Total clinical examinations score in different disease groups. The * indicates significance compared to clinically healthy horses.

4.6 Endoscopic assessment of the lower airways

Horses were examined endoscopically, the amount and viscosity of secretions and the tracheal bifurcation changes were recorded and scored according to a method described by Dieckmann (1987) and Gerber et al. (2004) (Table 25). In addition, the endoscopy score was included in the scoring-system according to Ohnesorge et al. (1998) (Tables 23 and 28).

In clinically healthy horses, the endoscopic image showed normal airways, no secretions in the trachea and the tracheal bifurcation was thin. RAO-affected horses in exacerbation had profuse white, milky-white to yellowish secretions in the trachea which was often located dorsally and extended into the lower third of trachea up to the main bronchi and to the tracheal bifurcation. The tracheal bifurcation was mildly to severely thickened. RAO-affected horses in remission showed few amounts of milky to yellowish secretions accumulated in the last third of trachea and up to the main bronchi. Tracheal bifurcation was mildly to moderately thickened. In horses with chronic interstitial pneumopathy, very little milky secretions were found in the trachea up to the distal third of the trachea. Tracheal bifurcation was mildly to moderately thickened. In horses

with acute to subacute respiratory infection, there were white to yellowish secretions in the trachea accumulated in the distal third, the tracheal bifurcation was mildly to moderately thickened (Figure 6). The endoscopy score according to Gerber et al. (2004) are listed in Tables 12 and 28, there were significant increase in the amount, viscosity of secretions and tracheal bifurcation scores in RAO-affected horses in exacerbation and remission and horses with chronic interstitial pneumopathy. While in horses with acute to subacute respiratory infection, the amount and viscosity of secretions were significantly increased but not the tracheal bifurcation score.

4.7 Thoracic radiography

Thoracic radiography was performed routinely for all horses used in the present study before endoscopy and BALF collection. In clinically healthy horses, a normal lung pattern was observed. RAO-affected horses in exacerbation and remission showed mild to moderate interstitial lung patterns with moderate to severe bronchial wall thickening and increased bronchial opacity. In horses with chronic interstitial pneumopathy, moderate to severe interstitial pattern, in some cases with a marked vascular pattern, represented increased interstitial opacity. In acute to subacute respiratory infection, there were increases in alveolar pattern density.

Table 12: Endoscopy score in different disease groups, the results are expressed as mean values ± SD. The * indicates significances at $p<0.05$, the detailed significances for the inter-groups comparisons are listed in Table 28.

	Amount of secretions score	**Viscosity of secretions score**	**Tracheal bifurcation score**
Group I	0.47±0.64 (0-2)	0.4±0.51 (0-1)	0.25±0.45 (0-1)
Group II	3.5±0.63$^{*\uparrow}$ (3-5)	3.88±0.5$^{*\uparrow}$ (3-5)	1.36±1$^{*\uparrow}$ (0-3)
Group III	2±1.33$^{*\uparrow}$ (0-4)	2.33±1.4$^{*\uparrow}$ (0-4	1.33±0.82$^{*\uparrow}$ (0-3)
Group IV	2±1.2$^{*\uparrow}$ (0-4)	1.8±1.2$^{*\uparrow}$ (0-4	1.56±0.88$^{*\uparrow}$ (0-3)
Group V	3.67±0.57$^{*\uparrow}$ (3-4)	3.67±0.57$^{*\uparrow}$ (3-4)	1.33±0.57 (1-2)

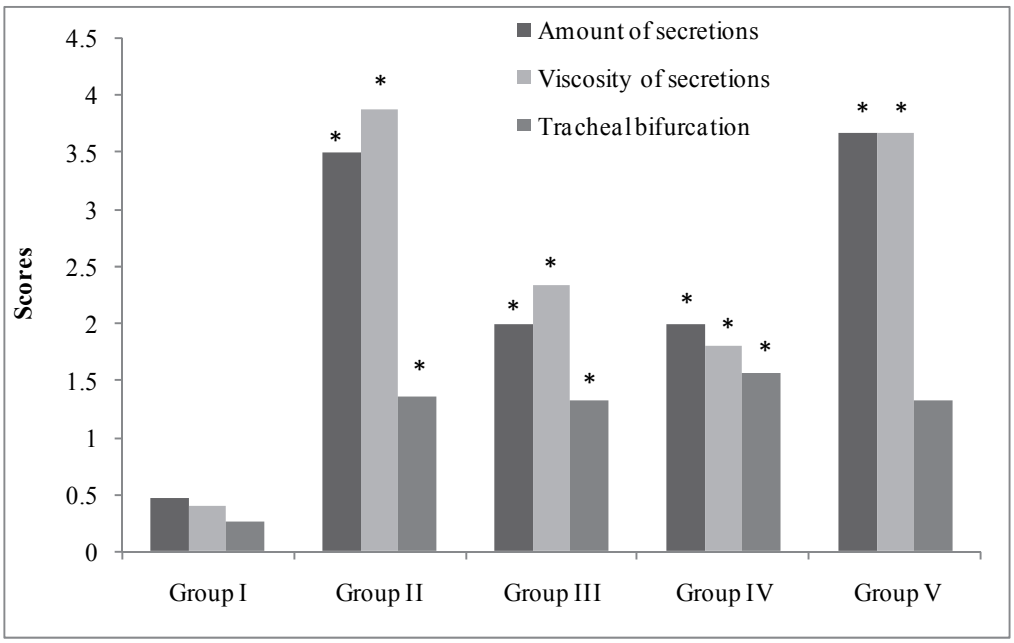

Figure 6: Endoscopy score in different disease groups. The * indicates significance compared to clinically healthy horses.

4.8 Lung function testing

The results of lung function testing, including blood gas analysis (pH, $PaCO_2$, PaO_2, $AaDO_2$ and HCO_3) and interpleural pressure measurements, are presented in Table 13. PaO_2, $AaDO_2$ and interpleural pressure showed significant differences between control horses and different disease groups.

The PaO_2 was highly significantly decreased in RAO-affected horses in exacerbation (87.9±12.15 mmHg) compared to clinically healthy horses (101.95±6.18 mmHg), while the $AaDO_2$ was significantly increased in RAO-affected horses in exacerbation (10.91±9.3 mmHg) and horses with acute to subacute respiratory infection (11.33±5.82 mmHg) compared to clinically healthy horses (0.52±1.03 mmHg) (Figures 7 and 8).

Interpleural pressure showed a significant increase in RAO-affected horses in exacerbation (5.67±2.06 cm H_2O) compared to clinically healthy horses (3.57±1.13 cm H_2O), while it was significantly increased in RAO-affected horses in exacerbation compared to RAO-affected horses

in remission (4.23±0.93 cm H_2O) (Figure 9). Other pulmonary function testing parameters showed no significant changes in other disease groups compared to clinically healthy horses.

Table 13: Blood gas analysis and interpleural pressure in different disease groups. The results are expressed as mean values ± SD. The * indicates significance at $p<0.05$, the detailed significances for the inter-groups comparisons are listed in Table 27.

	$PaCO_2$ (mmHg)	**PaO_2 (mmHg)**	**$AaDO_2$ (mmHg)**	**Interpleural pressure (cm H_2O)**
Group I (n=15)	43.87±2.53 (40-48.33)	101.95±6.18 (90.9-110.5)	0.52±1.03 (0-3.3)	3.57±1.13 (2-5)
Group II (n=17)	43.63±4.76 (39.1-58.4)	87.9±12.15$^{*\downarrow}$ (55.4-104.2)	10.91±9.3$^{*\uparrow}$ (0-29.8)	5.67±2.06$^{*\uparrow}$ (4-11)
Group III (n=18)	43.75±3.02 (37.7-48.2)	94.98±7.38 (74.2-109)	5.17±8.03 (0-30.9)	4.23±0.93 (2-6)
Group IV (n=11)	44.19±3.24 (39.5-50.6)	94.23±9.4 (74.8-103.1)	5.55±8.5 (0-24.5)	4.33±1.03 (3-6)
Group V (n=3)	41.37±6.27 (34.5-46.8)	89.17±11.9 (77.7-101.5)	11.33±5.82$^{*\uparrow}$ (6.1-17.6)	4±0 (4-4)

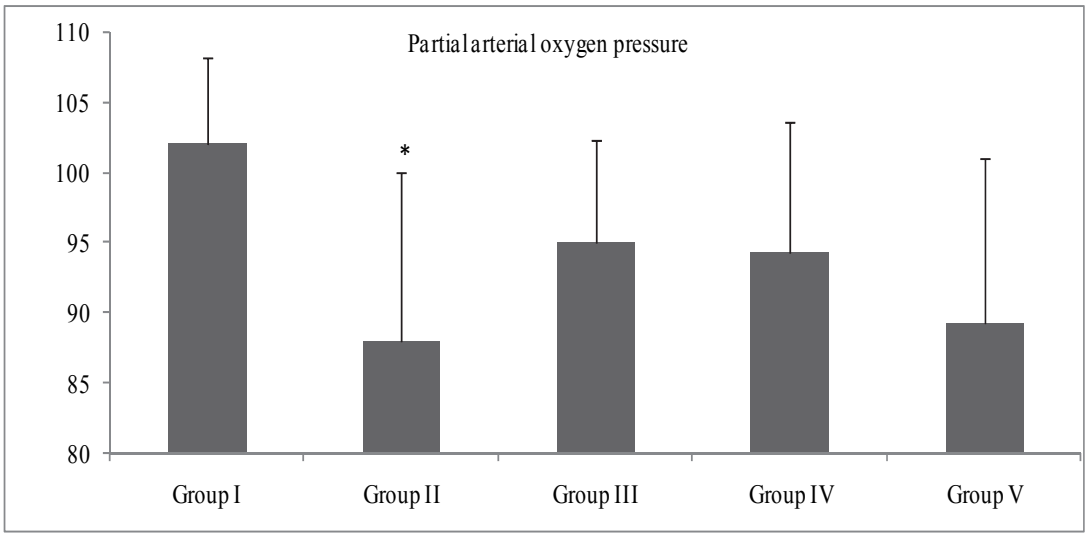

Figure 7: Results of PaO_2 in different disease groups. The * indicates significance compared to clinically healthy horses.

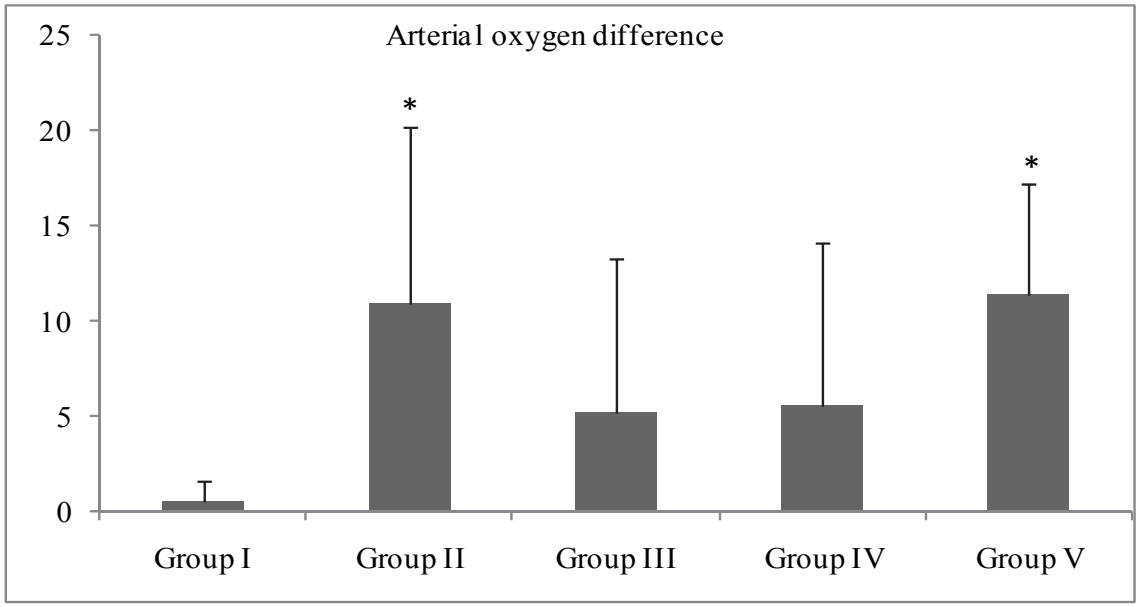

Figure 8: Results of $AaDO_2$ in different disease groups. The * indicates significance compared to clinically healthy horses.

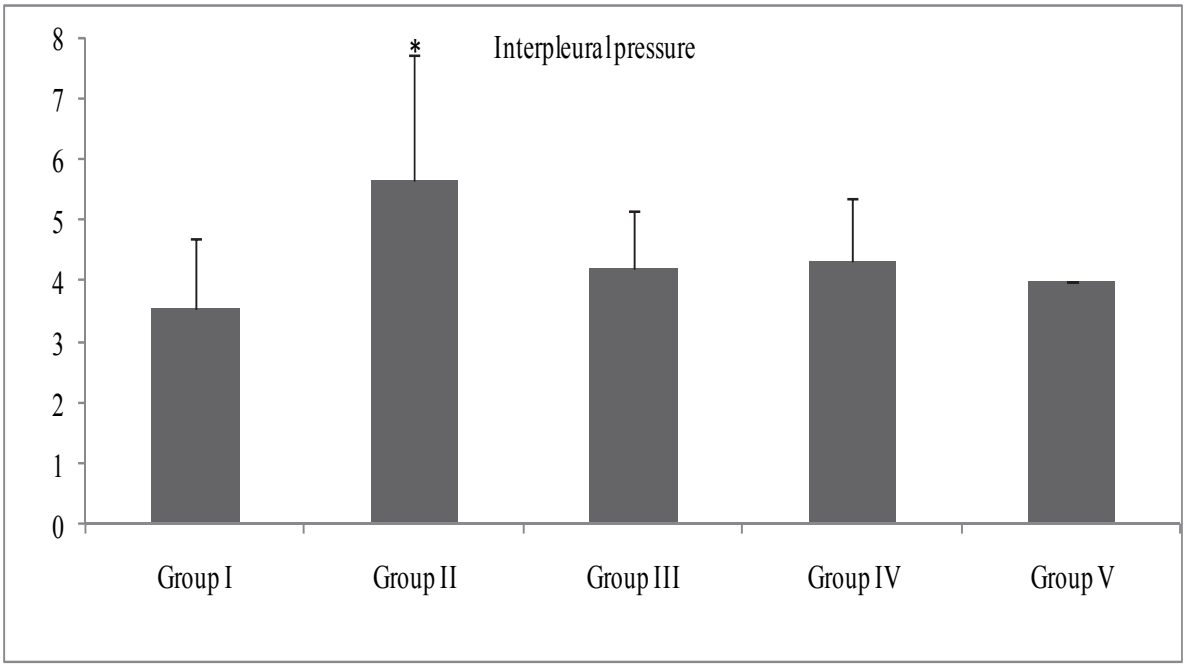

Figure 9: Results of interpleural pressure in different disease groups. The * indicates significance compared to clinically healthy horses.

