Inflammatory Response in Equine Airways

Cytokines in Bronchial Epithelium and Bronchoalveolar Lavage Cells

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Abstract

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Non-infectious airway inflammation in horses is a common problem worldwide when horses are housed indoors. In this thesis the inflammatory pathways in equine airways, with special regards to the role of bronchial epithelium, were investigated in horses with highly reactive airways (recurrent airway obstruction, RAO) and in clinically normal race horses under conventional management. In the former group the cytokine mRNA and protein expression in bronchial epithelium and were examined, whereas only the cytokine mRNA expression of bronchoalveolar lavage (BAL) cells and BAL cytology were included in the work on the stabled race horses. These latter horses unexpectedly developed transient pulmonary eosinophilia which allowed deeper investigation of eosinophil kinetics in horses. These studies showed that endobronchial biopsy was a safe and readily performed procedure to obtain airway tissue samples from the live horse. Moreover, biopsies were of sufficient size and quality for evaluation of cytokine mRNA expression, airway morphology and for immunohistochemical analysis.

The work on RAO horses strengthened earlier findings of increased mRNA expression of the neutrophil chemotaxic cytokine IL-8 in RAO horses in both BAL cell and bronchial tissues. Thus, use of bronchial tissues can provide a valued complementary tool to study serial events of inflammation in equine airway at closely timed intervals not possible with the sampling technique of BAL.

In the group of race horses, prolonged winter stabling was associated with subtle pulmonary alterations reflective of increased inflammatory response in equine airways. Specifically there was increased expression of IL-6 mRNA in BAL cells along with a trend for increased neutrophils in the BAL. No underlying cause of the transient pulmonary eosinophilia could be identified. Surprisingly, expression of mRNA of eosinophil associated IL-4 and IL-5 was not detectably altered in these horses. It appears that eosinophil influx to the equine lung can be subclinical apart from temporary exercise intolerance, and abate without specific treatment other than deworming.

These combined studies introduce and demonstrate future potential in examination of bronchial tissues as a complement to BAL cells studies in molecular biological analysis to increase the understanding of mechanisms of noninfectious airway inflammation in the horse.

Keywords: RAO, IAD, BAL, endobronchial biopsy, cytokine, horse, stable environment

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To my family

I am still under the impression that there is nothing alive quite so beautiful as a Thoroughbred horse.
-John Galsworthy

Riding: The art of keeping a horse between you and the ground.
-Author Unknown

There is something about the outside of a horse that is good for the inside of a man.
-Winston Churchill

Contents

Appendix

List of original papers **I-IV**
The thesis is based on the following papers, which will be referred to in the text by Roman numerals:

I. M. Riihimäki, A. Raine, T. Art, P. Lekeux, L. Couëtil and J. Pringle. Partial divergence of cytokine mRNA expression in bronchial tissues compared to bronchoalveolar lavage cells in horses with recurrent airway obstruction. Vet Immunol Immunopathol. In press: 2008. doi:10.1016/j.vetimm.2007.12.001

II. M. Riihimäki, A. Raine, J. Pourazar, T. Sandström, T. Art, P. Lekeux, L Couëtil and J. Pringle. Epithelial expression of mRNA and protein for IL-6, IL-10 and TNF-α in endobronchial biopsies in horses with Recurrent Airway Obstruction. BMC Veterinary Research. In press: 2008, 4:8, doi:10.1186/1746-6148-4-8

III. M. Riihimäki, I. Lilliehöök, A. Raine, M. Berg and J. Pringle. Clinical alterations and bronchoalveolar cell gene expression of mRNA IL-4 and IL-5 in horses with transient pulmonary eosinophilia. Res Vet Sci. In press: 2007. doi:10.1016/j.rvsc.2007.09.015

IV. M. Riihimäki, A. Raine, L. Elfman and J. Pringle. Markers of respiratory inflammation in horses in relation to seasonal changes in air quality in a conventional racing stable. Accepted. Can J Vet Res, 2008.

Abbreviations

Δ	Delta, a change or difference between mathematical values
BAL	Bronchoalveolar Lavage
BALF	Bronchoalveolar Lavage Fluid
cDNA	Complementary DNA
Cdyn	Dynamic compliance
C_T	Threshold cycle
COPD	Chronic Obstructive Pulmonary Disease
ΔPplmax	Maximum change in pleural pressure
FEV_1	Forced Expiratory Volume in one second
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GMA	Glycolmethacrylate
IAD	Inflammatory Airway Disease
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
LPS	Lipopolysaccharide
mRNA	Messenger Ribonucleic Acid
NF	Nuclear Factor
PaO_2	Partial pressure of oxygen
RAO	Recurrent Airway Obstruction
RL	Pulmonary resistance
RNA	Ribonucleic Acid
RR	Respiratory Rate
RT-PCR	Reverse Transcriptase-PCR
SD	Standard Deviation
SPAOPD	Summer Pasture Associated Obstructive Pulmonary Disease
TA	Tracheal Aspirate
TGF	Transforming Growth Factor
Th cell	T helper cell
TLR	Toll-like receptor
TNF	Tumour Necrosis Factor

Introduction

General background

Terminology

Non-infectious respiratory disease with airway inflammation in horses is a common clinical problem worldwide when horses are housed indoors. Horses with inflammatory respiratory diseases develop various degrees of clinical signs, from obvious respiratory distress while at rest in a sedentary horse to solely exercise intolerance in the athletic horse. The nomenclature for various disease states has been under continual revision. In the past, horses with asthma-like syndrome known in lay terms as "heaves," or broken wind, were given the diagnosis "chronic obstructive pulmonary disease" (COPD). However, COPD was a poor choice of terminology as it denotes a nonreversible pulmonary disease in man associated primarily with exposure to tobacco smoke. At an international workshop on equine airway disease in 2001 (Robinson, 2001) the terminology was clarified and COPD was replaced with the now generally accepted term "recurrent airway obstruction" (RAO), or heaves, to describe an older horse with airway obstruction that can be reversed with treatment or environmental changes. Horses falling outside the definition of RAO, yet having mild non-infectious airway inflammation of unspecified etiology were in turn described as having simply "inflammatory airway disease" (IAD) (Robinson, 2001). Thus other diagnostic terms, such as small airway disease, chronic bronchitis, and others were supplanted. With increased research and understanding of airway diseases, additional consensus statements on disease diagnosis terminology have been forthcoming (Couetil *et al.*, 2007; Robinson, 2003). A similar syndrome, but aetiologically distinct from RAO is "summer pasture associated obstructive pulmonary disease" (SPAOPD) (Beadle, Horohov & Gaunt, 2002; Dixon & McGorum, 1990; Horohov *et al.*, 2005; Mair, 1996). While the physiological effects of pulmonary inflammation in SPAOPD and RAO appear highly similar, the development of clinical signs in the former are related to being at pasture instead of being housed indoors, which suggests a very different airway trigger. Therefore, because of its unique clinical character and etiology. SPAOPD is retained to describe a disease separate and distinct from RAO (Beadle, Horohov & Gaunt, 2002; Dixon & McGorum, 1990; Mair, 1996).

Recurrent Airway Obstruction (RAO)

Horses with RAO develop neutrophilic inflammation and mucous accumulation in the airways, bronchospasm and impaired pulmonary function in response to inhaled particles or substances (Gerber *et al.*, 2004a; Robinson *et al.*, 1996). Bronchoalveolar lavage (BAL) cytology reveals neutrophilia (>20%) associated with decreased macrophage and lymphocyte percentages (Couetil *et al.*, 2001; Derksen *et al.*, 1985b; Mair, Stokes & Bourne, 1987). The ultrastructural changes associated with RAO include bronchial goblet cell metaplasia, peribronchial lymphocyte accumulation, airway neutrophilia, epithelial hyperplasia, loss of ciliated cells and, in some cases, subepithelial fibrosis (Kaup *et al.*, 1990; Kaup,

Drommer & Deegen, 1990; Lugo *et al.*, 2006; Naylor, Clark & Clayton, 1992; Nyman *et al.*, 1991). The clinical signs are similar to asthma, with airway obstruction, chronic intermittent cough, increased respiratory effort and mucus nasal discharge (Robinson, 2001; Robinson *et al.*, 2003). Signs of impaired pulmonary function can abate with optimal environmental management, but return after exposure to dusty stable environments; hence the term RAO describing the recurrent nature of the airway obstruction. The association between stable environment and feed quality and occurrence of neutrophilic airway inflammation in RAO has been well documented (Derksen*, et al.*, 1985b; Gerber *et al.*, 2004b; Holcombe *et al.*, 2001; Tremblay *et al.*, 1993). Typically, disease onset for RAO is in middle aged or older horses (Dixon, Railton & McGorum, 1995). Several studies suggest the presence of breed predisposition in combination with various environmental factors (i.e. different housing routines between breeds and/or genetic background) (Couetil & Ward, 2003; Ramseyer *et al.*, 2007). Recently, a genetic link to development of RAO has been demonstrated (Jost *et al.*, 2007). Management of the stable environment, in particular with respect to air quality, is considered to be the most important factor to attain clinical remission in horses with RAO (see below) (Couetil *et al.*, 2005; Derksen *et al.*, 1985a; Jackson *et al.*, 2000; Vandenput *et al.*, 1998b), whereas medical treatment is based on pharmacological reduction of bronchospasm and airway inflammation during active disease (Ammann, Vrins & Lavoie, 1998; Couetil*, et al.*, 2001; Giguere *et al.*, 2002; Rush, 2006; Rush *et al.*, 1998b).

The relationship between poor stable environment and feed quality and clinical exacerbation of RAO has been demonstrated in several studies (Gerber*, et al.*, 2004a; Holcombe*, et al.*, 2001; Laan *et al.*, 2005; Tremblay*, et al.*, 1993). Earlier studies indicate that RAO has a multifactorial background, with disease occurring in individuals with genetic predisposition in combination with exposure for specific allergens and pro-inflammatory agents (Pirie *et al.*, 2001; Pirie, Dixon & McGorum, 2002). While even presumed clinically normal horses can have mild pulmonary neutrophilia in selected stable environments (Holcombe*, et al.*, 2001; Tremblay*, et al.*, 1993) horses with RAO react much more markedly after short exposure to dusty stable air, with airway mucus accumulation, increased mucus viscoelasticity and pulmonary neutrophilia (Gerber*, et al.*, 2004a; Tremblay*, et al.*, 1993). Thus management tools for environmental control of RAO should include providing increased time outdoors at pasture or in paddocks, feeding with grass silage instead of hay and provision of low dust beddings in combination with improving ventilation and stable routines (Clarke, 1987; Vandenput*, et al.*, 1998b; Webster *et al.*, 1987; Woods *et al.*, 1993).

Inflammatory Airway Disease (IAD)

IAD is considered to be a common finding in racehorses in training (Burrell *et al.*, 1996; Moore *et al.*, 1995). However, the diagnosis of IAD is also applicable to horses at all ages, which, in contrast to RAO has a milder degree of inflammation based on BAL cytology and lack obvious clinical signs of respiratory disease (Couetil*, et al.*, 2007; Gerber *et al.*, 2003a; Robinson *et al.*, 2006). According to the most recent consensus statement the inclusion criteria for IAD are only loosely defined, but include poor performance or exercise intolerance and possibly cough

and excess tracheal mucus. Additional criteria include cytologic changes suggestive of nonseptic inflammation in BAL or detectable pulmonary dysfunction/airway hyperresponsiveness based on selected pulmonary function tests. Horses with clinical or haematological signs of infection and horses with signs of increased respiratory effort at rest are excluded (Couetil*, et al.*, 2007). Diagnostic criteria for IAD in its most subtle phase rest predominately on BAL cytological findings. Classically these are characterized by mild neutrophilia (usually <20%) (Couetil*, et al.*, 2007; Couetil*, et al.*, 2001; Hoffman, 1999).. However, BAL cytological profiles that include increased mast cells (>2%) (Hoffman, 1999) and/or increased eosinophils (>0.1 %) (Hare & Viel, 1998; Moore*, et al.*, 1995) are also considered as diagnostic for IAD (Couetil*, et al.*, 2007; Hare & Viel, 1998; Hoffman, 1999; Moore*, et al.*, 1995). Subtle pulmonary function impairment/airway hyperresponsiveness has also been observed in some horses, and suggested as an underlying reason for reduced exercise intolerance (Couetil*, et al.*, 2007; Hoffman, Mazan & Ellenberg, 1998; Sanchez *et al.*, 2005). Still others have shown that "subclinical" IAD according to BAL cytologic definitions is present in a large number of stabled horses, probably as a "normal" reaction to inhaled agents in the stable environment (Gerber*, et al.*, 2004a; Holcombe*, et al.*, 2001; Tremblay*, et al.*, 1993). Environmental factors such as a exposure to a dusty stable environment, viral and bacterial infections alone, or various combinations of these are suggested as potential causes of the inflammation observed with IAD. Clearly, the syndrome of IAD remains poorly understood (Burrell, Mackintosh & Taylor, 1986; Burrell*, et al.*, 1996; Christley *et al.*, 2001; Couetil*, et al.*, 2007; Moore*, et al.*, 1995; Wood *et al.*, 1997; Wood *et al.*, 2005a; Wood *et al.*, 2005b).

Stable environment

The horse stable environment contains a mixture of inorganic and organic components. Organic dust contains several factors such as, as endotoxin, (1→3)-β-D-glucan (structural cell wall component of most fungi and some bacteria), micro-organisms, allergens and noxious gases, which can potentially initiate airway inflammation in stabled horses (Clarke & Madelin, 1987; Woods*, et al.*, 1993). Feed and bedding are considered as main sources of dust in stable environment (Clarke & Madelin, 1987; Robinson*, et al.*, 2003; Vandenput *et al.*, 1998a; Vandenput *et al.*, 1997; Woods*, et al.*, 1993). In experimental inhalation challenges *Faenia retrivirgula* and *Aspergillus fumigatus* have been suggested to induce pulmonary inflammation with clinical signs similar to RAO in susceptible horses (McGorum, Dixon & Halliwell, 1993d; Pirie *et al.*, 2002; Pirie, Dixon & McGorum, 2002; Pirie, McLachlan & McGorum, 2002; Simonen-Jokinen *et al.*, 2005). However, it remains uncertain whether inhalation of these molds causes a true allergic response or simply a generalized non-allergic inflammation in equine airways (Derksen*, et al.*, 1985a; McGorum, Dixon & Halliwell, 1993d). Inhalation of lipopolysacharide (LPS) also induces neutrophil accumulation into equine lung and inhaled LPS together with inhaled hay dust components induce increased pulmonary reactivity in RAO horses (Nevalainen *et al.*, 2002; Pirie*, et al.*, 2001; Pirie, Dixon & McGorum, 2003). Challenge with poor quality hay dust suspension is a more potent inducer of inflammation than the isolated components of

endotoxin or inhaled molds alone, suggesting that factors in addition to endotoxin (McGorum, Dixon & Halliwell, 1993d) in the stable environment also contribute to airway inflammation (Pirie*, et al.*, 2001; Pirie, Dixon & McGorum, 2003).

In summary, exposure to various factors in the stable environment contributes substantially to lower airway inflammation in horses, but there is a lack of studies comparing hygienic measurements in the stable environment to inflammation markers.

Immunology

Provocation studies using moldy hay or other inhalable agents with the potential to induce airway inflammation, have been widely used in order to evaluate temporal physiologic and immunologic events following exacerbation of RAO. These studies often vary substantially in the methods of provocation, sample material studied, sampling time in relation to provocation, laboratory methods, and material being analyzed and possibly even disease phenotype of experimental horses. On the other hand, when horses in their own stable environment develop naturally occurring RAO are studied, additional problems arise that interfere with data analysis, such as variation in type, amount and timing of provocation and less stringent defined of inclusion criteria for RAO. The airway epithelium works partly as a mechanical barrier along with the mucociliary membrane but also has an important role in both the innate and adaptive immune systems. It is active in clearance of inhaled particles, attracts inflammatory cells through expression of different cytokines/chemokines and adhesion factors and regulates smooth muscle function (Adler *et al.*, 1994; Knight & Holgate, 2003; Takizawa, 1998). The various inflammatory cells, signals for their recruitment and other mediators of the cascade of inflammation have been the focus in the more recent research on RAO. These studies have concentrated on understanding the mechanism of observed neutrophil influx to the lung and attempted to determine if the underlying mechanism of inflammation is a true allergic response or a more non-specific inflammatory response to inhaled agents.