4.9 Cytology of respiratory secretions

4.9.1 Tracheobronchial secretions cytology

Tracheobronchial secretions were collected from 39 horses. TBS collection from the remaining 25 horses was not possible due to no or a too little amount of tracheal secretions in the airways. Macrophages, lymphocytes and neutrophils percentages in TBS were found to be highly significant different between clinically healthy and different disease groups (Tables 14 and 29). TBS Macrophages and lymphocytes percentages were highly significantly decreased in RAO-affected horses in exacerbation (9.5±12.36% and 7.02±4.12% respectively) compared to clinically healthy horses (65.54±10.84% and 18.16±9.04% respectively) due to the fact that neutrophils were more predominant in RAO-affected horses in exacerbation.

TBS neutrophils percentages were highly significantly increased in RAO-affected horses in exacerbation (83.12±16.23%) and remission (52.57±23.8%) compared to clinically healthy horses (12.07±4.88%). On the other hand, TBS neutrophils percentage in RAO-affected horses in exacerbation was highly significant increased compared to of RAO-affected horses in remission. TBS eosinophils percentage and mast cells percentage showed no significant changes (Figure 10).

4.9.2 Bronchoalveolar lavage fluid cytology

Bronchoalveolar lavage fluid was collected from all 64 horses. Macrophages, lymphocytes, neutrophils and mast cells percentages were highly significantly different between clinically healthy horses and different disease groups (Tables 15 and 29).

BALF macrophages percentage showed a highly significant decrease in RAO-affected horses in exacerbation (19.64±12.07%) and remission (43.78±12.98%) compared to clinically healthy horses (56.48±4.75%). On the other hand, BALF macrophages percentage in RAO-affected horses in exacerbation was significantly decreased compared to RAO-affected horses in remission and in horses with chronic interstitial pneumopathy (50.83±15.46%).

Table 14: Results of TBS cytology among different disease groups, the results are expressed as mean values ± SD. The * indicates significance at $p<0.05$. The detailed significances for the inter-groups comparisons are listed in Table 29.

Disease groups	TBS macrophages (%)	TBS lymphocytes (%)	TBS neutrophils (%)	TBS eosinophils (%)	TBS mast cells (%)
Group I (n=7)	65.54±10.84 (49.4-80.6)	18.16±9.04 (8.25-33.2)	12.07±4.88 (4-18.75)	0.29±0.42 (0-1)	0.94±1.38 (0-4)
Group II (n=15)	9.5±12.36$^{*\downarrow}$ (0.6-51)	7.02±4.12$^{*\downarrow}$ (1.5-19)	83.12±16.23$^{*\uparrow}$ (30-94.5)	0.17±0.4 (0-1.5)	0.25±0.31 (0-1)
Group III (n=9)	33.39±24 (5.75-88)	18.65±13.16 (4.7-43.5)	52.57±23.8$^{*\uparrow}$ (15.5-86.75)	1.44±3.46 (0-10.5)	0.48±0.76 (0-2)
Group IV (n=5)	37.94±26.9 (4.3-73.66)	13.8±9.66 (3.4-25.5)	46.29±32.49 (17.8-91.4)	1.1±1.2 (0-3)	0.87±1.49 (0-3.5)
Group V (n=3)	18.97±24.54 (3.25-47.25)	14.95±13.21 (5.25-30)	65.77±37.69 (22.5-91.5)	0.32±0.35 (0-0.7)	0±0 (0-0)

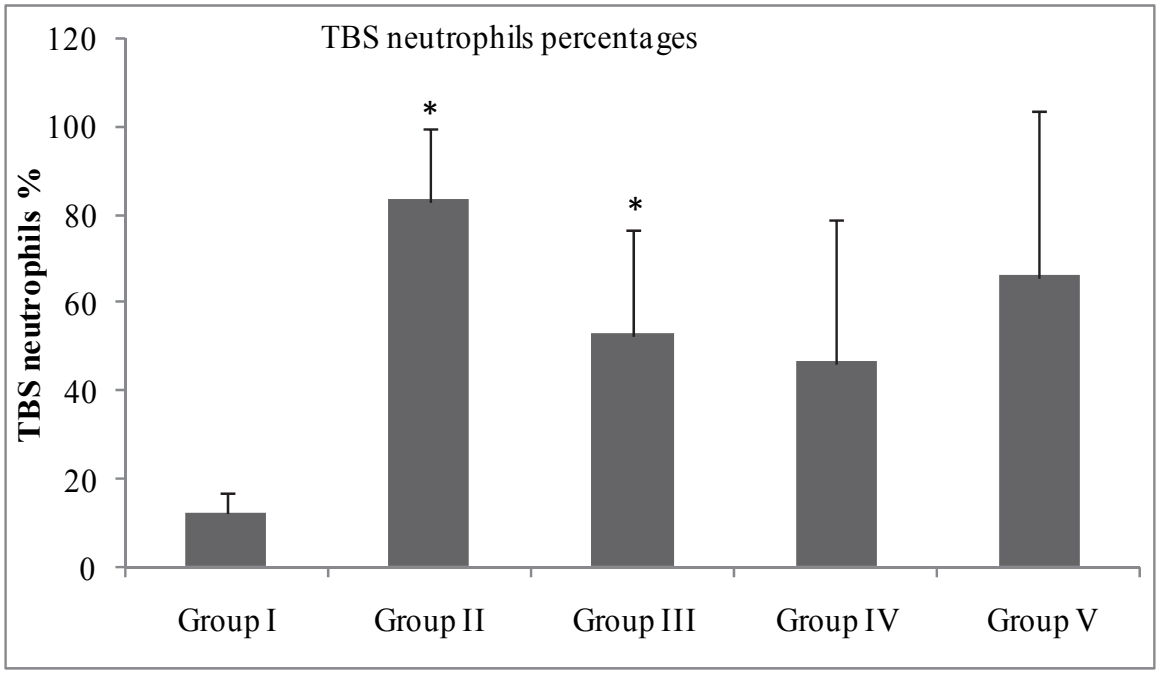

Figure 10: Neutrophils percentages of the TBS in different disease groups. The * indicates significant difference compared to clinically healthy horses.

BALF lymphocytes percentage was significantly decreased in RAO-affected horses in exacerbation (18.66±12.16%) compared to clinically healthy horses (38.15±6.41%). There was a

significant decrease in BALF lymphocytes percentage in RAO-affected horses in exacerbation compared to that in remission (34.63±13.65%).

BALF neutrophils percentage was significantly increased in RAO-affected horses in exacerbation (60.68±21.59%), remission (15.64±8.19%) and in horses with chronic interstitial pneumopathy (8.73±5.71%) compared to clinically healthy horses (3.02±2.41%). Also, BALF neutrophils percentage was significantly increased in RAO-affected horses in exacerbation compared to RAO-affected horses in remission and horses with chronic interstitial pneumopathy (Figure 11).

BALF mast cells were significantly decreased in RAO-affected horses in exacerbation (1.16±1.22%) compared to RAO-affected horses in remission (3.8±3.21%) and horses with chronic interstitial pneumopathy (5.03±4.34%). But, there was no significant difference from the clinically healthy horses group. BALF eosinophils percentage showed no difference between all groups.

Table 15: Results of BALF cytology, the results are expressed as mean values ± SD. The * indicates significance at $p<0.05$. The detailed significances for the inter-groups comparisons are listed in Table 29.

Disease groups	**BALF macrophages (%)**	**BALF lymphocytes (%)**	**BALF neutrophils (%)**	**BALF eosinophils (%)**	**BALF mast cells (%)**
Group I (n=15)	56.48±4.75 (46.5-64)	38.15±6.41 (27.8-50)	3.02±2.41 (0.5-7.6)	0.13±0.27 (0-1)	2.22±2.06 (0-8)
Group II (n=17)	19.64±12.07$^{*\downarrow}$ (5.6-47)	18.66±12.16$^{*\downarrow}$ (3.6-35.5)	60.68±21.59$^{*\uparrow}$ (27-90.4)	0.27±0.35 (0-1)	1.16±1.22 (0-4.6)
Group III (n=18)	43.78±12.98$^{*\downarrow}$ (22-66)	34.63±13.65 (22-69)	15.64±8.19$^{*\uparrow}$ (2.5-27.8)	1.95±3.9 (0-15.5)	3.8±3.21 (0.2-12.5)
Group IV (n=11)	50.83±15.46 (15-67)	34.47±11.87 (22.2-60)	8.73±5.71$^{*\uparrow}$ (1.2-24.1)	0.95±0.92 (0-2.5)	5.03±4.34 (0-15)
Group V (n=3)	47.23±21.93 (22-61.7)	22.82±12.15 (8.8-30.4)	28.57±34.7 (7.1-68.6)	0.15±0.13 (0-0.25)	1.23±1.12 (0.4-2.5)

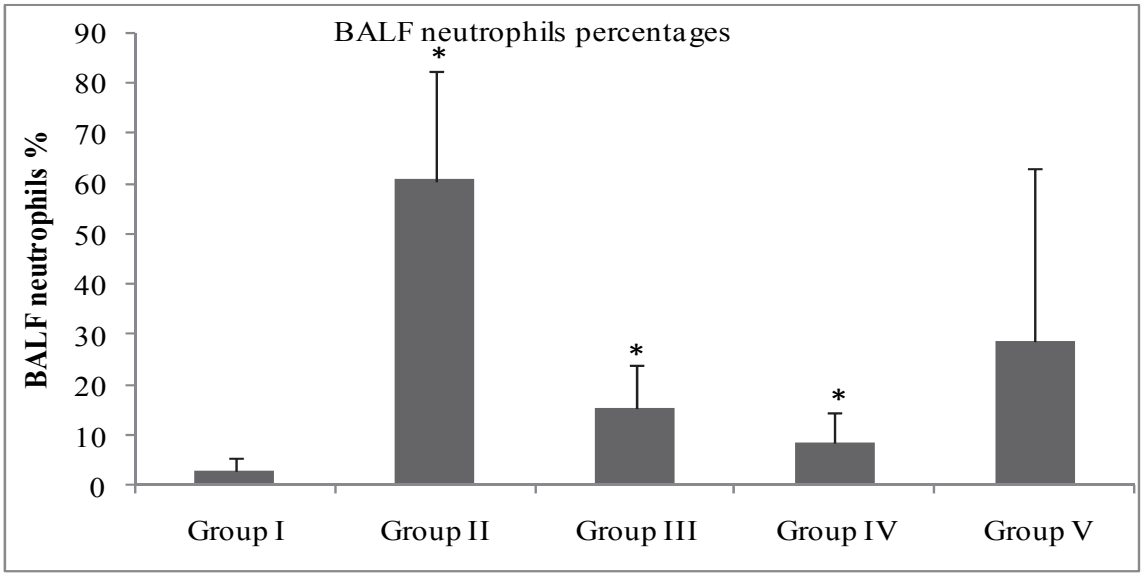

Figure 11: Neutrophils percentage of the BALF in different disease groups. The * indicates significant difference compared to clinically healthy horses.

4.10 Results of inflammatory markers

4.10.1 ELISA assays

4.10.1.1 IL-8 ELISA assay

Interleukin-8 concentration was measured by ELISA in 64 horses. There were highly significant increases of the IL-8 concentration in RAO-affected horses in exacerbation (32.83±33.93 pg/ml) and remission (17.6±9.92 pg/ml) compared to clinically healthy horses (6.15±2.17 pg/ml). Other disease groups showed no significant difference from controls (Tables 16, 30 and Figure 12).

4.10.1.2 MMP-2 ELISA assay

MMP-2 concentration was measured by ELISA in 64 horses and it showed significant differences between disease groups. RAO-affected horses in exacerbation (5.21±0.77 ng/ml) and remission (7.67±15.5 ng/ml) showed highly significant increases in MMP-2 concentration compared to clinically healthy horses (2.49±0.83 ng/ml). On the other hand, RAO-affected horses in remission showed a highly significant increase compared to RAO-affected horses in exacerbation and

horses with chronic interstitial pneumopathy (2.81±0.34 ng/ml). RAO-affected horses in exacerbation showed a highly significant increase in MMP-2 compared to horses with chronic interstitial pneumopathy. Other groups showed no significant differences (Tables 16, 30 and Figure 13).

4.10.1.3 MMP-9 ELISA assay

MMP-9 concentration was measured in 56 horses. Highly significant differences between different disease groups were detected. There was a highly significant increase in MMP-9 concentration in RAO-affected horses in exacerbation (433.34±89.05 pg/ml), remission (312.06±23.92 pg/ml) and in horses with chronic interstitial pneumopathy (263.2±23.85 pg/ml) compared to clinically healthy horses (176.29±60.22 pg/ml). RAO-affected horses in exacerbation showed a highly significant increase compared to RAO-affected horses in remission and chronic interstitial pneumopathy. On the other hand, RAO-affected horses in remission showed a significant increase compared to chronic interstitial pneumopathy horses. Other inter-groups differences were not significant (Tables 16, 30 and Figure 14).

4.10.2 Fluorescent method for MMP-8

Matrix metalloproteinase-8 activity was measured by the Sensolyte® 570 MMP assay kit based on the fluorimetric method which is ideal for detecting generic MMP-8 activity in biological samples. MMP-8 activity showed significant differences between all groups. In RAO-affected horses in exacerbation (0.84±1 µmol/l), remission (0.1±0.06 µmol/l) and in horses with chronic interstitial pneumopathy (0.02±0.01 µmol/l) there were highly significant increases in MMP-8 activity compared to clinically healthy horses (0.01±0.01 µmol/l). RAO-affected horses in exacerbation showed a highly significant increase in MMP-8 activity compared to RAO-affected horses in remission and horses with chronic interstitial pneumopathy. On the other hand, RAO-affected horses in remission showed a significant increase compared to chronic interstitial pneumopathy horses (Tables 16 and 30).

Table 16: Concentrations of MMP-2, MMP-9, IL-8 using ELISA and MMP-8 activity using fluorimetry; the results are expressed as mean values ± SD. The * indicates significances at $p<0.05$, the detailed significances for the inter-groups comparisons are listed in Table 30.