Exacerbation of disease in RAO is characterised by neutrophil influx to the lung together with appearance of clinical signs 4-5 hours after exposure to moldy hay (Fairbairn *et al.*, 1993). Neutrophils boost the elimination of inhaled particles by phagocytosis and release of peroxidases, proteases and nucleases. The early signals needed to attract these neutrophils and their potential roles in the disease RAO have been the subject of substantial research efforts over the past several decades. The gene and protein expression of the neutrophil chemotactic cytokine IL-8 is increased in RAO horses during disease exacerbation (Ainsworth *et al.*, 2003; Ainsworth *et al.*, 2006; Franchini *et al.*, 2000; Franchini *et al.*, 1998; Laan *et al.*, 2006). Ainsworth *et al*. (Ainsworth*, et al.*, 2006) recently showed an increase of IL-8 mRNA expression, not only in BAL cells or endobronchial brushings, but also increased IL-8 immunoreactivity in endobronchial biopsies in RAO horses after challenge. Additionally, mRNA for IL-17, a cytokine that indirectly contributes to the neutrophil recruitment to the airways through NF-κB (nuclear factor–κB activation), was elevated in BAL fluid (BALF) after long term

exposure to moldy hay (Ainsworth, *et al.*, 2006; Debrue *et al.*, 2005). However, the mRNA levels of IL-17 in these studies failed to correlate with observed airway neutrophilia. Additionally, even if epithelial driven IL-8 mRNA was shown to correlate wiyh BALF neutrophil counts and epithelial Yoll-like receptor, TLR4, it appeared that BAL neutrophilia is present in advance of the increase in IL-8, indicating that signals apart from IL-8 are more likely responsible for initiating neutrophil influx to the lung in RAO (Ainsworth, *et al.*, 2003; Berndt *et al.*, 2006).

Initiation of airway inflammation in RAO horses is also associated with increased activity of the transcription factors for protein expression. An increased NF-κB p65 homodimer activity in bronchial epithelium in RAO horses has been shown to correlate with altered lung function (Bureau *et al.*, 2000a). This increase has been observed 24 hour after disease exacerbation and remarkably persisted at least 21 days into remission, indicating that the presence of the initiating factor/s for RAO are not necessary required for persistent inflammatory response (Bureau, *et al.*, 2000a). Interestingly, in later *in vitro* studies this activity of NF-κB in bronchial brushing samples was maintained as long as viable granulocytes were present. The eventual autoregulatory feedback system is mediated by cytokines such as IL-1β and TNF-α (Bureau *et al.*, 2000b). Additionally, there are indications that spontaneous neutrophil apoptosis (Brazil *et al.*, 2005) can be delayed due to protective mechanisms (Bureau, *et al.*, 2000b), leading to increased life spans for neutrophil granulocytes in airway lumen.

As part of innate immunity the alveolar macrophages (AM) can, after stimulus, be a source of the neutrophil chemo-attractant IL-8 and of macrophage inflammatory protein (MIP-2) which stimulates neutrophil influx into the airway lumen (Franchini, *et al.*, 1998). More recent studies have confirmed the role of AM in pathogenesis of RAO by demonstrating increased gene expression of different pro-inflammatory cytokines in BALF isolated AM's from RAO horses (Laan, *et al.*, 2006; Laan, *et al.*, 2005). Additionally, inhaled dust components are recognized by Toll-like receptors (TLR), expressed in a variety of cells including alveolar macrophages and pulmonary epithelial cells (Berndt, *et al.*, 2006). Endotoxins are also recognized by TLR's, specifically TLR4. Interestingly an increased amount of TLR4 mRNA (potentially leading to increased IL-8 mRNA expression) together with lack of upregulation of zinc finger protein A20 (involved in negative regulation of TLR4) have been shown in bronchial epithelial cells collected from stabled RAO horses (Berndt, *et al.*, 2006). However, others studies have found that the increase of TLR4 gene expression was observed in BAL cells but not in endobronchial biopsies (Ainsworth, *et al.*, 2006). As a further response to inhaled dust gelatinolytic and collagenolytic matrix metalloproteinases (MMPs) increase in equine airways. These substances are associated with airway remodelling (Nevalainen, *et al.*, 2002; Raulo *et al.*, 2001a; Raulo *et al.*, 2001b; Raulo, Sorsa & Maisi, 2000; Simonen-Jokinen, *et al.*, 2005). In summary, these findings regarding innate immunity suggest that a specific allergic reaction is not required for neutrophil influx to the lung in RAO horses.

The contribution of T-cells to development of RAO, through cytokines related to disease exacerbation and cell influx to the lung of the horses has also been widely studied. The occurence of T lymphocyte phenotypes (Ainsworth *et al.*, 2002; Kleiber *et al.*, 1999; McGorum, Dixon & Halliwell, 1993b; Watson *et al.*, 1997) and cytokine expression in BAL fluid and peripheral blood samples in RAO horses are contradictory in earlier studies (Cordeau *et al.*, 2004; Giguere*, et al.*, 2002; Kleiber *et al.*, 2005; Lavoie *et al.*, 2001). In support of a Th2 response increased IgE anti-*Aspergillus fumigatus* and *Micropolysopra faeni* in BAL fluid and increased IgE against recombinant mould allergens in serum have been found in RAO horses (Eder *et al.*, 2000; Halliwell *et al.*, 1993; Schmallenbach *et al.*, 1998). Additionally, increased expressions of IL-4 and IL-5 mRNA, and decreased amount of IFN-γ mRNA in BAL fluid from horses with RAO have suggested a predominance of Th2 type (hypersensitivity type) response in acute exacerbation of RAO (Cordeau*, et al.*, 2004; Lavoie*, et al.*, 2001). Conversely, during the chronic phase of the disease an increased IFN-γ mRNA expression in BAL (Ainsworth*, et al.*, 2003; Giguere*, et al.*, 2002) has been measured. Others even suggest a mixed Th1/ Th2 picture, depending on timing of sampling (Giguere*, et al.*, 2002). When cytokine profiles from peripheral blood and BAL fluid purified populations of CD4+ and CD8+ lymphocytes from RAO horses were quantified 48h after provocation there was no association with Th1 or Th2 responses, indicating that differences in earlier studies can partly be explained with differences in proportions of lymphocytes or mixed cell populations in BAL samples (Kleiber*, et al.*, 2005). Expected time dependent factors (discussed above) and the type of pro-inflammatory agents in the hay or straw can influence the immunological pathways activated (Rivera *et al.*, 2006). In summary, it remains uncertain whether the immunological mechanisms behind RAO in horses are due to Th1 or Th2 cell mediated reactions or a mixture of the two.

Mast cells, although comprising a minor part of the cell population in BALF, can potentially contribute to the inflammatory response associated with challenge with dusty hay (Dacre et al., 2007; McGorum, Dixon & Halliwell, 1993c; van der Haegen et al., 2005). Inhalation challenge increases the quantity of mast cell protein tryptase (indicating mast cell degranulation) (Dacre, et al., 2007) in BALF and histamine concentration in pulmonary epithelial lining fluid (McGorum, Dixon & Halliwell, 1993c). Mast cell tryptase in BAL has been shown to be elevated in both controls and RAO horses as response to inhalation challenge, but an increased number of mast cells in bronchial epithelium appears to occur only in RAO affected horses (Dacre*, et al.*, 2007). A significant association with BALF mast cell percentage and airway reactivity in horses with IAD supports further the potential role of mast cells in the inflammatory response in horse airways (Hoffman, Mazan & Ellenberg, 1998).

In summary, the immunological mechanisms behind RAO and factors initiating neutrophil influx to the equine lung remain incompletely understood. More focus on early events in cell signalling of the immune triggers and events hold promise to clarify this murky situation and resolve some of the conflicts in reports surrounding the immune processes in this disease.

Pulmonary eosinophilia

Pulmonary eosinophilic accumulation in man is best known for its clear association with asthma, but has also been related to parasitic infections, anaphylactic reactions, and a number of heterogeneous lung diseases distinguished by pulmonary eosinophilia (Chitkara & Krishna, 2006; Kroegel, 2003). In human medicine an idiopathic syndrome of self limiting eosinophilia with minimal respiratory symptoms was described by Löffler in 1932 (Loffler, 1932). The term "Löfflers Syndrome" is still used to describe transient blood and BAL eosinophilia of unknown cause. However, closer inspection occasionally reveals underlying parasitic infection or reaction to extrinsic agents such as drugs, chemicals or inhaled smoke (Chitkara & Krishna, 2006; Kroegel, 2003). The etiology and pathogenesis underlying eosinophilic inflammation has been studied extensively in medical research, but is complex and much remains undefined. In veterinary medicine, the best described eosinophilic disorder is idiopathic canine eosinophilic bronchopneumopathy (EBP) which is characterised by eosinophil accumulation in pulmonary interstitium and bronchial mucosa in dogs (Peeters *et al.*, 2006; Rajamaki *et al.*, 2006). The predominant research on lung diseases in horses has focused on biology of well known neutrophilic inflammation (Robinson, 2001; Robinson, 2003) whereas scant attention has been afforded horses with pulmonary eosinophilia. Possible causes of eosinophilic pulmonary inflammation in horses include parasitic pulmonary disease (parascaris migration or lungworm infection), hypersensitivity pneumonitis and multisystemic eosinophilic epitheliotrophic disease (Singh *et al.*, 2006). There are also several case-reports in horse describing various syndromes with unknown etiology characterised by eosinophil accumulation in lungs and other organs (Latimer *et al.*, 1996).

Bronchoalveolar lavage fluid eosinophilia has been described in IAD horses (Hare & Viel, 1998; Moore*, et al.*, 1995) but its significance is as yet unclear. Hare and Viel (Hare & Viel, 1998) found increased airway responsiveness to histamine challenge post exercise in a selected group of race horses with elevated eosinophils in BALF. While the genesis of the pulmonary eosinophilia was not established they hypothesised it to be allergic in nature, persistent, and potentially an early manifestation of RAO.

Since the eosinophil appears to play a central role in human asthma (Foster *et al.*, 2001; Hamelmann & Gelfand, 2001; Hogan *et al.*, 1997; Hogan *et al.*, 1998; Johansson *et al.*, 2004; Kroegel, 2003; Rennick *et al.*, 1990; Tomaki *et al.*, 2002; Wang *et al.*, 1998; Webb *et al.*, 2001), molecular mechanisms regulating eosinophil differentiation, survival and regulation of apoptosis have been extensively studied *in vivo* and *in vitro* in human and animal models. In the complex of eosinophilic inflammation IL-5 is a key signal for eosinophil recruitment from bone marrow (Benarafa *et al.*, 2002; Coffman *et al.*, 1989; Foster *et al.*, 1996; Hamelmann & Gelfand, 2001; Hogan*, et al.*, 1997; Hogan*, et al.*, 1998; Johansson*, et al.*, 2004; Rankin, Conroy & Williams, 2000; Rennick*, et al.*,

1990; Tomaki, *et al.*, 2002; Wang, *et al.*, 1998). Interleukin-3, granulocyte-macrophage stimulating factor (GM-CFS) and in particular, IL-5 stimulate eosinophil maturation, survival, priming and activation (Hamelmann & Gelfand, 2001; Rankin, Conroy & Williams, 2000; Tomaki, *et al.*, 2002). Interleukin-5 together with eotaxin co-operate locally in pulmonary tissue and provoke pulmonary eosinophilia (Foster, *et al.*, 2001). Another important signal for eosinophil accumulation is increased levels of IL-13 and IL-4 that upregulate IgE synthesis and co-operate to enhance eosinophil transmigration to pulmonary tissue (Foster, *et al.*, 2001; Hamelmann & Gelfand, 2001).

While less directly connected to eosinophil trafficking, IL-4 has a major role in induction of different allergic responses. It regulates the T-cell switch to the CD4+Th2-type phenotype, and plays an important role in IgE isotype switching in B cells, and regulates eotaxin production. It has also a marked inhibitory effect on some proinflammatory cytokines (Opal & DePalo, 2000). The cytokines IL-4 and IL-13 up-regulate VCAM-1 expression in endothelial cells and eotaxin generation needed for eosinophil recruitment (Rankin, Conroy & Williams, 2000), thus promoting transmigration of eosinophils to the pulmonary tissue (Foster, *et al.*, 2001; Gleich, 2000). It has been shown that IL-5 in horses is produced by activated blood mononuclear cells (Giguere & Prescott, 1999) and that IL-5 stimulates equine eosinophils in vitro (Cunningham *et al.*, 2003; Sepulveda *et al.*, 2005). Further, IL-5 and IL-4 mRNA expression in BAL cells have been studied in horses with RAO, associated with neutrophilic rather than eosinophilic pulmonary influx (Cordeau, *et al.*, 2004; Giguere, *et al.*, 2002; Lavoie, *et al.*, 2001). Despite the central role of these cytokines in a Th2 response their connection to eosinophil kinetics in equine lung diseases has not been examined.

Diagnostic tools

There are several techniques in equine practice and research to detect, quantify, and characterise the inflammatory process in the respiratory tract, including physiologic measurements, and various analyses to examine cellular and noncellular immunologic components. Diagnosis of RAO/IAD is determined on the combined basis of history, clinical examination, endoscopy and, in many instances, BAL cytology. Results from pulmonary function tests are used in research and for clinical purposes for disease monitoring and in grading of disease severity. Arterial blood gas tension is a simple but relatively insensitive way to mirror the gas exchange in the lung. For research purposes scintigraphy (Votion *et al.*, 1999) or the multiple inert gas elimination technique (Nyman, Bjork & Funkquist, 1999) can be used for sensitive evaluation of ventilation-perfusion mismatching in the lung. The conventional measurement of pleural pressure changes in horses has traditionally been used for dynamic lung function testing with measurement of pleural pressure and airflow after placement of oesophageal balloon catheter (Derksen, *et al.*, 1985a). However, recent more sensitive techniques including forced oscillatory mechanics (FOM) with monofrequency method (Art *et al.*, 1989; Young, Tesarowski & Viel, 1997) or impulse oscillometry (van Erck *et al.*, 2004), which requires only placement of a facemask

on the horse, can be used to detect subtler airway physiologic aberrations. Other techniques for lung-function testing include plethysmography (Hoffman *et al.*, 2007), forced expiratory flow volume curves (Couetil*, et al.*, 2001; Couetil, Rosenthal & Simpson, 2000) and even tests of airway reactivity (Hoffman, Mazan & Ellenberg, 1998). Additionally, exhaled breath condensate has also been introduced as a tool to evaluate of inflammation markers in lung (Wyse *et al.*, 2005). In the search for potential allergens involved in RAO an intradermal skin testing and serum IgE analysis have been studies, but these have not been proven clinically reliable in horses (Lebis, Bourdeau & Marzin-Keller, 2002; Lorch *et al.*, 2001a; Lorch *et al.*, 2001b; McGorum, Dixon & Halliwell, 1993a). In order to collect material for investigation of the inflammatory process in the equine lung bronchial brushings, BAL, tracheal lavage, exhaled breath condensate, and even lung tissue samples have been of value (Ainsworth*, et al.*, 2003; Bureau*, et al.*, 2000a; Couetil *et al.*, 2006a; Gerber *et al.*, 2003b; Giguere*, et al.*, 2002; Lugo*, et al.*, 2006; Schatzmann *et al.*, 1974; Wyse*, et al.*, 2005) Imaging techniques, such as radiographic examination of equine lung, however, have little value for diagnostics of lower grade inflammation (Mazan, Vin & Hoffman, 2005), but can be useful in order to exclude other causes for respiratory disease in RAO and IAD horses that fail to respond to appropriate treatment.

Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) cytology has been a standard diagnostic method to examine in horses for lower respiratory inflammation since the early 1980's (Viel, 1983). In addition for routine diagnostics for investigation of diffuse pulmonary disorders in equine practise it has been widely used in research. BAL cytology and various molecular markers for inflammation in BAL fluid are traditionally used in pharmacological studies (Couetil *et al.*, 2006b; Moore *et al.*, 2004; Robinson *et al.*, 2002; Rush *et al.*, 1998a; Traub-Dargatz *et al.*, 1992), immunological and etiological studies (Balson, Smith & Yager, 1997; Couetil*, et al.*, 2006a; Turlej *et al.*, 2001; Watson*, et al.*, 1997). Additionally BAL has also been the main tool in order to investigate the effect of stabling in airway inflammation in horses (Derksen*, et al.*, 1985b; Gerber*, et al.*, 2004b; Holcombe*, et al.*, 2001; Tremblay*, et al.*, 1993). Finally, BAL methodology plays an important role as a research tool in the area of exercise physiology and exercise intolerance in horses (Raidal *et al.*, 2000; Raidal, Rose & Love, 2001). The method for BAL sampling and analysis has not been standardised, but generally a total of 300 -500 ml of lavage fluid (generally isotonic saline, NaCl, in 37ºC), is used in one or several aliquots in order to obtain true bronchoalveolar lavage rather than bronchial lavage (Sweeney *et al.*, 1992; Viel, 1983). Guidelines for upper limits of inflammatory cells in BALF have been described (Couetil*, et al.*, 2007; Robinson, 2003), As the sampling method (endosope/blind tube), use of sedatives and local anesthesia, volume and aliquots, fixatives, cell preparation and other factors can vary between research centres, the methodological aspects of both sampling and sample preparation must be taken into consideration for interpretation of BAL findings (Lapointe, Vrins & Lavoie, 1994; Pickles *et al.*, 2002a; Pickles *et al.*, 2002b; Pickles *et al.*, 2002c; Sweeney*, et al.*, 1992).