Disease groups	MMP-2 ELISA (ng/ml)	MMP-9 ELISA (pg/ml)	MMP-8 fluorimetry (µmol/l)	IL-8 ELISA (pg/ml)
Group I (n=15)	2.49±0.83	176.29±60.22	0.01±0.01	6.15±2.17
Group II (n=17)	5.21±0.77$^{*\uparrow}$	433.34±89.05$^{*\uparrow}$	0.84±1$^{*\uparrow}$	32.83±33.93$^{*\uparrow}$
Group III (n=18)	7.67±15.5$^{*\uparrow}$	312.06±23.92$^{*\uparrow}$	0.1±0.06$^{*\uparrow}$	17.6±9.92$^{*\uparrow}$
Group IV (n=11)	2.81±0.34	263.2±23.85$^{*\uparrow}$	0.02±0.01$^{*\uparrow}$	10.56±5.97
Group V (n=3)	3.6±0.27	253.29±34.84	0.07±0.06	13.27±3.28

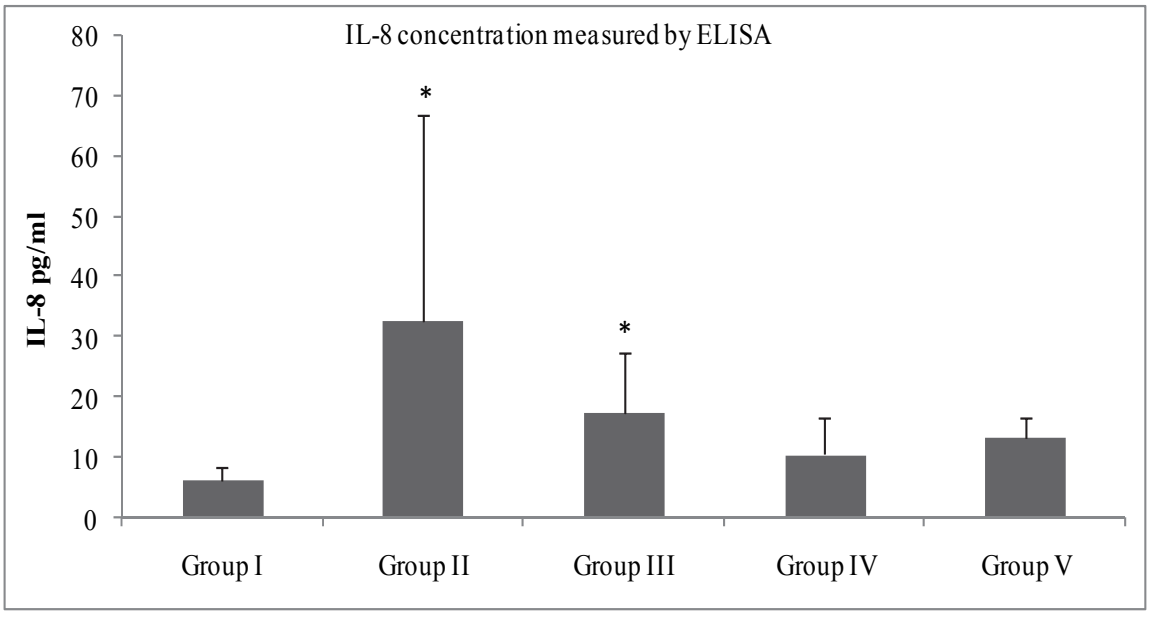

Figure 12: Concentration of IL-8 by ELISA in different disease groups. The * indicates significance compared to clinically healthy horses.

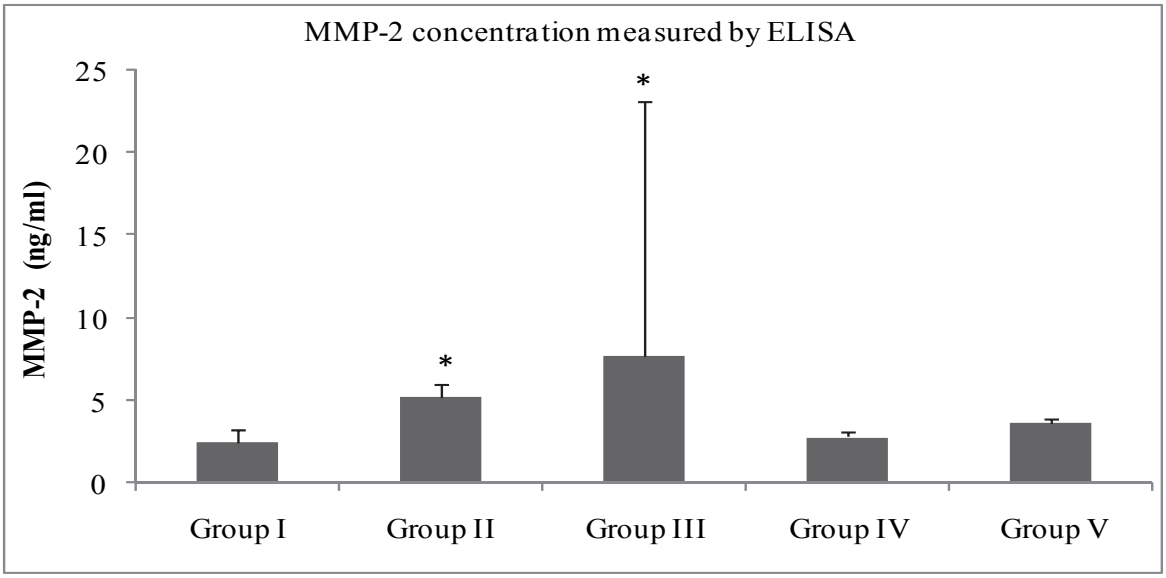

Figure 13: Concentration of MMP-2 by ELISA in different disease groups. The * indicates significance compared to clinically healthy horses.

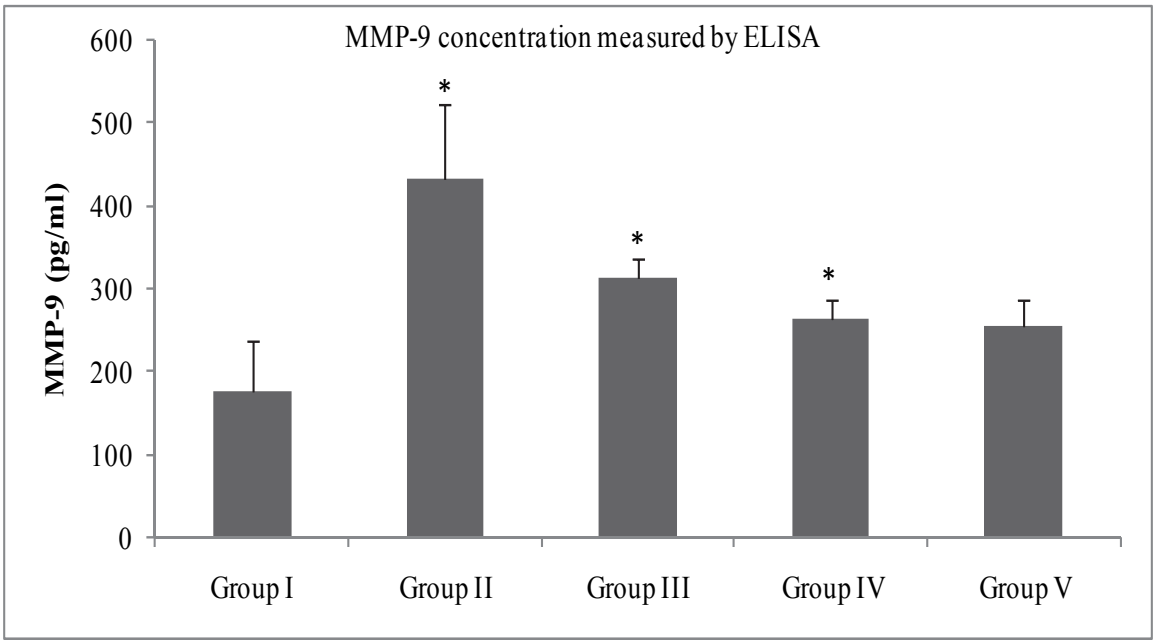

Figure 14: Concentration of MMP-9 by ELISA in different disease groups. The * indicates significance compared to clinically healthy horses.

4.10.3 Gelatin zymography

Gelatin zymography was performed on 55 samples from control horses (n=13), RAO-affected horses (exacerbation n=17; remission, n=14), horses with chronic interstitial pneumopathy (n=8) and horses with acute to subacute respiratory infection (n=3). Gelatinolytic activity bands were detected at 70 kDa for MMP-2 (activated form) and at 100 and 140 kDa for MMP-9 (proenzyme and activated form) (Figure 15).

In addition, MMP-2 bands showed significant differences between groups, where RAO-affected horses in exacerbation (17288.53±8927.59 peak area) and remission (3530.94±2894.15 peak area) showed highly significant increases in peak areas compared to healthy horses (1114.76±672.72 peak area). On the other hand, MMP-2 peak area of RAO-affected horses in exacerbation was significantly increased compared to RAO-affected horses in remission and to those with chronic interstitial pneumopathy (2799.45±2592.28 peak area) (Tables 26, 30 and Figure 16). Other inter-group differences were not significant.

MMP-9 bands showed significant differences between groups. RAO-affected horses in exacerbation (10967.31±9530.07 peak area) showed a highly significant increase in peak area compared to healthy horses (619.29±996.32 peak area), and also showed a highly significant increase in peak area compared to RAO-affected horses in remission (1832.16±2111.29 peak area) and horses with chronic interstitial pneumopathy (864.06±767.93 peak area) (Tables 26, 30 and Figure 17). Other inter-group differences were not significant.

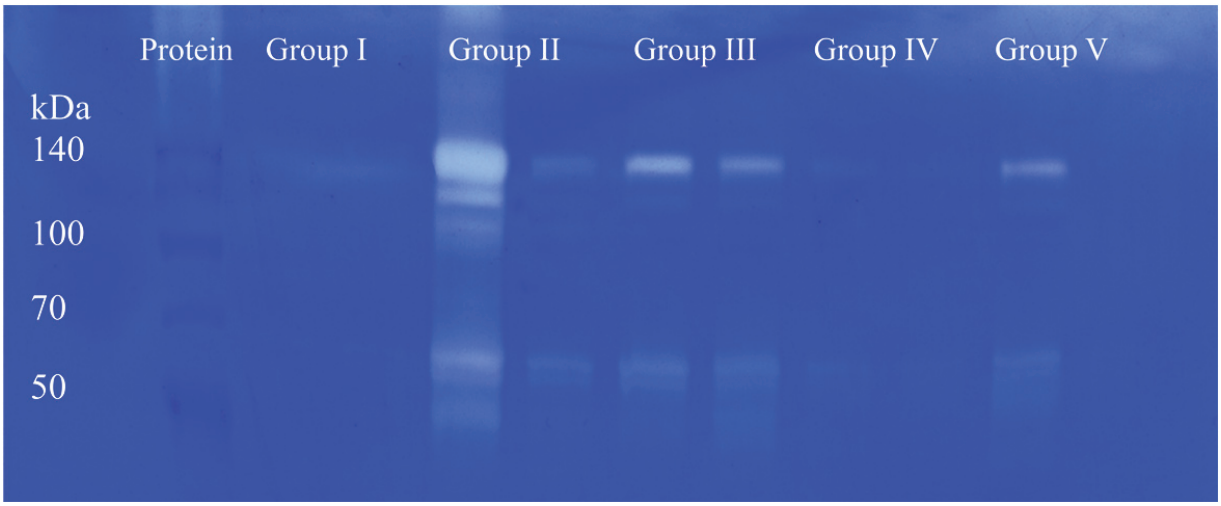

Figure 15: Gelatin zymography result in control, RAO horses in exacerbation and remission, horses with chronic interstitial pneumopathy and horses with acute to subacute respiratory infection. The 70 kDa bands are representative for MMP-2 activity and that at 140 kDa were considered representative for MMP-9 activity. The molecular weight of protein marker has the bands of 140, 100, 70 and 50 kDa.

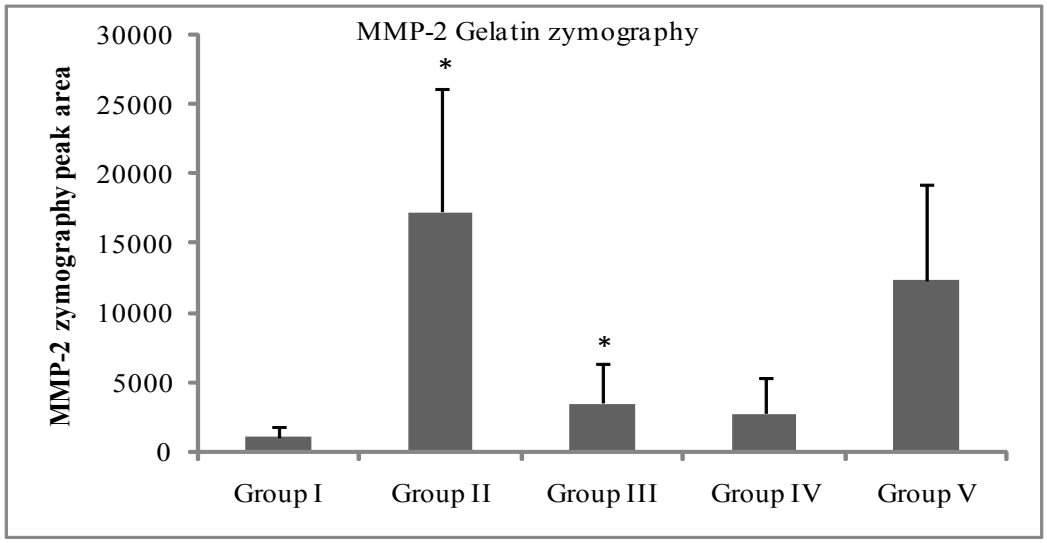

Figure 16: MMP-2 peak areas by zymography in different disease groups. The * indicates significance compared to clinically healthy horses.

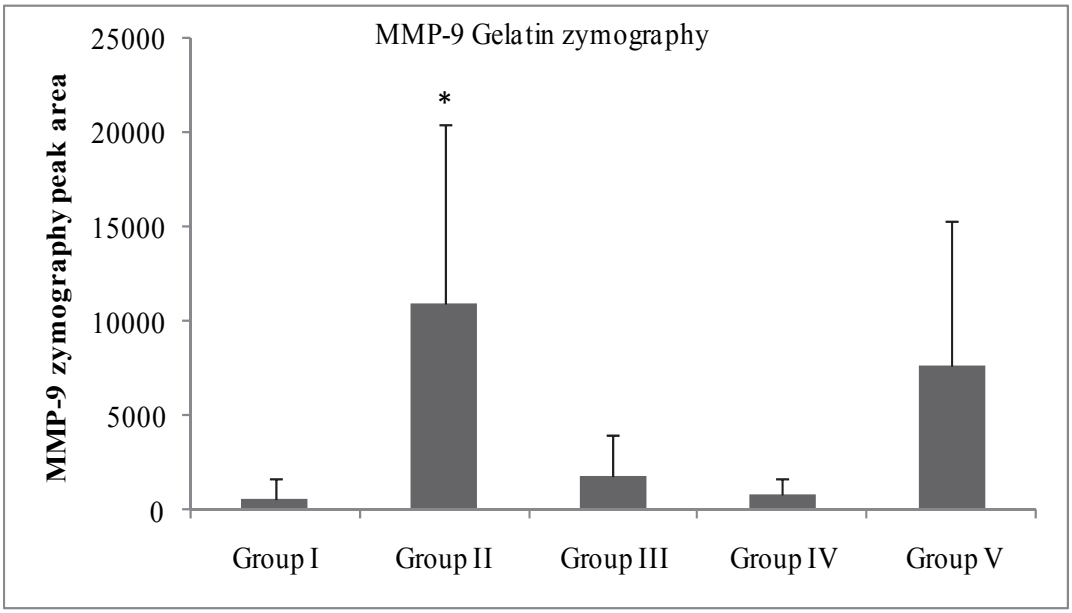

Figure 17: MMP-9 peak areas by zymography in different disease groups. The * indicates significance compared to clinically healthy horses.