Tracheal aspirate

Tracheal aspirate (TA), usually gathered by an endoscopically guided catheter, can be used complementary to BAL, especially when bacteriological samples are collected. However, as these samples represent a blend of secretions from the entire lower respiratory tract, there is poor correlation between cytological findings in TA and BAL. Moreover there is poor correlation between TA and histopathological findings of lowers airway disease (Derksen *et al.*, 1989; Hughes *et al.*, 2003; Larson & Busch, 1985; Malikides *et al.*, 2003). Thus TA alone should not be used as a main diagnostic criterion for IAD or RAO (Couetil*, et al.*, 2007). The cut off value for neutrophil numbers or percentage in TA has not been validated, but in samples collected from young racehorses the level of 20% neutrophils in TA has been proposed (Christley*, et al.*, 2001; Malikides, Hughes & Hodgson, 2007). In other examinations of nonracing horses up to 70% of the horses have higher neutrophil % in TA than the aforementioned suggested cut off level (Robinson*, et al.*, 2003). Bacterial growth in TA samples in RAO horses is a common finding and can be related to impaired airway clearance with mucus accumulation, contamination from upper airway bacteria or in the worse case, true bacterial colonization and respiratory infection. As TA represents a pooled sample from the whole lung and BAL represents only a lung segment lavaged, bacteriological samples should be collected with TA (Naylor, Clark & Clayton, 1992).

Tissue sampling

Airway tissue samples provide an opportunity to further study pathological changes in airways in correlation to structural and cellular morphology. Further advantages of tissue samples are only now being appreciated, with continual development of molecular techniques to dissect the cellular and subcellular events in airway inflammation. Tissue biopsies are used in human medicine for several different fields: for further studies of pathogenesis of different respiratory diseases (asthma and COPD), as a complementary method for BAL and induced sputum, for evaluation of effect of different drug treatments, for definition of the state of the disease and for investigation of environmental effects in the respiratory system. A classical way to obtain tissue material from the distal airways from RAO horses, has been transthoracic lung biopsies (Pusterla *et al.*, 2007; Schatzmann*, et al.*, 1974) and more recently thoracoscopically guided pulmonary wedge resection (Lugo*, et al.*, 2006; Lugo *et al.*, 2002). However, both these techniques are invasive, and the former associated with fatal complications (Savage, Traub-Dargatz & Mumford, 1998). As a safer alternative, endobronchial biopsies are used increasingly in human medicine as a complementary method to BAL and induced sputum. Bronchial biopsies are used for study of the underlying pathogenesis of diseases such as asthma and chronic obstructive pulmonary disease, and for evaluation of effects of different treatments (Behndig *et al.*, 2006; Jeffery, Holgate & Wenzel, 2003; Jeffery, 1998b). Endobronchial biopsy sampling in humans is considered as a safe invasive method (Djukanovic *et al.*, 1991; Elston *et al.*, 2004; Hattotuwa *et al.*, 2002; Jeffery, Holgate & Wenzel, 2003; Payne *et*

al., 2001; Saglani *et al.*, 2003) and it has been suggested that BAL accounts for more of the adverse events than biopsy procedures (Elston*, et al.*, 2004). In contrast to BAL bronchial biopsies provide tissues from which the airway morphology can be assessed (e.g. epithelial damage in asthma), resident and infiltrating inflammatory cells can be quantified, and gene products identified. Resident airway epithelial cells play an important role in inflammation through interaction with inflammatory cells, acting as a source of various inflammatory cytokines and mediators, expression of adhesion molecules for recruitment of inflammatory cells, and contributing to airway remodelling (Djukanovic *et al.*, 1990; Holgate, 2000; Jeffery, Laitinen & Venge, 2000; Knight & Holgate, 2003; Rennard *et al.*, 1995; Takizawa, 1998). Additionally, in some instances, an indication of severity or prognosis can be discerned (Fabbri *et al.*, 1998; Holgate, 2000; Jeffery, 1998a; Jeffery, Holgate & Wenzel, 2003; Jeffery, 1996; Jeffery, Laitinen & Venge, 2000; Saetta *et al.*, 1998). Surprisingly, even if the airway inflammation and obstruction of RAO has many similarities to airway alterations in asthma in man, the utility of bronchial biopsy for study of RAO in the horse has only recently been reported (Ainsworth*, et al.*, 2006). The major reason for this is probably the selection of relatively proximal biopsy sites, whereas in RAO the currently detectable airway changes are primarily in the bronchioles and not in the bronchi. This limitation is further discussed in chapter aspects on material and methods.

Aims

The overall aim of this thesis was:

- To examine inflammatory pathways in equine airways, with special regards to the role of airway epithelium in relation stable air environment.

The specific aims were:

- To compare the cytokine mRNA expression in bronchial epithelium and BAL cells in RAO horses during respiratory crisis and on remission.
- To observe whether there is a difference in mRNA and protein expression of the same cytokines in bronchial tissue during acute inflammation and in remission in RAO horses.
- To evaluate the clinical signs and cytokine mRNA expression related to eosinophil kinetics in horses with transient pulmonary eosinophilia.
- To investigate the seasonal influence of stable environment on inflammation markers in BAL cells of stabled horses.

Material and methods

A brief summary on animals, study protocols, used sampling methods, processing techniques and analyses are presented below.

Animals

The studies included detailed clinical data and results from BAL cytology and bronchial biopsy samples collected from both experimental horses in association with provocation tests and also from privately own horses exposed to natural challenge during stabling periods. A summary of the signalment of the horses included in studies and study protocols are shown in table 1. Horses with RAO as well as horses with varying degrees of IAD and healthy controls were included in these studies. Studies I and II were performed in collaboration with Equine Sports Medicine Centre, Faculty of Veterinary Medicine, University of Liège, Belgium and involved RAO horses and healthy controls owned by the University. Studies III and IV involved young privately owned Standardbred trotters from one trainer that were followed longitudinally, of which 11 were included in study III and 9 in study IV. The studies were approved by the institutional animal care and use committee, University of Liège, Belgium (studies I-II) and by the Ethical Committee for Animal Experiments, Uppsala, Sweden (studies III, and IV).

Study design

Paper I

The study was designed in order to investigate the cytokine mRNA expression in bronchial epithelium versus BAL cells in RAO horses as response to inhalation provocation. Endobronchial biopsies and BALF were obtained from seven well documented RAO horses after provocation with moldy hay, straw bedding and poor stable ventilation that induced impairment of pulmonary function. The RAO horses were reexamined after remission when horses had been at pasture for two months, at which time even healthy controls on pasture were also included in the study. The cytokine mRNA expression in bronchial mucosal biopsies and BAL-cells were analyzed with quantitative real-time PCR regarding cytokines related to inflammatory response; IL-5, IL-6, IL-8, IL-10, IL-17 and TGF-β1.

Paper II

To broaden the knowledge gathered in the previous study (I), the bronchial tissue mRNA levels and protein expression of selected cytokines (IL-6, IL-10 and TNF-α) in bronchial tissues were compared. The mRNA levels for these selected cytokines in bronchial mucosal biopsies were analyzed by quantitative real-time PCR and protein levels of the same cytokines were measured by immunohistochemistry (IHC).

Paper III

Transient pulmonary eosinophilia was detected in the group of Standardbreds, which were part of an ongoing study presented in paper IV. In order to assess clinical changes related to pulmonary eosinophilia in horses and to examine some of the immunological aspects of pulmonary eosinophil kinetics in horses, the detailed clinical signs and cytokine mRNA in BAL (IL-4 and IL-5 mRNA, real time-PCR) as well as morphology of bronchial epithelium were investigated before, during, and after the episode of pulmonary eosinophilia. Additionally, the results from parasitological samples and stable environmental measurements were included in the analysis for the possible the etiology of the observed pulmonary eosinophilia.

Fig. 1. Transient pulmonary eosinophilia was observed in a group Standardbreds in training. Photo: Carl-Henry Bergström (left) and John Pringle (right).

Paper IV

The original aim of this study was to observe whether seasonally associated factors in stable air could be related to indices of airway inflammation of stabled horses. Twelve young Standardbred trotters and their stable environment were investigated twice during the winter stabling period (Feb 2004, March 2005) and once following a prolonged period at pasture (Sept 2005). The pulmonary response in the horses was evaluated using detailed clinical examinations, endoscopy, BAL cytology and quantitative real-time PCR analysis of cytokine mRNA (IL-6 and IL-10) expression in BAL cells. The results from stable measurements, total and respirable dust, endotoxin and (1→3)-β-D-glucan, were compared to inflammatory signs in the respiratory tract of the horses.

Fig. 2. The stable environment and the horses were investigated during one summer and two winter samplings. Photo: Carl-Henry Bergström.

Table 1. Summary of material and methods presented in papers I-IV

Study	Animals	Sampling methods	Analysis methods	Factors analyzed
I	7 RAO horses on crisis/pasture Age 19.7±SD 6.6 years 6 mares, 1 gelding 6 Controls on pasture Age 16.8±SD 7.9 years 5 mares, 1 gelding	Pulmonary function tests BAL Endobronchial biopsies	Oesophageal balloon catheter technique, pneumotachography Real-time PCR	Pleural pressure changes, respiratory airflow BAL cytology Real-time PCR BAL cells and biopsies for cytokines: IL-5, IL-6, IL-8, IL-10, IL-17 and TGF-β1
II	7 RAO horses on crisis/pasture Age 19.7±SD 6.6 years 6 mares, 1 gelding 6 Controls on pasture Age 16.8±SD 7.9 years 5 mares, 1 gelding	Pulmonary function tests BAL Endobronchial biopsies	Oesophageal balloon catheter technique, pneumotachography Real-time PCR IHC	Pleural pressure changes, respiratory airflow BAL cytology Real-time PCR and IHC in biopsies for IL-6, IL-10 and TNF-α
III	11 Standarbred trotters in training Age 4.4 ± SD 1.4 years 7 mares, 3 geldings and 1 stallion Three examinations; Prior, during and after observed pulmonary eosinophilia	Arterial and Venous blood sampling BAL Endobronchial biopsies Faecalsampling Environmental measurements	Real-time PCR Biopsy morphology	Haematology and blood gases BAL cytology Real-time PCR for in BAL cells: IL-4 and IL-5 Parasitological examinations Total and respirable dust, endotoxin and glucan
IV	9 Standarbred trotters in training Age 4.4± SD 1.6 years 5 mares, 3 geldings and 1 stallion One summer and two winter assessments	Arterial and Venous blood sampling BAL Environmental measurements	Real-time PCR	Haematology and blood gases BAL cytology Real-time PCR for in BAL cells: IL-6 and IL-10 Total and respirable dust, endotoxin and glucan

Sampling methods

Clinical examination and hematology

In all studies the horses received detailed clinical and respiratory examinations and in studies I and II, the clinical signs in the horses were scored by a blinded observer according to a previously published scoring system (Couetil*, et al.*, 2006a; Rush*, et al.*, 1998a). In studies III-IV all horses received detailed clinical and respiratory examinations, which included chest auscultation following rebreathing. The clinical signs were scored from I-IV (from normal to markedly abnormal values) by the same two veterinarians in all horses. Routine blood sample analysis for white and red blood cell parameters (Cell-Dyn 3500, Abbott Laboratories, North Chicago, IL, USA) and plasma fibrinogen (Konelab 30, Thermo Fisher Scientific, Waltham, USA) were performed in studies III and IV.

Fig. 3. Horses received detailed clinical and respiratory examinations. Photo: Roland Thunholm and John Pringle.

Pulmonary function tests

In studies I and II, pleural pressure changes were measured with an oesophageal balloon catheter connected to a calibrated pressure transducer and the air flow and volume were measured through a facemask by a Fleish pneumotachograph (Model NR4; Gould Electronics, Eichstetten, Germany). The maximum change in transpulmonary pressure (ΔPpl_{max}), pulmonary resistance (R_L) and dynamic compliance (C_{dyn}) were obtained from lung a function computer (Haemodynamics Respiratory System; ACEC, Namur, Belgium). Each measurement period averaged 2 min, and an average value of 10 representative breaths was selected for analyses. In studies III-IV, the arterial blood gas partial pressures (PaO_2 and $PaCO_2$) were used as general indices of pulmonary function.

Endoscopy

Upper and lower airway endoscopy (Pentax, Breda, The Netherlands, 170 cm x 12.9 mm (studies I and II) or VES vet vision, XiON matrix FX 100, endoscope

PV-G28, XiON medical, Berlin, Germany, 180cm x 10,4mm (study III and IV)), was performed after sedation in all studies. The pharynx, guttural pouches (study III and IV only), trachea and parts of the bronchial tree of one lung (during biopsy sampling) were examined endoscopically. Grading of mucus in trachea or nasal discharge in studies III-IV were scored on a scale of grade 1-3 as described in paper III. In studies I and II the mucus was quantified using a previously published 5-grade system (Gerber*, et al.*, 2004b).

Fig. 4. Upper and lower airway endoscopy was performed after sedation in all studies. Photo: Roland Thunholm.

Bronchoalveolar Lavage

BAL and biopsy samples were collected by bronchoscopy after intravenous premedication with sedative (romifidine, Sedivet® vet; Boehringer Ingelheim, Ingelheim, Germany, or detomidin, Domosedan® vet, Pharma AB, Espoo, Finland and butorphanol tartrate, Torbugesic®; Fort Dodge, Wyeth, Madison, New Jersey, USA). The BAL was performed either with 6 x 60 ml (studies I, II and III) or 3 x 100 ml (studies III and IV) sterile isotonic saline (studies I, II and V) solution (at 37°C). Fluid recovered was placed on ice, samples pooled and an aliquot collected for cytology. In studies III and IV a local anesthetic (Carbocain®, AstraZeneca, Södertälje, Sweden) was instilled endoscopically at the tracheal bifurcation as well as at several levels of the bronchial tree before performing endobronchial biopsy and BAL.

Fig, 5. Bronchoalveolar lavage sampling. Photo: Roland Thunholm.

Biopsies

In all studies six biopsies (approximately 1-2 mm in diameter) were collected from each horse via endoscopy from the bronchial epithelium from subsegmental and segmental subcarinae with a biopsy forceps (reusable, 240cm, Pentax, KW3433S, Pentax Corporation, Tokyo, Japan). To avoid blood contamination of the BAL, biopsies were taken from the non-lavaged lung. In order to avoid artifacts created by earlier biopsy locations the contralateral site was chosen for second bronchoscopy when sampling was performed at 6 month intervals as in study III and IV. The first two biopsies in each horse were taken at the first generation bronchus and immediately frozen in liquid nitrogen and stored at -80°C until analysis for cytokine mRNA with real-time PCR. The remaining four biopsies collected from subsegmental and segmental subcarinae were immediately fixed in chilled acetone containing protease inhibitors (20mM iodoacetamide and 2mM phenyl methyl sulfonyl fluoride) at -20°C overnight for further embedding. After fixation, biopsies were processed into glycolmethacrylate (GMA) resin (Britten, Howarth & Roche, 1993), and stored at –20°C in airtight tubes in -20°C until cutting and immunostaining. Transthoracic lung biopsies obtained from one horse in study III were sampled according to an earlier described technique (Schatzmann*, et al.*, 1974) and embedded with the same method as the endobronchial biopsies.

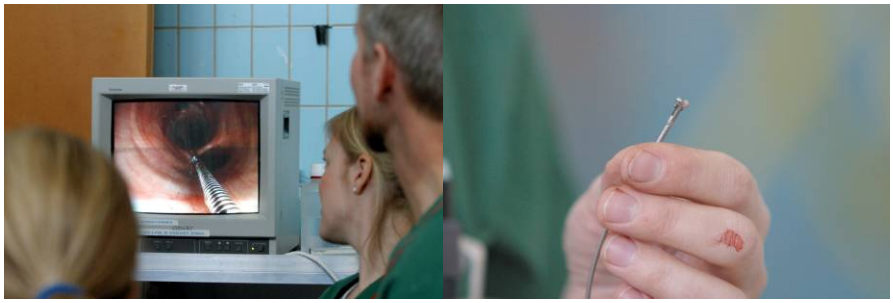

Fig. 6. Bronchial biopsy sampling endoscopically with a biopsy forceps. Photo: Roland Thunholm.