4.11 Correlations between different examined parameters

Spearman correlations were performed between basic data, clinical examination scores after Ohnesorge et al. (1998), cytology of respiratory secretions (TBS and BALF), blood gas parameters, endoscopic score after Gerber et al. (2004), MMPs and IL-8. Strong correlation exists if the spearman correlation coefficient (r) is above 0.6, while moderate correlation exists, when r ranged between 0.4 – 0.6 and a weak correlation exists, when r ranged between 0.2 – 0.4. The complete tables of correlation are presented in the Appendix (Tables 17, 18 and 19).

4.11.1 Correlation of repiratory rate and clinical scores

Respiratory rate was moderately correlated with dyspnea score (r=0.55), endoscopy score (r=0.51) and BAL score (r=0.46), while a strong correlation with the total examination score (r=0.61) was observed. On the other hand, there was a weak correlation between respiratory rate and lung percussion and auscultation score and blood gas analysis score (Table 17).

4.11.2 Correlation between BAL, TBS cytology and clinical score

Correlations between BALF, TBS cytology and clinical examination score are presented in Table 17. BALF neutrophils percentage was strongly correlated with endoscopy score (r=0.72), BAL score (r=0.94) and total examination score (r=0.87); it was moderately correlated with dyspnea score (r=0.51) and a weak correlation was found with the cough score (r=0.29), lung percussion score (r=0.27) and blood gas score (r=0.33). TBS neutrophils percentage was strongly correlated with dyspnea score (r=0.68), endoscopy score (r=0.64), BAL score (r=0.78) and total examination score (r=0.85), a weak correlation was evident with the cough score (r=0.36). BALF neutrophils percentage was strongly correlated with TBS neutrophils percentage (r=0.81).

4.11.3 Correlation of BALF and TBS cytology, MMPs and IL-8

BAL neutrophils percentage was strongly correlated with MMP-2 concentration (r=0.75), MMP-9 concentration (r=0.81) and MMP-8 activity (r=0.74); a moderate correlation was found with IL-8 (r=0.4). TBS neutrophils percentage was strongly correlated with MMP-2 concentration (r=0.72), MMP-9 concentration (r=0.73) and MMP-8 activity (r=0.65) (Table 18).

4.11.4 Correlation of clinical scores, MMPs and IL-8

The correlations of the clinical score, MMPs and IL-8 are listed in Table 18. Cough score was weakly correlated to MMP-2 concentration (r=0.31), MMP-9 concentration (r=0.3) and MMP-8 activity (r=0.25). Dyspnea score was strongly correlated to MMP-2 concentration (r=0.61), MMP-9 concentration (r=0.68) and MMP-8 activity (r=0.63), but weakly correlated to IL-8 concentration (r=0.32). Endoscopy score was strongly correlated to MMP-9 concentration (r=0.66) and MMP-8 activity (r=0.62), and moderately correlated to MMP-2 concentration (r=0.57), weak correlation was observed with the IL-8 concentration (r=0.36). The total examination score showed strong with MMP-2 concentration (r=0.75), MMP-9 concentration (r=0.79) and MMP-8 activity (r=0.78), while moderate correlation was observed with IL-8 concentration (r=0.46).

4.11.5 Correlation of lung function and clinical score

Oxygen tension was negatively and weakly correlated with cough score (r=-0.27) and dyspnea score (r=-0.38), and a moderate negative correlation was found with the endoscopy score (r=-0.47). Arterial oxygen differences was moderately correlated with dyspnea score (r=0.47) and endoscopy score (r=0.58). Interpleural pressure was moderately correlated with dyspnea score (r=0.47) (Table 17).

4.11.6 Correlations between MMP methods

MMP-2 concentration measured by ELISA was strongly correlated with the results of MMP-2 peak areas obtained by gelatin zymography (r=0.72). Moreover, MMP-9 concentration measured by ELISA was moderately correlated with the results of MMP-9 peak areas obtained by gelatin zymography (r=0.58) (Table 19).

4.11.7 Correlations between endoscopy scoring methods

Endoscopy score was strongly correlated with the amount of secretions score (r=0.9), viscosity of secretions score (r=0.92) and moderately correlated with the tracheal bifurcation score (r=0.58) (Table 19).

Table 17: Correlations between clinical examination scores, TBS and BALF neutrophils and lung function testing. The results are presented as correlation coefficients with the p-value in brackets. *= significance at <0.05 and **= significance at 0.01.

	Respiratory rate	BAL neutrophils percentage	TBS neutrophils percentage	PaO_2 (mmHg)	$AaDo_2$ (mmHg)	Interpleural pressure (cm H_2O)
Cough score	0.10 (0.418)	0.29* (0.019)	0.36* (0.025)	-0.27* (0.032)	0.24 (0.065)	-0.13 (0.412)
Dyspnea score	0.55** (0.000)	0.51** (0.000)	0.68** (0.000)	-0.38** (0.002)	0.47** (0.000)	0.47** (0.002)
Lung percussion score	0.38** (0.002)	0.22 (0.087)	0.41** (0.01)	-0.42** (0.001)	0.38** (0.003)	0.3 (0.058)
Lung auscultation score	0.35** (0.005)	0.27* (0.031)	0.36* (0.023)	-0.28* (0.028)	0.28* (0.025)	0.3 (0.058)
Endoscopy score	0.51** (0.000)	0.72** (0.000)	0.64** (0.000)	-0.47** (0.000)	0.58** (0.000)	0.37* (0.021)
BAL score	0.46** (0.000)	0.94** (0.000)	0.78** (0.000)	-0.42** (0.001)	0.46** (0.000)	0.31 (0.054)
Blood gas score	0.39** (0.001)	0.33** (0.009)	0.24 (0.147)	-0.6** (0.000)	0.65** (0.000)	0.51** (0.001)
Total examination score	0.61** (0.000)	0.87** (0.000)	0.85** (0.000)	-0.61** (0.000)	0.67** (0.000)	0.48** (0.002)
BAL neutrophils percentage			0.81** (0.000)	-0.44** (0.000)	0.45** (0.000)	0.4* (0.011)
TBS neutrophils percentage				-0.56** (0.000)	0.54** (0.000)	0.29 (0.128)

Table 18: Correlations between clinical examination scores, TBS and BALF cytology and different inflammatory biomarkers measured in the present study. The results are presented as correlation coefficients with the p-value in brackets. *= significance at <0.05 and **= significance at 0.01.

	MMP-2 ELISA (ng/ml)	MMP-9 ELISA (pg/ml)	MMP-8 fluorimetry (µmol/l)	IL-8 ELISA (pg/ml)	MMP-2 zymography (peak area)	MMP-9 zymography (peak area)
Cough score	0.31* (0.012)	0.3* (0.026)	0.25* (0.044)	0.2 (0.111)	0.25 (0.068)	0.36** (0.006)
Dyspnea score	0.61** (0.000)	0.68** (0.000)	0.63** (0.000)	0.32** (0.01)	0.68** (0.000)	0.63** (0.000)
Lung percussion score	0.1 (0.437)	0.13 (0.345)	0.16 (0.207)	-0.05 (0.702)	0.28* (0.042)	0.13 (0.364)
Lung auscultation score	0.18 (0.152)	0.23 (0.091)	0.25* (0.043)	0.14 (0.269)	0.29* (0.034)	0.23 (0.086)
Endoscopy score	0.57** (0.000)	0.66** (0.000)	0.62** (0.000)	0.36** (0.003)	0.69** (0.000)	0.56** (0.000)
BAL score	0.77** (0.000)	0.81** (0.000)	0.74** (0.000)	0.41** (0.001)	0.78** (0.000)	0.67** (0.000)
Blood gas score	0.21 (0.093)	0.19 (0.167)	0.2 (0.117)	0.2 (0.109)	0.4** (0.003)	0.4** (0.002)
Total examination score	0.75** (0.000)	0.79** (0.000)	0.78** (0.000)	0.46** (0.000)	0.8** (0.000)	0.71** (0.000)
BAL neutrophils percentage	0.75** (0.000)	0.81** (0.000)	0.74** (0.000)	0.4** (0.001)	0.79** (0.000)	0.76** (0.000)
TBS neutrophils percentage	0.72** (0.000)	0.73** (0.000)	0.65** (0.000)	0.37* (0.002)	0.77** (0.000)	0.69** (0.000)

Table 19: Correlations between MMP-2 and MMP-9 measured by both ELISA and gelatin zymography and endoscopy score with amount and the viscosity of secretions. The results are presented as correlation coefficients with the p-value in brackets. *= significance at <0.05 and **= significance at 0.01.

	MMP-2 zymography (peak area)	MMP-9 zymography (peak area)	Amount of secretions	Viscosity of secretions	Tracheal bifurcation
MMP-2 ELISA (ng/ml)	0.72** (0.000)	0.66** (0.000)	0.53** (0.000)	0.6** (0.000)	0.2 (0.144)
MMP-9 ELISA (pg/ml)	0.72** (0.000)	0.58** (0.000)	0.58** (0.000)	0.65** (0.000)	0.25 (0.097)
Endoscopy score	0.69** (0.000)	0.56** (0.000)	0.9** (0.000)	0.92** (0.000)	0.58** (0.000)

5 Discussion

Respiratory disease affecting horses is one of the most common problems affecting performance. As a chronic inflammatory condition, it is usually accompanied by hypersensitivity reactions resulting in neutrophils recruitment into the airway lumen with development of signs of airways inflammation (Robinson et al., 1996). The present study evaluated various inflammatory markers including MMP-2, MMP-8, MMP-9 and IL-8 concentration and/or activity. The clinical and diagnostic importance of these parameters was investigated in BALF of horses with RAO, chronic interstitial pneumopathy and acute to subacute respiratory infection. For this purpose, selected assays have been performed. The results are discussed separately for the methodology and the evaluation of distinct parameters in BALF.

5.1 Discussion of the methods

5.1.1 Collection methods for TBS and BALF

The respiratory fluid samples obtained in the present study were TBS and BALF which is representing the lower airways, but the amount of fluid recovered by TBS is much smaller than the amount from BAL. The dilution of BAL samples was unknown due to the difficulty in measuring the dilution factor. To avoid misleading results, a standard volume of PBS, standard number of aliquots and a standard sampling site was recommended by Haslam and Baughman (1999). Although, in another study by Rennard et al. (1986), McGorum et al. (1993c) the use of urea and albumin has been recorded to estimate the dilution factor of BAL but the time from infusion until aspiration back of PBS has an effect on the concentration of urea and albumin in BALF. The amount of PBS used for collecting BAL samples in the present study were 500 ml and this was recommended by the International Workshop on Equine Chronic Airway Disease (Robinson, 2001). The application of a local anesthetic (20 ml of 2% lidocaine) to the mucosa of the larynx and bronchi until the level of the tracheal bifurcation is carried out in our study to decrease the chance of coughing and also was carried out by different authors (Robinson, 2001, Koblinger et al., 2013, Hoffman, 2008). Various other authors do not consider the general use of local anesthetics (McGorum and Dixon, 1994, Lapointe et al., 1994). The BALF samples were gently mixed and the cytology preparation was performed immediately after collection to obtain

optimal cytological valuable specimens (Hoffman, 2008). The differential cell count in TBS and BALF samples were performed in our study in 500 cells for every sample and the mean was taken, similar recommendations to examine 500 cells were reported by Viel and Hewson (2003). In the contrast, 200 to 400 cells in equine BALF were used by other authors for cell differentiation (Sweeney et al., 1994, McGorum and Dixon, 1994). Although, the total cell count was performed by Sweeney et al. (1994) and others, we did not perform it based on the previous results of McGorum and Dixon (1994) who stated that the total cell count is of a little diagnostic importance due to the unknown dilution factor of TBS and BALF and the relatively inaccurate measurement (Lapointe et al., 1994).

5.1.2 Discussion of gelatin zymography

Gelatinolytic activity was measured by SDS-page gelatin zymography, where the bands were identified based on the molecular weights represented by complex forms >110 kDa, proMMP-9 90-110 kDa, active MMP-9 75-85 kDa, proMMP-2 65-75 kDa and active MMP-2 <65 kDa (Raulo et al., 2001a, Nevalainen et al., 2002). We have found similar bands, but our results were based on the molecular weight of the samples compared to that of protein marker, MMP-2 and MMP-9 human standards. Meanwhile, the inhibition with EDTA was used to identify the gelatinolytic bands as MMPs in a previous study by Raulo et al. (2001a). Various samples dilutions were reported in previous studies, TELF samples were diluted to 1:6 on the basis of serum/tracheal fluid urea ratio, then samples were mixed 2:1 with sample buffer (Raulo et al., 2000). Synovial fluid samples were diluted 1:10 (Fietz et al., 2008). In our study, 1:20, 1:10, 1:5 and 1:1 were tested, of which we found the dilution of 1:1 with sample buffer was the most suitable dilution for running the gels and to give clear activity bands and this was in agreement to the report of Hu and Beeton (2010), the difference in samples dilutions compared to other authors may be attributed to the difference in the initial sample dilution during collection of samples. In synovial fluid samples, MMP-2 activity was indicated at 72 kDa and MMP-9 activities at 92, 120, and 250 kDa (Fietz et al., 2008) which was similar to our own results. Further analysis of the MMP-2 and MMP-9 bands obtained from BALF samples were performed using densitometry software *ImageJ* for objective quantification of the bands and the results were calculated based on peak area (Hu and Beeton, 2010), we also aimed for direct quantitative measurements using

ELISA kits validated for equine plasma. To our knowledge this is the first record to use ELISA kits for measuring MMP-2, MMP-9 in BALF samples of RAO horses.

5.1.3 Discussion of fluorimetry method

Since the difficulties occurred in measuring the MMP-8 activity by gelatin zymography, a fluorimetric method has been introduced for the first time for a more sensitive determination of MMP-8 in equine BALF. It contains 5-FAM/QXL™520 fluorescence resonance energy transfer peptide that is cleaved by active MMP-8 (Stryer, 1978).