Environmental measurements

Environmental measurements in study III and IV were collected, over a 4-7 hours period during the daytime routines in the investigated stable. Air sampling was performed with pumps (SKC Inc., Eighty Four, PA, USA) placed in the breathing zone approximately 1-1.5 m above ground level at three points in the stable corridor. Total and respirable dusts in air were collected in separate cassettes with a 25 mm (pore size 0.8 µm) membrane filter. Total-dust samplers had open faced cassettes with no size-selection. In the case of respirable dust, a metal cyclone separator (SKC Inc.; USA) was added before the filter cassette, removing particles of > 3.5 µm. The equipment for respirable dust was attached to the personnel's clothing in the breathing zone. Sampling was done with a flow of 2 L/min for 4-7 hours. Airborne endotoxin and (1→3)-β-D-glucan were sampled in separate cassette-holders with 25 mm nucleopore filters (pore size 0.4 µm, 2.0 L/min; 4 hours). Horse allergen was collected with an IOM-sampler attached to a pump and operating with airflow of 2.0 L/min and sampling was performed during 4-7 hours.

Fecal sampling

Due to laboratory delay in reading of BAL cytology in study III fecal samples that were collected in that study were unfortunately obtained only two months after the BAL sampling in which pulmonary eosinophilia was observed. At the first sampling occasion individual samples were collected from three horses with highest number of eosinophils in BAL and from the remaining horses the samples were pooled in three groups. The second sampling was performed in connection to re-examination approximately 14 weeks after deworming, when fecal egg count was done on individual samples.

Processing and Analyses

BAL cytology

The total cell count was determined manually in studies I and II (THOMA cell; VWR International, Fontenay-sous-Bois, France). In these studies BAL samples for cytological analysis were centrifuged at 200 x g for 15 minutes (Shandon Cytospin Centrifuge, Thermo Electron Corporation, Pittsburgh, Pennsylvania,

USA) and stained with May-Grünwald staining. Differential cell counts were determined based on counting 200 cells per slide.

In studies III and IV, total leukocyte counts were determined using Cell-Dyn 3500 (Abbott Laboratories, North Chicago, IL, USA). When the optical and impedance leukocyte counts of the Cell-Dyn disagreed, values recorded were from a manual count using a counting chamber. For cytological evaluation of BAL samples in these two studies cytospin preparations were prepared using two different concentrations of BAL-fluid. The first was prepared by centrifuging a 10 ml aliquot at 400 x g for 5 min and resuspending the resulting cell pellet with 50 µl albumin solution (1 g bovine serum albumin and 0.002 g NaN3 dissolved in 10 ml of 0.9% NaCl and stored at 4^{o}C). The uncentrifuged preparation was made by adding 50 µl of the albumin solution to a 250 µl aliquot of the BAL-fluid. Cytospin preparations with 100 µl aliquots were prepared in the cytocentrifuge cassettes. After the smears had dried, they were stained with modified Wright stain in an automatic stainer (Hematec, Bayer Diagnostic Division, Tarrytown, NY, USA). Where possible, 400 cells were counted (200 cells on each slide) and the average number was used. Epithelial cells, which often appear in aggregates, and nonintact cells were excluded from the differential count.

Quantitative real-time PCR for bronchoalveolar lavage and biopsies

In studies I and II quantitative real-time PCR using previously synthesized cDNA was performed with both BAL and biopsy samples. In studies III and IV, quantification of equine mRNA expression was performed by one step quantitative real-time PCR.

After sampling, BAL cells and biopsies were stored at -80°C until RNA was isolated. Total RNA was isolated with Trizol reagent (Invitrogen, Stockholm, Sweden) according to the manufacturer's instructions. When used, cDNA was synthesized from 500 ng total RNA using Superscript III reverse transcriptase (Invitrogen) and gene specific primers. Quantitative real-time PCR was performed on a 7300 Real Time System (Applied Biosystem, Foster City, CA, USA). Primer and probe sequences used are presented in the papers following. In the two step reactions PCR were run with Platinum Taq polymerase and in one step reactions rTth polymerase were used. Reaction conditions were optimized for each primer set.

The RNA levels of the target genes were normalized against GAPDH mRNA levels and the comparative Ct ($2^{-\Delta\Delta Ct}$) method was used for calculating relative cytokine mRNA expression (Livak & Schmittgen, 2001). The PCR efficiencies, as determined by assaying serial dilutions of RNA or from raw fluorescence data by using the DART-PCR software (Peirson, Butler & Foster, 2003), were approximately equal for the target genes and the housekeeping genes.

Immunohistochemistry

Biopsies collected in study II were processed with appropriate antibodies in the same batch of immunostaining. Two sections from one biopsy with optimal morphologic structure from each horse were cut at 2 µm thickness and placed on poly-L-Lysine treated slides. Endogenous peroxidases were inhibited using 0.1% sodium azide and 0.3% hydrogen peroxide for 30 min. After 3x5 min. washes in TRIS-buffered saline with 0.1% Triton-X-100 (TBST), non-specific antibody binding was blocked with undiluted culture medium, Dulbecco´s Modified Eagle´s Medium (Sigma, Missouri, US) containing 10% fetal calf serum and 1% bovine serum albumin (BSA) for 30 minutes. Further blocking of non-specific antibody binding was then done by incubation with swine normal serum for 30 min. The primary antibodies used in paper II, diluted in 0.05% TBST with 1% BSA, and incubated overnight. After rinsing with TBST for 3x5 min, biotinylated swine anti goat IgG F(ab´)2 (Dako Glostrup Denmark) was applied for 2 h, followed by the streptavidin-biotin horseradish peroxidase complex (Dako) for another 2 h. The sections were then visualized with diaminobenzidine (DAB) to yield a brown colour and counter-stained with Mayer Haematoxylin. TBST treated biopsies were used as negative controls.

Quantification of immunostaining

Cytokine immunoreactivity was quantified using a colour video camera (Sony DXC-950P - Sony, Tokyo, Japan) containing 380,000 effective picture elements (pixels). The camera was connected to a Leica Imaging Workstation, with highly specific PC software (Leica Q500IW, Leica Cambridge UK). The image setting included a possibility to carefully adjust the individual colour components being displayed by the system in order to ensure a close match between the direct image and that being displayed. Detection of an appropriate colour was quantified using binary definition of colour images as displayed on the screen. The binary image required the user to define which pixel in the image that was to be considered for measurement. The positively stained area was managed as a grid of pixels containing the binary value 1 (the pixel is "set") and remaining area contains the binary value 0 (the pixel is not "set"). All intact epithelium from both sections was used for quantification of epithelial area, from which positive immunoreactivity area was compared to the total measured area.

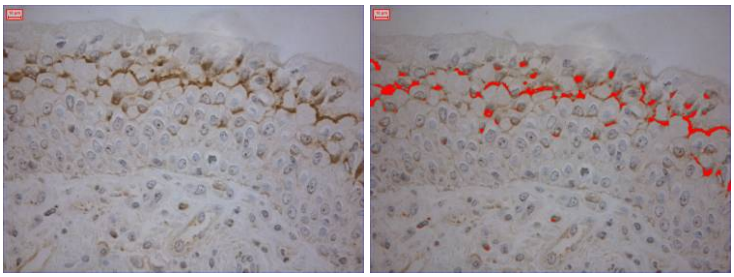

Fig. 7. Immunohistochemical staining of IL-6 in an RAO horse during remission (left) and the staining as quantified with Leica Imaging Workstation (right). Photo; Jamshid Pourazar.

Environmental measurements

The total and respirable dust samples collected for study IV were analyzed by a gravimetric method, and the organic proportion was calculated after combustion of the filter and weighing of the remaining inorganic material (Occupational and Environmental Medicine Laboratory, Orebro University Hospital, Sweden). The detection limit was 0.1 mg/sample and results presented as mg/m^3.