5.2 Discussion of the results

In our study, we successfully collected 64 BALF samples, from 15 clinically healthy horses and 49 horses with signs of respiratory tract disorders, while patients with other illness were excluded from the study. The selection criteria for classifying horses into diseased or healthy groups was based on the anamnestic signs, percentages of neutrophils in BALF, clinical score, interpleural pressure, endoscopy and blood gas analysis findings. In human medicine, the selection criteria for COPD are based on the clinical and cytological examination of sputum and BALF (Barnes, 2000). RAO, like other chronic respiratory diseases of horses, is more common in older horses. Dixon et al. (1995b) diagnosed RAO at 4-24 years and Robinson et al. (2000) at a mean age of 19.5 years. Previous knowledge of RAO includes that horses at the age of 6-10 years or older are the most likely to be affected by RAO (Lavoie, 2007, McPherson et al., 1979b) and this was consistent with our results, where the average age of RAO-affected horses in exacerbation and remission was 17.44 and 12.49 years, respectively. On the other hand, the gender of horses was found to have no influence on the occurrence of the disease occurrence as both sexes were equally affected, this was similar to a study by Hotchkiss et al. (2007).

The cough and exercise intolerance are the main reason for presenting the horse to the clinic for further clinical examination, although previous studies recorded 84% of horses with chronic lung disease to have cough (McGorum and Dixon, 1994, Dixon et al., 1995b, Laumen et al., 2010) and therefore it is not a suitable distinguishing feature alone, it should be accompanied by other

clinical examinations (Burrell et al., 1996) beside detecting the level and activity of MMPs in BALF of different disease groups and with different techniques.

The respiratory rate measurement was used as an indicator of the severity of the diseases although it lacks specificity and sensitivity according to a study by Dixon et al. (1995a), where they recorded a mean of 16 breaths per minute in RAO horses and in healthy horses 12 breaths per minute. In our own study we found 14 breaths per minute in healthy horses and a mean of 21 breaths per minute in RAO-affected horses in exacerbation.

In RAO-affected horses during exacerbation we observed painful cough, labored expiratory effort, exercise intolerance, flaring of nostrils and a heaves line in some patients, and this was in agreement with previous studies by Laumen et al. (2010), Moran and Folch (2011), McPherson et al. (1978). In contrast to exacerbation, RAO-affected horses in remission were commonly presented with normal breathing patterns at rest, but developed increasing expiratory dyspnea during exercise, similarly to observations by McGorum and Pirie (2008), Gerber (1973). Additionally, horses with chronic interstitial pneumopathy showed exercise intolerance, increased breathing, sporadic cough and severe dyspnea and this was similarly observed in studies by Nout et al. (2002), Donaldson et al. (1998). In contrast to this, horses with acute to subacute respiratory infection were presented mainly with moist cough and nasal discharge and this was in agreement with MacKay and Urquhart (1979). Our observations regarding temperature and heart rate showed no significant differences in all diseased horses compared to clinically healthy horses and this might support the hypothesis that these conditions are of chronic nature (Couetil et al., 2001).

Scoring system was used during clinical examinations, the score range in our study was from 0-15 points and this score was modified after studies by Ohnesorge et al. (1998), Kampmann et al. (2001), Dieckmann (1987), where they used the percentage of neutrophils in TBS as a determinant for the respiratory secretion score while we used neutrophils percentage in BALF instead. On the other hand, Tesarowski et al. (1996) used a score system with a maximum 25 points which include all criteria of the respiratory system including auscultation. Due to the significant correlations between the clinical score parameters including blood gases, interpleural pressure, BALF neutrophils percentage, we confirm the use of the method used for lung scoring as an appropriate way for scoring the lung. The total examination score ranged from 0-1 in

clinically healthy horses, 2-6 in RAO-affected horses in remission and 6-14 in RAO-affected horses in exacerbation, this is in agreement with the studies of Ohnesorge et al. (1998), Herholz et al. (2002). In our study, the cough score had a weak correlation with the MMP-2 and MMP-9 concentrations and MMP-8 activity, while dyspnea and endoscopy scores were strongly correlated with the MMP-2, MMP-8 and MMP-9 concentrations. On the other hand, our results support the importance of MMP-2, MMP-8, MMP-9 and IL-8 in assessing horses with respiratory inflammations where good correlation of the clinical score to these inflammatory markers was observed. Fischer (1980), Fey (2004) reported that there were correlations of the clinical score with different examined parameters.

Endoscopy was considered as a part of the clinical evaluation of horses. The endoscopy score used in our study included the amount, viscosity of tracheal secretions and the thickening of tracheal bifurcation according to Gerber et al. (2004), Fischer (1980), who scored the amount, viscosity of secretions in a scale range of 0-5 points and the tracheal bifurcation had a scale range of 0-3 points which is consistent with our own method. In a study by Fey (2004), a score of 4 points was used. The criteria used in our own study for endoscopic assessment of airways in healthy and diseased horses were modified according to Ohnesorge et al. (1998), Gerber et al. (2004). In previous literatures, the presence of secretion in the airways was a consequence of housing horses (Holcombe et al., 2001). The endoscopic appearance of the lower airways of clinically healthy horses revealed normal tracheal mucosa, no secretions, and a thin tracheal bifurcation. In contrast to this, RAO-affected horses in exacerbation had high amounts of seromucoid to mucoid secretions in the trachea, which were very viscous sticking dorsally to the mucosa and spreading to the lower third of trachea. A mild to severe thickening of the tracheal bifurcation was observed in a study performed by Koblinger et al. (2011). Our results were in agreement with that of Robinson et al. (2003), who reported an accumulation of mucus in the trachea of affected horses. In general, the endoscopy score was strongly correlated with TBS and BALF neutrophils percentages, MMP-8 activity and MMP-9 concentration and moderately correlated with respiration rate, arterial oxygen differences and MMP-2 concentration. On the other hand, a strong correlation was found between endoscopy score after Ohnesorge et al. (1998) and the amount, viscosity of secretions and moderately correlated to the thickening of the tracheal bifurcation scores after Gerber et al. (2004).

The radiographic appearance of horses with chronic interstitial pneumopathy and in horses with acute to subacute respiratory infection was identified by its bronchial and interstitial pattern with increased interstitial opacity. These results were in agreement with Lakritz et al. (1993), who reported an increase in the interstitial opacity with the presence of interstitial infiltration forming a nodular appearance in foals with interstitial pneumonia. Although thoracic radiography is not specific for RAO in exacerbation and remission, it may be important to rule out other types of lung disease (Robinson, 2001).

The differential count of TBS and BALF involved inflammatory cells that were expressed as percentages; it includes mainly neutrophils, macrophages, lymphocytes, eosinophils and mast cells, with presence of goblet cells and pulmonary epithelial cells. The cytologic evaluation of respiratory secretions was focused basically on the percentage of neutrophils due to their importance during respiratory disease progress, where a minimum of 8% was used in our study as an inclusion criterion differentiating between healthy and diseased horses, while in a study by Dixon et al. (1995a) 5% is a margin, additionally, the percentage of neutrophils in TBS and BALF are considered an indicative of the severity of airway disease (Nuytten et al., 1983, Franchini et al., 1998). However, the neutrophils were found in higher percentages in TBS than that in BALF.

Our results further support the clinical examination and lung function findings, and give a suggestive clue for the presence of airway inflammation and this was also proved by the increase in neutrophils chemotactic cytokine IL-8 (Riihimaki et al., 2008b, Riihimaki et al., 2008a), where TBS and BALF neutrophils percentages were highly significant increased in RAO-affected horses. Additionally, there was no significant changes in eosinophils and mast cells percentages and this was agreed to a previous study performed by Tesarowski et al. (1996). On the other hand, TBS and BALF neutrophils percentages were strongly correlated with endoscopy score and total examination score, and were moderately correlated with $AaDO_2$ values. IL-8, MMP-2, MMP-9 concentrations and MMP-8 activity were correlated to the neutrophils percentages in TBS and BALF, supporting their role as inflammatory markers for different types of respiratory disease in horses.

The arterial blood gas analysis was performed in non sedated horses (Davis and Rush, 2002). In our investigations the measurements were performed on 3 samples on 3 successive days and then the mean value was calculated. We found that PaO_2 was decreased in RAO-affected horses in exacerbation with a mean value of 87.9 mmHg, this result was in agreement with a study by McPherson et al. (1978), where they recorded PaO_2 of less than 82 mmHg in RAO affected horses and in control horses was >86 mmHg, and also in agreement with a study by Davis and Rush (2002), who reported PaO_2 level of 80.8 mmHg in RAO affected horses and this confirms that RAO leading to changes in ventilation which results in inefficient blood gas exchange (Dixon et al., 1995c). On the other hand, PaO_2 was negatively correlated with TBS and BALF neutrophils percentage, MMP-2, MMP-9 and IL-8 concentrations as well as MMP-8 activity, this suggesting the role of PaO_2 and inflammatory markers in the evaluation of functional impairment of the lung tissue. $PaCO_2$ showed no significant differences in diseased groups compared to clinically healthy horses. There was a very weak or no correlation with other clinical findings, while in results by Herholz et al. (2002) PaO_2 is closely correlated with the degree of dyspnea. Additionally, the $AaDO_2$ was measured by the same analyzer not manually calculated, in our study it was increased in diseased horses compared to healthy horses and this was in agreement with a previous study by Raulo et al. (2006), who found that $AaDO_2$ in RAO affected horses exceeded the reference value of 10 mmHg with a median value of 24.1 mmHg.

According to previous studies by Costa et al. (2000), Erichsen et al. (1994), the interpleural pressure provided a valid measurement of the severity of the bronchial obstruction and this was in agreement to our study, where the interpleural pressure showed significant increase in diseased horses compared to clinically healthy horses (<4 cmH_2O), additionally, the moderate correlation between interpleural pressure and clinical scores and inflammatory biomarkers indicates a valid estimate of the severity of the disease.

The IL-8 concentration was increased in RAO-affected horses in exacerbation and remission compared to clinically healthy horses, and this supports the hypothesis that neutrophils are considered as active elements in the inflammatory response and may influence the immune processes through cytokine secretion, where neutrophils percentage was increased in TBS and BALF (Joubert et al., 2001, Cassatella, 1995) and this is also suggesting the effect of initial neutrophilic inflammatory reaction and potential irreversible airway remodeling (Art et al., 2006,

Ainsworth et al., 2006). The concentration of IL-8 was weakly correlated with dyspnea and endoscopy score, TBS neutrophils percentage and arterial oxygen difference, while it was moderately correlated with BALF neutrophils percentage.

In the present study, matrix metalloproteinases as inflammatory biomarkers were evaluated including MMP-2, MMP-9 concentrations as well as MMP-8 activity. The diagnostic importance of these markers was investigated in BALF of clinically healthy horses and those with lower airway diseases. The levels of MMP-2, MMP-9 and MMP-8 in our own study were increased in RAO-affected horses in exacerbation and remission, chronic interstitial pneumopathies and in horses with acute to subacute respiratory infection, this was similar to previous studies by Koivunen et al. (1997a), (1997b), Raulo et al. (2001b) who found an elevated levels of gelatinases and collagenases in TBS and BALF of RAO horses. These elevated gelatinolytic and collagenolytic activities in the extracellular compartment of the BALF were highly correlated with the results of the clinical examinations and BALF cytology. The concentration and activity of these MMP members were very low in healthy horses compared to diseased group, and this might be attributed to the normal remodeling process in the lungs (Mautino et al., 1997) where MMP activity were countered by the action of TIMPs activity. However, during the active phase of pulmonary inflammation, the extracellular levels of TIMPs were suggested to be insufficient to counteract the activated MMPs in the patients used in the present study, additionally, the imbalance between MMP activation and inhibition is usually associated with tissue destruction in inflammatory lung diseases (Clutterbuck et al., 2010).

The MMP-2 was evaluated by ELISA and gelatin zymography, our results addressed significant increase of MMP-2 concentration measured by ELISA in RAO-affected horses in exacerbation and remission compared to clinically healthy horses. In contrast, Raulo et al. (2001a), Simonen-Jokinen et al. (2005b) found that MMP-2 did not play a major role in chronic lung inflammation, where it is not frequently detected in inflammatory responses. Meanwhile, the concentration of MMP-2 measured by ELISA showed highest values in horses suffering from RAO in remission, which are characterized by a low grade inflammation and pulmonary dysfunction compared to RAO in exacerbation, therefore, a role of this enzyme remains obscure and needs further study.

The MMP-9 was the main MMP present in the airways of RAO susceptible horses following hay dust inhalation challenge (Simonen-Jokinen et al., 2005a, Nevalainen et al., 2002). The increases in MMP-9 were found to be correlated with the increases in TBS and BALF neutrophils percentages and this supports the hypothesis of the role of MMP-9 for neutrophils migration through the basement membrane (Delclaux et al., 1996) and its role in airway inflammation and remodeling (Prause et al., 2004).

Consistent with the above results, SDS-page gelatin zymography was performed for the detection of MMP-2 and MMP-9 activities in BALF samples. The MMP-2 activity at 70 kDa was identified by comparing it with the molecular weight of a human MMP-2 standard running on the same gel; and the MMP-9 activity was defined at 100 to 140 kDa, which is equal to the activity of MMP-9 standards. The results of gelatin zymography are in agreement with a previous study that has identified MMP activity bands depending on the molecular weight (Simonen-Jokinen et al., 2005b, Raulo et al., 2001a).

To our knowledge, the measurement of the peak areas of MMP-2 and MMP-9 bands in equine BALF by densitometry using ImageJ software is a novel approach for detecting MMP activity in a quantitative method. Since MMP-9 is secreted from neutrophils and its main function is to enable migration of leucocytes to the site of inflammation (Watanabe et al., 1993), the results of the present study indicate that MMP-2 and MMP-9 were significantly increased in RAO-affected horses in exacerbation and remission compared to clinically healthy horses and this is supported by the significantly increased neutrophils percentages in BALF as previously described.

Since collagen zymography for the detection of MMP-8 activity in BALF samples was very difficult to perform, a photometric activity assay was performed instead (Slanina et al., 2011). To our knowledge, the present study introduced for the first time the use of a fluorimetric activity assay for more sensitive determination of MMP-8 in equine BALF. In a study by Raulo et al. (2001b), the collagenolytic activity was approximately 7 times greater in TELF samples from horses with RAO compared to healthy horses. Meanwhile, immunoreactivity of MMP-8 and MMP-13 was significantly increased in the same samples of horses with RAO compared to healthy horses and this was similar to our own results, where the MMP-8 activity was found to be increased in RAO-affected horses in exacerbation, remission and horses with chronic interstitial

pneumopathy compared to clinically healthy horses. Additionally, the activity of MMP-8 in the present study in horses during disease remission was apparently decreased in comparison to that in exacerbation, and this suggests that MMP-8 participate in pulmonary tissue destruction during exacerbation which was in agreement with the results of Raulo et al. (2001b) and may indicate an ongoing disease stage that may reflect pathologically elevated activity takes place in the airways of diseased horses.

6 Conclusions

We concluded that:

1. There was a clear difference between clinically healthy horses and diseased horses concerning the clinical score, endoscopic findings, respiratory secretion cytology and inflammatory biomarkers. The BALF and TBS samples were most representative of respiratory disease. The results of respiratory secretions cytology remain very important data, when the diagnosis is unclear to differentiate the type of disease present.
2. BALF samples could be utilized to indicate the total gelatinolytic activity of the whole respiratory tract, especially active forms of MMP-9, and therefore would be a useful diagnostic tool for equine respiratory disorders and airway inflammation.
3. Strong correlations were present between clinical scores, concentrations of inflammatory biomarkers (MMP-2, MMP-8, MMP-9 and IL-8), TBS and BALF cytology. The increased concentrations of MMP-2, MMP-8, MMP-9 and IL-8 in different chronic pneumopathies suggest the useful role of these biomarkers to evaluate the severity of respiratory disease and impairment of lung tissue during the active ongoing inflammation and may have prognostic value.
4. As a result of the impossibility of performing gelatin zymography for detecting MMP-8, a new fluorimetric method, was used successfully for measuring of MMP-8 in BALF of horses in the present study.

Finally, further studies should focus on the balance between MMPs and TIMPS, their progression during disease and possible improvement during therapy.

7 Summary

Tarek Shety (2014):

Determination of Matrix Metalloproteinases and Interleukin-8 in Bronchoalveolar Lavage Fluid in Horses with Different Lung Diseases

The aim of the present study was to investigate the clinical importance of inflammatory markers in the pathogenesis of RAO, chronic interstitial pneumopathy and acute to subacute respiratory infections in horses in parallel with the clinical examination scores, TBS and BALF cytology and pulmonary function testing.

To our knowledge, the detection of the concentration of MMP-2 and MMP-9 in BALF by ELISA was the first trial performed in our study and was found to be an easy, accurate and reliable method. In addition, a semi-quantitative densitometry method based on the gelatin zymography bands for estimating the MMP-2 and MMP-9 activity were performed. As the collagen zymography for measuring MMP-8 was impossible to perform; the concentration of MMP-8 was detected in BALF by a fluorescent method.

A total of 64 warmblood horses, admitted to the Equine Clinic, Freie Universität Berlin, were used in the present study. Fifteen clinically healthy horses were used as the healthy standard which had no history of respiratory disease and BALF neutrophils percentage ≤8%. Clinical history, physical examination, endoscopy, radiography of the lung and percentage of neutrophils in BALF and TBS were used as the basis for grouping of diseased horses. RAO-affected horses in exacerbation (n=17), in remission (n=18), horses with chronic interstitial pneumopathy (n=11) and horses with acute to subacute respiratory infection (n=3) are the main disease groups studied.

The total examination score were increased significantly in diseased horses compared to controls. The percentage of neutrophils in BALF and TBS was correlated with the concentration of MMP-2, MMP-8, MMP-9 and IL8 and this support the role of these inflammatory markers in the pathogenesis of lung disease.

MMP-2 concentration was significantly increased in RAO-affected horses in exacerbation (5.21±0.77 ng/ml) and remission (7.67±15.5 ng/ml) compared to clinically healthy horses (2.49±0.83 ng/ml). MMP-9 concentration was significantly increased in RAO-affected horses in exacerbation (433.34±89.05 pg/ml), remission (312.06±23.92 pg/ml) and horses with chronic interstitial pneumopathy (263.2±23.85 pg/ml) compared to healthy horses (176.29±60.22 pg/ml). These results were confirmed by zymography for MMP-2 and MMP-9 activity in 55 horses. MMP-8 concentration was also significantly increased in RAO-affected horses in exacerbation (0.84±1 µmol/l), remission (0.1±0.06 µmol/l) and horses with chronic interstitial pneumopathy (0.02±0.01 µmol/l) compared to healthy individuals (0.01±0.01 µmol/l) as evaluated by fluorescent method. BALF neutrophil percentage showed a high positive correlation with the concentration of MMP-2, MMP-8 and MMP-9. Also, a highly positive correlation was found with the MMP-2 and MMP-9 activity measured by gelatin zymography. These results were suggestive for its role in the pathogenesis of lung disease. In addition, IL-8 was significantly increased in RAO-affected horses in exacerbation and remission.

The observations of the present study provide new insights into the diagnostic potential of various biomarkers in equine airway disease and could be used as an important tool for detecting lung tissue remodeling. Further studies should be focused on the role of TIMPs as a therapeutic approach.

8 Zusammenfassung

Tarek Shety (2014):

Bestimmung von Metalloproteinasen und Interleukin-8 in bronchoalveolären Lavageflüssigkeit bei Pferden mit verschiedenen Pneumopathien

Ziel der vorliegenden Studie war es, die klinische Bedeutung verschiedener Entzündungsmarker in der Pathogenese der Recurrent Airway Obstruction (RAO) in Exazerbation und Remission, der chronischen interstitiellen Pneumopathie und akuter bis subakuter Infektionen der tiefen Atemwege bei Pferden auf eine Korrelation zu den Ergebnissen der klinischen Untersuchung, Tracheobronchialsekret (TBS) und bronchoalveolärer Lavageflüssigkeit (BALF) Zytologie und Tests zur Lungenfunktion zu untersuchen.

Soweit wir wissen, wurden die Konzentrationen von MMP-2 und MMP-9 im Rahmen dieser Studie erstmals mittels ELISA ermittelt und diese Methode erwies sich als einfach, akkurat und zuverlässig. Außerdem erfolgte eine semi-quantitative densitometrische Auswertung der Banden der Gelatin-Zymographie zur Abschätzung der Aktivitäten von MMP-2 und MMP-9. Da sich die Kollagen-Zymographie zur Bestimmung der MMP-8 als wenig praktikabel erwies, erfolgte die Auswertung der MMP-8 in der BALF fluorimetrisch.

Insgesamt wurden 64 Warmblüter an der Klinik für Pferde, Allgemeine Chirurgie und Radiologie der Freien Universität Berlin im Rahmen dieser Studie untersucht. 15 dieser Pferde waren vorberichtlich und klinisch gesund, der Anteil der neutrophilen Granulozyten in der BALF lag ≤ 8% und sie dienten somit als gesunde Kontrollgruppe. Die Ergebnisse der Vorberichtserhebung, der klinischen, endoskopischen und röntgenologischen Untersuchungen, der Lungenfunktionstests und der zytologischen Untersuchunen von TBS und BALF dienten der Einordnung der Probanden in die verschiedenen Erkrankungsgruppen: RAO in Exazerbation (n=17), RAO in Remission (n=18), chronisch interstitelle Pneumopathie (n=11) und akute bis subakute Atemwegsinfektionen (n=3).

Der Gesamtuntersuchungsscore war bei den erkrankten Pferden gegenüber den gesunden Kontrolltieren signifikant erhöht. Der Anteil neutrophiler Granulozyten in TBS und BALF korrelierte mit den Konzentrationen von MMP-2, -8, -9 und IL-8, was die Rolle dieser Entzündungsmarker in der Pathogenese equiner Pneumopathien unterstützt.

Die MMP-2 Konzentration war bei RAO in Exazerbation (5,21 ± 0,77 ng/ml) und Remission (7,67 ± 15,5 ng/ml) gegenüber der Kontrollgruppe (2,49 ± 0,83 ng/ml) signifikant erhöht. Die MMP-9 Konzentration war bei RAO in Exazerbation (433,34 ± 89,05 pg/ml), RAO in Remission (312,06 ± 23,92 pg/ml) sowie den chronisch interstiellen Pneumopathien (263,2 ± 23,85 pg/ml) gegenüber der Kontrollgruppe (176,29 ± 60,22 pg/ml) signifikant erhöht. Diese Ergebnisse wurden für die MMP-2 und -9 in 55 BALF Proben zymographisch bestätigt. Die MMP-8 Konzentration war bei RAO in Exazerbation (0,84 ± 1 µmol/l), RAO in Remission (0,1 ± 0,06 µmol/l) sowie den chronisch interstiellen Pneumopathien (0,02 ± 0,01 µmol/l) gegenüber der Kontrollgruppe (0,01 ± 0,01 µmol/l) fluorimetrisch signifikant erhöht. Der Anteil neutrophiler Granulozyten in der BALF zeigte eine hochsignifikante positive Korrelation zu den zymographisch gemessenen Aktvititäten von MMP-2 und -9, was die Bedeutung der gelatinolytischen Aktvität bei verschiedenen Pneumopathien des Pferdes unterstützt. Außerdem war IL-8 signifikant erhöht bei RAO in Exazerbation und Remission.

Die Ergebnisse der vorliegenden Arbeit ermöglichen neue Einblicke in eine mögliche diagnostische Verwendung verschiedener Biomarker bei equinen Atemwegserkrankungen und diese könnten genutzt werden, um Umbauprozesse in der pulmonalen extrazellulären Matrix besser einschätzen zu können. Weitere Studien sollten sich mit der Rolle der natürlichen Inhibitoren der MMPs (tissue inhibitors of metalloproteinases, TIMPs) als mögliche Therapieoption beschäftigen.

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10 Appendix

Table 20: Numbers and percents of horses according to predicted and original diagnostic groups based on total examination score, BALF and TBS neutrophil percentage, MMP-2, MMP-8, MMP-9 and IL-8 concentrations using a discriminant analysis function.

Original diagnostic group	Predicted diagnostic group					
	I	II	III	IV	V	Total
Group I	10 (66.7)	0 (0)	0 (0)	5 (33.3)	0 (0)	15 (100)
Group II	0 (0)	15 (88.2)	2 (11.8)	0 (0)	0 (0)	17 (100)
Group III	0 (0)	1 (5.6)	16 (88.9)	1 (5.6)	0 (0)	18 (100)
Group IV	3 (27.3)	0 (0)	1 (9.1)	7 (63.6)	0 (0)	11 (100)
Group V	0 (0)	0 (0)	2 (66.7)	0 (0)	1 (33.3)	3 (100)

Table 21: The gender of horses expressed as the number of horses and the % from the total population used [n (%)].

	Group I	Group II	Group III	Group IV	Group V	Total
Gelding	8 (53.3%)	10 (58.8%)	11 (61.1%)	6 (54.5%)	-	35 (54.7%)
Mare	7 (46.7%)	7 (41.2%)	7 (38.9%)	5 (45.5%)	3 (100%)	29 (45.3%)

Table 22: The average age and weight of horses in different groups used in the present study, data were presented as mean values ± SD and (Min.-Max.).

	Age (years)	Weight (kg)
Group I (n=15)	8.75±3.49 (3.92-16.75)	425.33±111.92 (290-650)
Group II (n=17)	17.44±4.5 (10.08-23.25)	452.06±63.07 (300-550)
Group III (n=18)	12.49±4.23 (4.5-21.67)	519.33±63.04 (440-620)
Group IV (n=11)	12.25±3.71 (6.75-18.5)	510±131.28 (250-650)
Group V (n=3)	9.42±8.77 (1-18.5)	433.33±28.87 (400-450)
Total (n=64)	12.74±5.26 (1-23.25)	473.79±96 (250-650)

Table 23: Clinical examination scores in different groups, the results are expressed as mean values ± SD. The * indicates significance at $p<0.05$. The detailed significances for the inter-groups comparisons are listed in Table 28.

	Cough score	Dyspnea score	Lung percussion score	Lung auscultation score	Endoscopy score	BAL score	Blood gas score	Total examination score
Group I (n=15)	0.07±0.26 (0-1)	0±0 (0-0)	0±0 (0-0)	0±0 (0-0)	0±0 (0-0)	0±0 (0-0)	0.2±0.4 (0-1)	0.27±0.46 (0-1)
Group II (n=17)	0.47±0.5$^{*\uparrow}$ (0-1)	1.65±1.1$^{*\uparrow}$ (0-3)	0.18±0.39 (0-1)	0.35±0.78 (0-2)	1.82±0.39$^{*\uparrow}$ (1-2)	3±0$^{*\uparrow}$ (3-3)	0.76±0.75$^{*\uparrow}$ (0-2)	8.12±2.23$^{*\uparrow}$ (6-14)
Group III (n=18)	0.44±0.51$^{*\uparrow}$ (0-1)	0.33±0.76 (0-3)	0.11±0.32 (0-1)	0.22±0.65 (0-2)	1.17±0.71$^{*\uparrow}$ (0-2)	1.39±1.04$^{*\uparrow}$ (0-3)	0.17±0.38 (0-1)	3.88±1.41$^{*\uparrow}$ (2-6)
Group IV (n=11)	0.18±0.4 (0-1)	0.27±0.9 (0-3)	0.18±0.4 (0-1)	0±0 (0-0)	0.91±0.7$^{*\uparrow}$ (0-2)	0.45±0.69 (0-2)	0.36±0.81 (0-2)	2.36±2.66$^{*\uparrow}$ (0-9)
Group V (n=3)	0.67±0.58$^{*\uparrow}$ (0-1)	0±0 (0-0)	0±0 (0-0)	0±0 (0-0)	2±0$^{*\uparrow}$ (2-2)	1.33±1.53$^{*\uparrow}$ (0-3)	0.33±0.58 (0-1)	4.33±2.52$^{*\uparrow}$ (2-7)

Table 24: Frequencies of clinical examination scores in different disease groups; the details of scores identification were listed in Table 4.

		Number of horses				
Scores		**Group I (n=15)**	**Group II (n=17)**	**Group III (n=18)**	**Group IV (n=11)**	**Group V (n=3)**
Cough score	0	14	9	10	9	1
	1	1	8	8	2	2
Dyspnea score	0	15	2	14	10	3
	1	-	8	3	-	-
	2	-	1	-	-	-
	3	-	6	1	1	-
Lung percussion score	0	15	14	16	9	0
	1	-	3	2	2	-
	2	-	-	-	-	-
Lung auscultation score	0	15	14	16	11	3
	2	-	3	2	-	-
Endoscopy score	0	15	-	3	3	-
	1	-	3	9	6	-
	2	-	14	6	2	3
BAL score	0	15	-	4	7	1
	1	-	-	6	3	1
	2	-	-	5	1	-
	3	-	17	3	-	1
Blood gas score	0	12	7	15	9	2
	1	3	7	3	-	1
	2	-	3	-	2	-

Table 25: Frequencies of endoscopy score in different disease groups.

	Amount of secretions						**Viscosity of secretions**						**Tracheal bifurcation**			
	0	**1**	**2**	**3**	**4**	**5**	**0**	**1**	**2**	**3**	**4**	**5**	**0**	**1**	**2**	**3**
Group I	9	6	-	-	-	-	10	5	-	-	-	-	12	3	0	0
Group II	-	-	-	10	6	1	-	-	-	4	12	1	3	6	1	4
Group III	4	1	6	5	2	-	4	-	4	6	4	-	1	7	4	1
Group IV	1	2	4	2	1	-	1	4	2	2	1	-	-	3	5	-
Group V	-	-	-	1	2	-	-	-	-	1	2	-	-	2	1	-

The amount of secretions: (0) No secretions, (1) Little, multiple small streaks, (2) Moderate secretions located ventrally with larger streaks, (3) Marked secretions located ventrally, (4) Large pool forming and (5) Extreme profuse secretions. Viscosity of secretions: (0) no secretions, (1) fluid, serous secretions, (2) serous to seromucoid secretion, (3) intermediate seromucoid secretions, (4) viscous mucous to mucopurulent secretions and (5) very viscous dry, sticky mucous. Tracheal bifurcation: (0) thin, (1) mildly thickened, (2) moderately thickened and (3) severely thickened.

Table 26: Peak areas of the MMP-2 and MMP-9 zymography in different disease groups, the results are expressed as mean values ± SD. The * indicates significances at $p<0.05$. The detailed significances for the inter-groups comparisons are listed in Table 30.

	MMP-2 zymography (peak area)	**MMP-9 zymography (peak area)**
Group I (n=13)	1114.76±672.72 (195.61-2249.56)	619.29±996.32 (57.95-3776.25)
Group II (n=17)	17288.53±8927.59$^{*\uparrow}$ (4980.59-32307.09)	10967.31±9530.07$^{*\uparrow}$ (1603.48-36940.89)
Group III (n=14)	3530.94±2894.15$^{*\uparrow}$ (824.5-10034.7)	1832.16±2111.29 (75.36-7555.13)
Group IV (n=8)	2799.45±2592.28 (379.68-7726.73)	864.06±767.93 (91.02-2311.49)
Group V (n=3)	12438.71±6889.51 (6825.8-20127.45)	7673.57±7605.88 (1249.43-16071.89)

Table 27: The p-values for temperature, respiratory, heart rate and lung function testing to illustrate the inter-group differences tested by kruskal-Wallis test followed by Mann-Whitney with Bonferoni adjustment.

		Temp. (^oC)	Respiratory rate (breaths/min)	Heart rate (beats/min)	pH	$PaCO_2$	PaO_2	$AaDO_2$	HCO_3	IPP
Kruskal-Wallis statistics		2.465	23.183	9.114	2.912	1.525	14.969	18.217	3.068	9.763
P-value		0.651	0.000	0.058	0.573	0.822	0.005	0.001	0.546	0.045
Mann-Whitney test	Group I & II	No difference	0.000	No difference	No difference	No difference	0.001	0.000		0.012
	Group I & III		0.017				0.007	0.006		0.207
	Group I & IV		0.199				0.052	0.069		0.311
	Group I & V		0.365				0.086	0.002		0.726
	Group II & III		0.022				0.106	0.059		0.031
	Group II & IV		0.011				0.167	0.124		0.119
	Group II & V		0.007				0.874	0.874		0.125
	Group III & IV		0.443				0.598	0.778		1.000
	Group III & V		0.055				0.421	0.070		0.542
	Group IV & IV		0.242				0.499	0.170		0.703

Table 28: The p-values for clinical examination score and the endoscopy examination score to illustrate the inter-group differences tested by kruskal-Wallis test followed by Mann-Whitney with Bonferoni adjustment.

		Cough score	Dyspnoea score	Lung percussion score	Lung auscultation score	Endoscopy score	BAL score	Blood gas score	Total examination score	Amount of secretions	Viscosity of secretions	Tracheal bifurcation
Kruskal-Wallis		9.793	35.296	3.533	4.934	40.709	46.891	9.803	49.919	36.29	38.23	15.63
p-value		0.044	0.000	0.473	0.294	0.000	0.000	0.044	0.000	0.000	0.000	0.004
Mann-Whitney test	Group I & II	0.013	0.000	No difference	No difference	0.000	0.000	0.019	0.000	0.000	0.000	0.004
	Group I & III	0.017	0.056			0.000	0.000	0.808	0.000	0.001	0.000	0.001
	Group I & IV	0.373	0.243			0.000	0.013	0.910	0.001	0.001	0.002	0.001
	Group I & V	0.013	1.000			0.000	0.001	0.622	0.002	0.004	0.004	0.012
	Group II & III	0.878	0.000			0.003	0.000	0.008	0.000	0.000	0.000	0.908
	Group II & IV	0.126	0.001			0.001	0.000	0.102	0.000	0.001	0.000	0.553
	Group II & V	0.542	0.012			0.442	0.001	0.357	0.041	0.569	0.513	0.893
	Group III & IV	0.156	0.432			0.337	0.015	0.758	0.011	0.882	0.280	0.503
	Group III & V	0.486	0.378			0.049	0.876	0.507	0.797	0.034	0.095	1.000
	Group IV & IV	0.112	0.602			0.024	0.256	0.745	0.123	0.037	0.037	0.618

Table 29: The p-values for TBS and BALF cytology to illustrate the inter-group differences tested by kruskal-Wallis test followed by Mann-Whitney with Bonferoni adjustment.

		TBS Cytology					**BAL Cytology**				
		TBS Macro.	**TBS Lympho.**	**TBS Neutro.**	**TBS Eosino.**	**TBS Mast cells**	**BAL Macro.**	**BAL Lympho.**	**BAL Neutro.**	**BAL Eosino.**	**BAL Mast cells**
Kruskal-Wallis		20.849	14.146	24.214	5.905	6.041	34.112	20.537	48.902	7.542	15.725
p-value		0.000	0.007	0.000	0.206	0.196	0.000	0.000	0.000	0.110	0.003
Mann-Whitney test	Group I & II	0.000	0.001	0.000	No difference	No difference	0.000	0.000	0.000	No difference	0.066
	Group I & III	0.010	0.711	0.002			0.001	0.058	0.000		0.138
	Group I & IV	0.088	0.291	0.007			0.716	0.169	0.003		0.038
	Group I & V	0.017	0.425	0.017			0.813	0.021	0.015		0.373
	Group II & III	0.003	0.004	0.002			0.000	0.003	0.000		0.003
	Group II & IV	0.023	0.116	0.016			0.000	0.012	0.000		0.002
	Group II & V	0.813	0.214	0.314			0.044	0.560	0.101		0.790
	Group III & IV	0.641	0.423	0.641			0.132	0.928	0.016		0.485
	Group III & V	0.405	0.518	0.405			0.513	0.365	0.880		0.096
	Group IV & IV	0.297	0.881	0.456			0.815	0.186	0.208		0.102

Table 30: The p-values for MMP-2, MMP-9, IL-8 ELISA, MMP-8 fluorimetry and MMP-2, MMP-9 zymography to illustrate the inter-group differences tested by kruskal-Wallis test followed by Mann-Whitney with Bonferoni adjustment.

		MMP-2 ELISA	**MMP-9 ELISA**	**MMP-8 fluorimetry**	**IL-8 ELISA**	**MMP-9 zymography**	**MMP-2 zymography**
Kruskal-Wallis		50.371	47.79	50.329	19.694	31.982	38.794
p-value		0.000	0.000	0.000	0.001	0.000	0.000
Mann-Whitney test	Group I & II	0.000	0.000	0.000	0.000	0.000	0.000
	Group I & III	0.000	0.000	0.000	0.000	0.065	0.004
	Group I & IV	0.092	0.000	0.000	0.019	0.247	0.128
	Group I & V	0.086	0.026	0.011	0.011	0.013	0.009
	Group II & III	0.000	0.000	0.000	0.355	0.000	0.000
	Group II & IV	0.000	0.000	0.000	0.074	0.000	0.000
	Group II & V	0.007	0.008	0.030	0.711	0.491	0.368
	Group III & IV	0.000	0.000	0.000	0.072	0.453	0.413
	Group III & V	0.078	0.017	0.688	0.482	0.101	0.017
	Group IV & IV	0.010	0.815	0.102	0.243	0.041	0.025

10.1 Chemicals used in the present study.

Name	Label and company
Brij	Brij® 35, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Bromophenol blue	Bromophenol blue, Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Calcium chloride	Calcium chloride dihydrate, Serva, Feinbiochemica GmbH & Co., Heidelberg, Germany
EDTA tubes	Sarstedt, Nümbrecht, Germany
ELISA Kit for Equine IL-8	ELISA Kit for Equine IL-8 (E90080Eq 96 Tests), Uscn Life Science Inc., Wuhan, China
ELISA Kit for Equine MMP-2	ELISA Kit for Equine MMP-2 (E901100Eq 96 Tests), Uscn Life Science Inc., Wuhan, China
ELISA Kit for Equine MMP-9	ELISA Kit for Equine MMP-9 (E90553Eq 96 Tests), Uscn Life Science Inc., Wuhan, China
Fluorimetric Assay Kits	Fluorimetric Assay Kits (Sensolyte® 520 MMP-8 Assay Kit), Anaspec, Inc. Fermont, United States
Gelatin Zymogram Gels	Novex® 10% Gelatin Zymogram Gels, 1.0 mm, 12 wells, Novex® by life technologies, Van allen way carlsbad, USA
Glycerin	Glycerin (Rotipuran® ≥99.5%), Carl Roth GmbH + Co.KG, Karlsruhe, Germany
Lidocaine® 2%	Lidocainhydrochloride 2%, bela-Pharm GmbH, Vechta, Germany
May-Grünwald Giemsa stain	May-Grünwald Giemsa staining method, Sigma-Aldrich Chemie GmbH, Germany
Phosphate Buffered Saline (PBS)	BioWittaker® PBS Phosphate Buffered Saline, Lonza, Verviers, Belgium
Protein marker	Spectra™ Multicolor Broad Range Protein Ladder, Thermo Scientific, Rockford, USA
Protein Staining Solution	PageBlue™ Protein Staining Solution, Fermentas Life Sciences, Vilnius, Lithuania
Pulmicort® Suspension	*Pulmicort®* (1,0 mg Budesonid /2 ml), AstraZeneca GmbH, 22876 Wedel, Hamburg, Germany
Renaturing buffer	Triton® X-100, Ferak Berlin GmbH, Berlin
SDS	SDS Pellets >99%, Carl Roth GmbH + Co.KG, Karlsruhe, Germany
Sodium chloride	Natriumchlorid >99.8%, Carl Roth GmbH + Co.KG, Karlsruhe, Germany
TRIS	TRIS (Bufferan®>99.9%, p.a.), Carl Roth GmbH + Co.KG, Karlsruhe, Germany
Glycin	Carl Roth GmbH + Co.KG, Karlsruhe, Germany

10.2 Equipments used in the present study

Procedures	Label and company
Balance MC1	Sartorius AG, Goettingen, Germany
Blood gas analyser	Cobas b 123 POC system®,Roche, Swisslab, Germany
Differential blood cell count	pocH-100 iv Diff, Sysmex Europe GmbH, Norderstedt, Germany
Digital image analyzing software	ImageJ v1.47, Wayne Rasband, National institutes of health, USA, http://imagej.nih.gov/ij
Electrophoresis Cell	Novex XCELL, Novex Experimental Technology, Japan
Electrophoresis power supply	Novex Programmable Power Supply Model-3540, San Diego, USA
Video Endoscope®	Videomed GmbH, Munich, Germany
Fluorescence microplate reader	FLUO Star Optima, BMG Labtech GmbH, Ortenberg, Germany
Gel loading tips	Pippetor tips MµltiFlex®-tips, Carl Roth GmbH & Co.KG, Karlsruhe, Germany
Heating magnetic stirrer	Ikamag® Rct, Janke & Kunkel GmbH & CO.KG, IKA-Labortechnik, Staufen, Germany
Lab-Shaker	Lab-Shaker (Lab-Therm), Adolf Kühner AG, Basel, Switzerland
Microplate manager	Microplate Manager Version 5.2, Build 103, BioRad Laboratories, Hercules, CA 94547, USA
Microplate Reader	Bio Rad Mode 550 Microplate Reader, BioRad Laboratories, Hercules, CA 94547, USA
PH-Meter	PH-Meter 761 Climatic, Knick, West Germany
Silicone BAL Catheter	Smiths Medical ASD, Inc, USA
SPSS Statistics 17.0	SPSS Statistics for Windows, Version 17.0 released 2008, SPSS Inc., Chicago
Table Top Refrigerated Centrifuge	Hermle Z326K, Hermle Labortechnik GmbH, Germany
Universal Centrifuge	Hermle Z300, Hermle Labortechnik GmbH, Germany
Ventigraph®	model PG100/REC, BoehringerIngelheim, Ingelheim, Germany
Reichert total solid refractometer	Reichert GmbH, Seefeld, Germany
Microhematocrit centrifuge	Haemofuge Heraeus Sepatech, Germany
SaHoMa™-II mobile ultrasonic nebulizer	Nebu-Tec International, Eike Kern GmbH, Germany

10.3 Clinical examination reports and forms

Examination sheet 1:

Lung examination

1. History and signalment

Owner's data:

Last name: ……. First name: …….

Referent veterinarian:

Last name: ……. First name: …….

Veterinarians:

Assistant: ……. Supervisor: …….

Patient's data:

Name: ……. Breed: …….

Sex: ……. Age: …….

Weight: ……. Height: …….

Horse ID: ……. Color: …….

Head marks: ……

Limb marks: ……

History:

……

……

……

Previous examinations and treatments:

……

……

……

Examination sheet 2:

Lung examination

2. Clinical investigations

Horse's name: ……. Owner's name: …….

Investigators: ……. Date: …….

Clinical examination scores modified after **Ohnesorge et al. (1998)**.

Clinical score	Findings	Score	Real score
Cough score (max. 1)	No cough after manual compression of larynx	0	
	Coughing during manual larynx compression	1	
	Very frequent coughing	1	
	Spontaneous coughing	1	
Dyspnea score (max. 3)	Normal	0	
	Prolonged expiration	1	
	Abdominal breathing	1	
	Nostril flare during inspiration and returns to normal at end inspiration	2	
	Sinking of the intercostal area	3	
	Nostril flare during inspiration and expiration	3	
	Heaves line	3	
	Anal pumping	3	
Lung percussion score (max. 2)	3 fingers	0	
	Handbreadth	1	
	Damping	2	
Lung auscultation score (max. 2)	Normal lung sound	0	
	Rattling	2	
	Crackle	2	
	Wheezing	2	
Endoscopy score (max. 2)	Normal endoscopy	0	
	Significantly increased secretions with moderate viscosity	1	
	Highly increased secretions with high viscosity	2	
	Thickened tracheal bifurcation	1	
BALF score (max. 3)	Neutrophils <8%	0	
	Neutrophils 8-15%	1	
	Neutrophils 15-25%	2	
	Neutrophils >25%	3	
Blood gases score (max. 2)	$AaDo_2$: 0-7 mmHg	0	
	$AaDo_2$: 7-14 mmHg	1	
	$AaDo_2$: >14 mmHg	2	

Total score: …….…….

Examination sheet 3:

BALF cytology

Horse's name: ……. Case No.: ……..
Amount of fluid infused: ……. Amount of fluid collected: …….

Macrophages					
Lymphocytes					
Neutrophils					
Eosinophils					
Mast cells					
Remarks					
Result	Macrophages %	Lymphocytes %	Neutrophils %	Eosinophils %	Mast cells %

Examination sheet 4:

TBS cytology

Horse's name: ……. **Case No.:** …….

Macrophages					
Lymphocytes					
Neutrophils					
Eosinophils					
Mast cells					
Remarks					
Result	Macrophages %	Lymphocytes %	Neutrophils %	Eosinophils %	Mast cells %

Examination sheet 5:

Patient investigation sheet

Horse's name: ……. Date and Case No.: …….

1st Day	Clinical examination	
	Blood gas analysis	
2nd Day	Exercise tolerance test	
	Lactate before exercise tolerance	
	Lactate after exercise tolerance	
	Blood picture and hematocrite	
	Clinical examination	
	Blood gas analysis	
	BALF collection	
	TBS collection	
	BALF cytology	
	TBS cytology	
3rd Day	Clinical examination	
	Blood gas analysis	
Remarks		

10.4 Database software interface

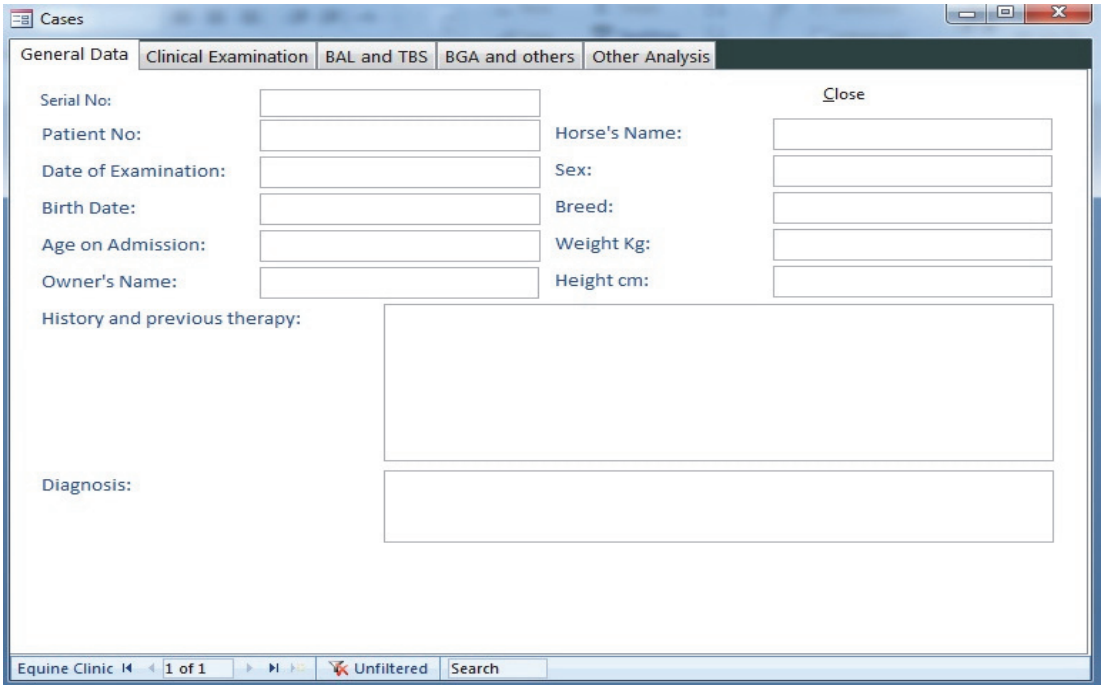

Figure 18: Caption of the database for data entry: General data.

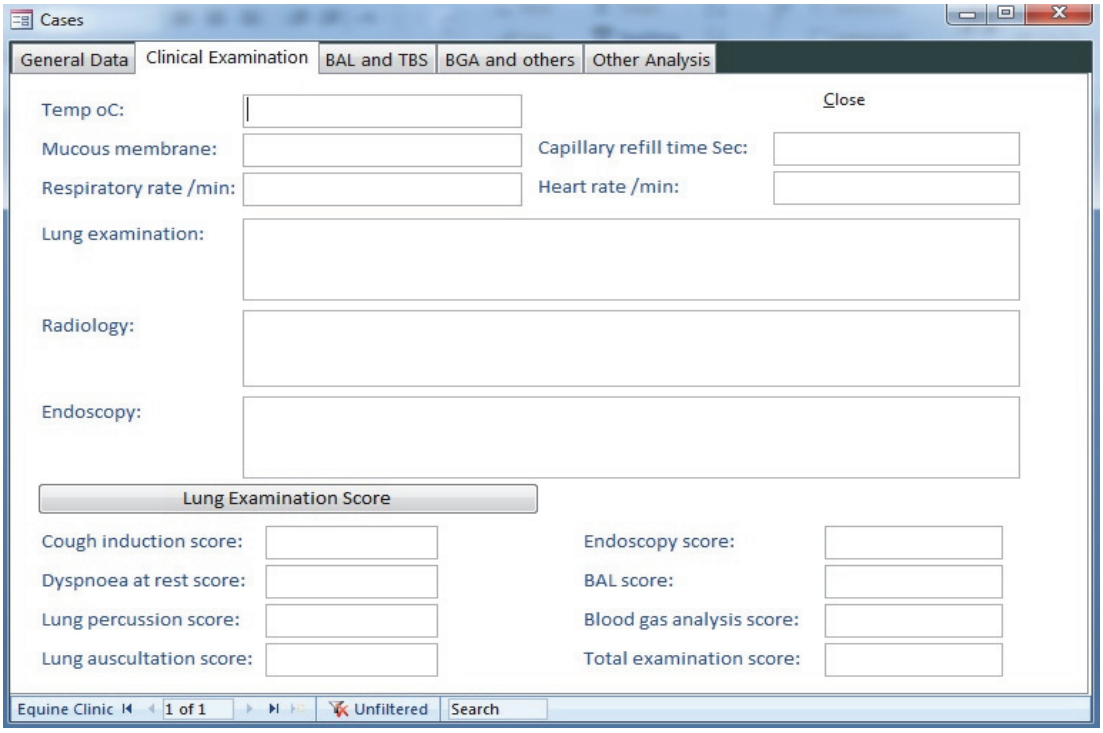

Figure 19: Caption of the database for data entry: Clinical examination data.

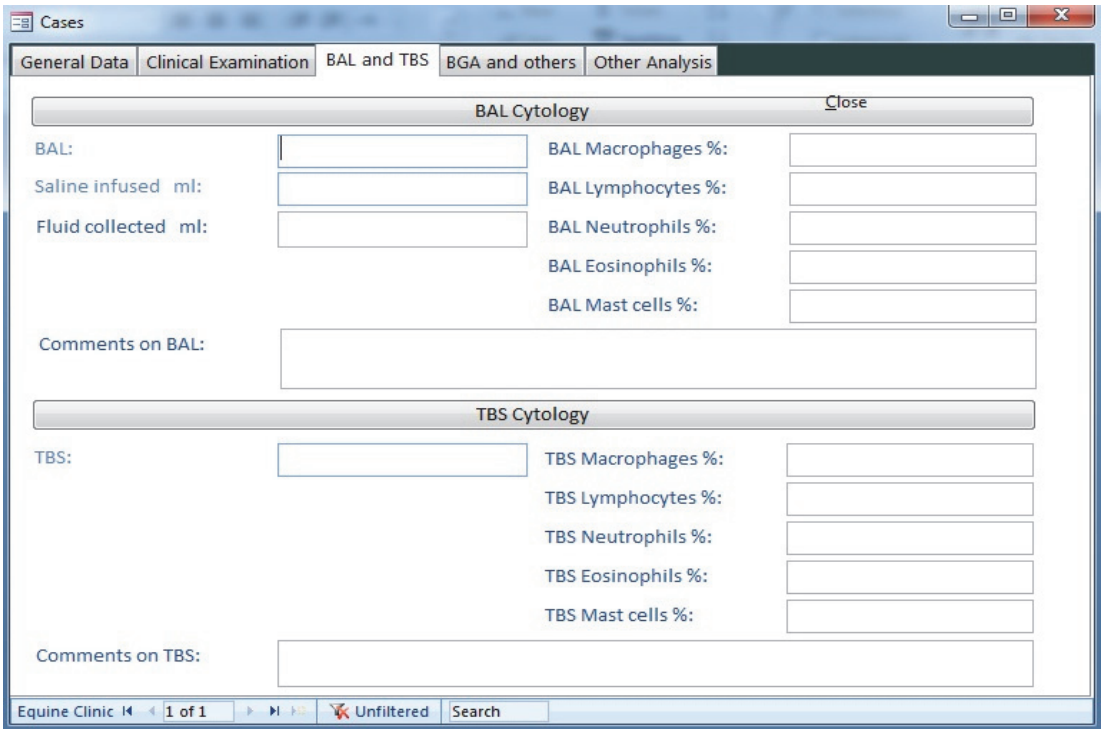

Figure 20: Caption of the database for data entry: TBS and BALF cytology.

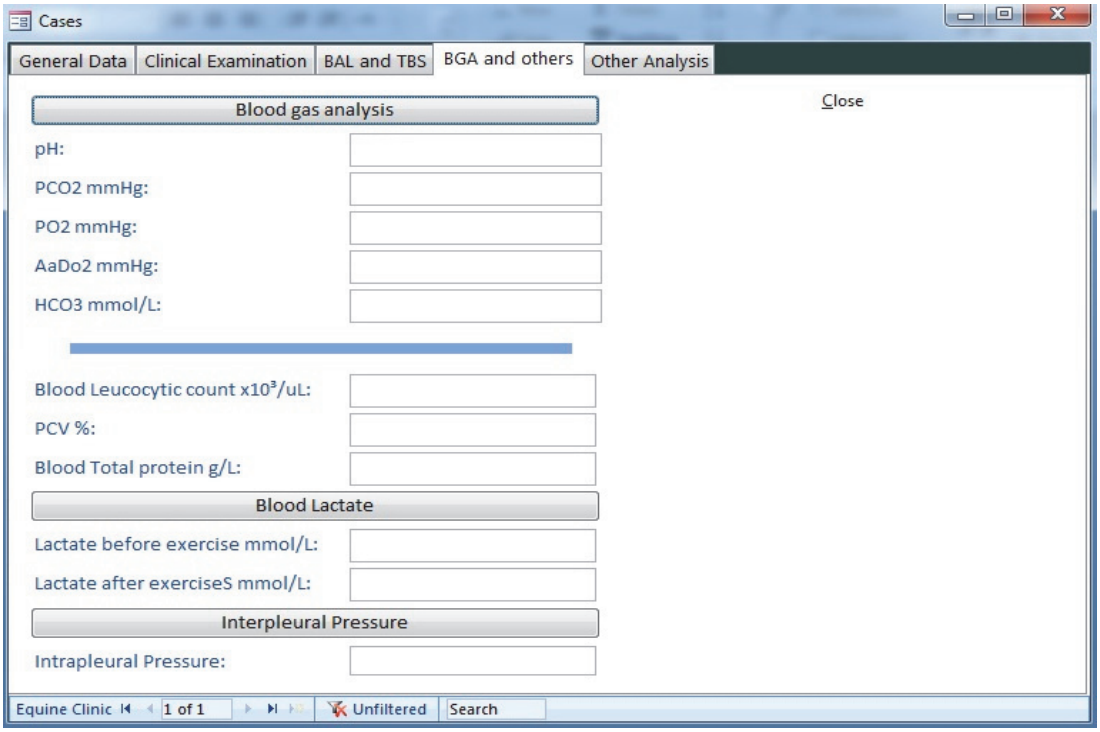

Figure 21: Caption of the database for data entry: Lung function testing.

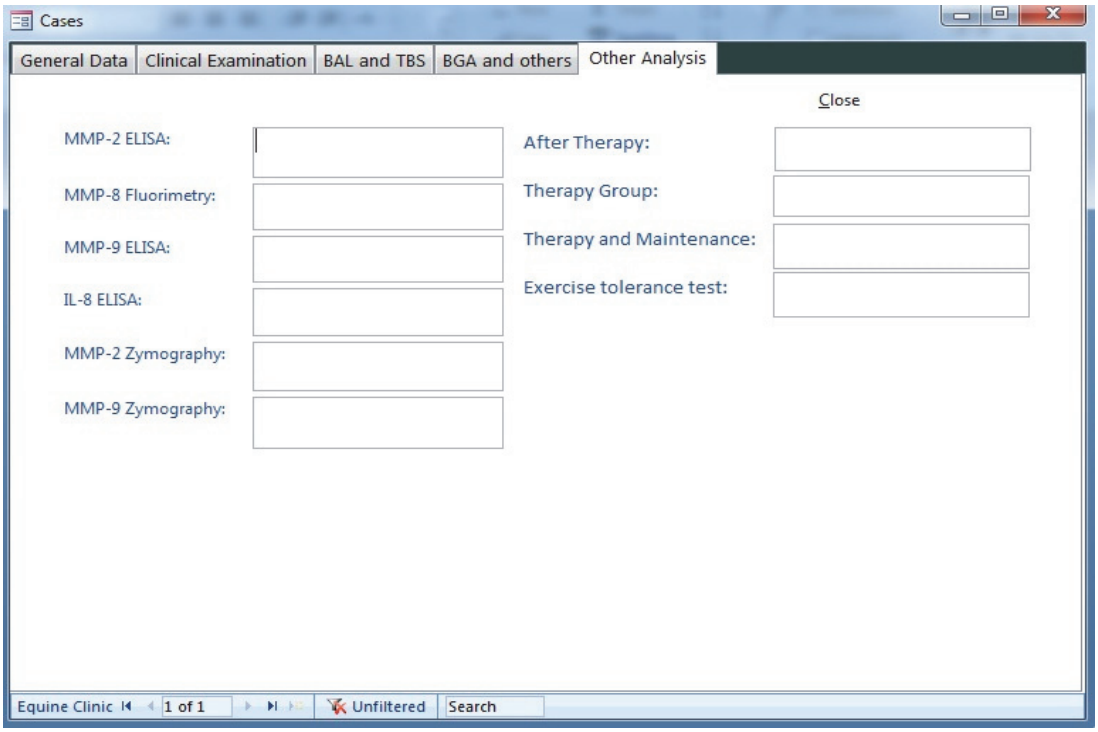

Figure 22: Caption of the database for data entry: Inflammatory markers.

11 Acknowledgements

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12 Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 21.10.2014

Tarek Soliman Shety