For analysis of endotoxin and (1→3)-β-D-glucan the filters were extracted with pyrogen-free water. Endotoxin was determined by the Department of Environmental Medicine, University of Gothenburg (Feb 2004) and Department of Infection Control, Uppsala University Hospital (Sep 2004) using the kinetic turbidimetric method with the Limulus test (Cape Cod Inc MA USA and Endosafe; Charles River Endosafe, Charleston, USA, respectively). The results are presented as ng/m^3 and the detection limit was 0.147 ng/m^3.

The amount of (1→3)-β-D-glucan was determined by the Department of Environmental Medicine using the Limulus test with glucan specific lysate (Cape Cod, MA, USA) in the chromogenic, kinetic version. The results are presented in ng/m3 and the detection limit was 0.1 ng/m^3. Due to laboratory problems the determinations of endotoxin and (1→3)-β-D-glucan from March 2005 were not included.

Fecal flotation

Fecal egg counts in study III were carried out using a modified McMaster technique. The fecal samples from the first sample collection were analyzed by a qualitative method due the low sampling volume and the quantitative method was at analysis of second sampling. The examination for *Dictyocaulus* spp. was carried out with the Baermann funnel technique (Anon, 1986).

Histopathological assessment of biopsies

In study III, endobronchial biopsies and the transthoracic lung biopsy were processed for further histopathological analysis; they were cut at 2 µm thick sections and stained with Mayers' haematoxylin for microscopic assessment of inflammatory changes. The pathologist was unaware of the BAL results associated with each biopsy.

Statistical analyses

Statistical comparisons were performed using a commercially available statistical software program (MINITAB). Data were treated as being nonparametric because of the low number of animals. Descriptive statistics from each sampling period were expressed as median ± SD. In all studies the Wilcoxon signed rank test was also used for relative cytokine expression for individual horses, where the theoretic median value 1 was used (theoretically no difference between measurements, or $\Delta\Delta C_T=0$, $2^{-\Delta\Delta C_T}=2^0=1$). The ΔC_T value is the difference between the CT value for the target gene and housekeeping gene, and the $\Delta\Delta C_T$ value is the

difference between ΔC_T values that are compared. In all studies a p value < 0.05 was selected as the level of significance.

In studies I and II, the Wilcoxon signed rank test was used to assess for differences between provocation and remission in RAO-horses, using a two tailed test (IHC, BAL cytology, clinical score and pulmonary function). The difference between mean ΔC_T values, for RAO horses in remission and controls on pasture for each cytokine, was used to count differences in cytokine mRNA expression and statistically tested using the Mann Whitney test. The Mann-Whitney test also was used to determine if there was a difference in BAL cell counts and pulmonary function between controls and principal animals during pasture. In study I, the Spearman rank order correlation was used to evaluate the correlation in cytokine expression in BAL cells and biopsies, and in study II the same test was used to investigate the correlation between PCR and IHC in biopsies as well ass assessing for possible correlation between cytokine expression and BAL cytology.

The initial comparisons in study III were made between summer time sampling and separately against both winter samplings. Subsequently, the results from winter sampling measurements were combined and averaged for statistical comparison against summer sampling, in order to reduce inherent background variation in results obtained during winter stabling period. The Wilcoxon signed rank test was used to assess for differences between the findings, using a two tailed test. Ratios of mRNA expression for mRNA IL-4 and IL-5 (means of winter versus summer time) for each horse were compared to bronchoalveolar lavage cytology changes by Spearman rank order correlation using a one sided test for significance as upregulation of these cytokines could be anticipated.

Environmental parameters measured in study IV were generally presented as median values and, where appropriate, as mean ± (SD). Three of the horses with marked elevated BAL eosinophilia, presented in study III, were excluded from all analyses in study IV. Two horses were not available for sampling on all occasions, one of which was missing for the second winter period (March 2005) and the other missing for the summer sampling (Sept 2005). The horse missing from the summer sampling was therefore also excluded from the cytokine mRNA analyses. The Wilcoxon signed rank test was used to assess statistical differences between individual sampling occasions, to compare between seasons after averaging the results from the two winter samplings and for comparison of relative cytokine expression similar to all studies.

Aspects on material and methods

Animals and study design

The horses with well defined clinical and experimental history of RAO or lung health (controls) were selected to the experimental inhalation study (papers I and II). The RAO horses were exposed to inhalation provocation that resulted in a defined respiratory crisis, following which they were allowed to go into remission through removal of the environmental challenge and be maintained continually on pasture. This careful selection ensured a well defined, but somewhat numerically limited horse group for experimental studies. As there are only a limited number of research centres where experimental inhalation studies are performed and the same limited numbers of horses are often used for several studies, the experimental inhalation studies are statistically based on low number of individuals. One additional problem in experimental horses compared to race horses is the lack of strenuous exercise. Exercise potentially increases impaction of inhaled particles within the lung (Art, McGorum & Lekeux, 2002) and can thus influence the immune response in the lung (Raidal*, et al.*, 2000).

Selection of the sampling time is one of the main questions in the study design in the experimental inhalation provocation studies where the time-dependent changes in cytokine mRNA expression after provocation are investigated. When provocation studies are designed, the sampling time can be decided either with a fixed time after the start of provocation (and in this case, the clinical and functional alteration will be different from one horse to the other); or the sampling can be carried out when the functional and clinical changes are sufficient. In our experimental studies (papers I and II) the sample collection was performed after the moldy hay challenge, created by housing all horses in a barn and feeding them moldy hay, until all RAO horses responded to provocation with clinical signs of respiratory exacerbation (blinded observer clinical score higher than 5 (Rush*, et al.*, 1998a)) and pulmonary function measurements that coincide with the occurrence typical signs of dyspnea, including maximum change in pleural pressure (ΔPpl_{max}) more than 1.5kPa (Art *et al.*, 2006). In our horses, these changes occurred after 2 days challenge but the predefined criteria to begin sampling was occurrence of sufficient degree of expression of the disease phenotype. It can be argued that sampling should be done at a precise set time post challenge for consistency in signal, or conversely, that sampling commence only when the phenotype of the disease is sufficiently expressed. We chose to sample at the point the phenotype was sufficiently expressed, which also can be used as a marker of sufficient expression of inflammation in airways. Both strategies- sampling after a predefined time delay in challenge exposure, -or sampling once signs of airway disease appear- influence the results and the optimal way to investigate inflammatory response in airways is repeated sampling serially over time. Sampling at a precise time point post challenge can capture early events in gene expression- if properly timed. However, as there is individual variation in development of disease, sampling once clinical signs are sufficiently developed ensures that gene expression upregulation has had time to occur. However, a disadvantage of our strategy is that early events may not have been captured since

development of phenotype may lag substantially after cytokine production and thus key events setting off the cascade may have been missed. The horses included in this study had also been provoked in earlier studies, which potentially, despite ample wash-out time, could have influenced the robustness of the inflammatory response in the lung. Finally, when provoked with moldy hay and dusty stable environment the antigens and inhaled particles differ between studies. These aspects alone can obviate attempts for direct inter-study comparison.

While there are many publications on experimental inhalation provocation test of horses highly reactive to dusty environments (RAO horses) and also on detailed analysis of stable environments, studies on how and if normal variations in conventional stable environment affect the equine respiratory system are lacking. Thus the design of study IV aimed to examine qualitative indices of indoor stable air during winter versus late summer conditions in a typical Standardbred racing stable and to assess whether air quality or season was associated with clinically detectable respiratory signs or alterations to selected biomarkers of airway inflammation in horses. One additional aim was to evaluate methods and their suitability for further clinical studies of stable environment, for both horses and people working in these stables. Unfortunately, because of low compliance we were unable to include data on respiratory health indices of the people working in the stable. Thus design of this study was by necessity an observational study that followed the respiratory indices of healthy athletic horses during their normal stable environment in Swedish summer and winter conditions. In this study we wanted to follow what is representative for a group of horses that equine veterinarians deal with on a daily basis. As the horses in this group were not clinically ill this study can be viewed as focusing on "equine environmental medicine", where the aim was to follow the healthy stabled athletic horses in their normal environment in Swedish summer and winter conditions and provide a basic data from the various environmental measurements in relation to respiratory health of the stabled horses for use as a basis for further field studies. The privately owned Standardbreds in training (papers III and IV), stabled in their normal environment, represent healthy athletic horses on their natural training and stabling conditions. One of these horses had a history and BAL cytological profile consistent with IAD, which is not surprising given the generous scope allowable for defining this syndrome in young race horses (Couetil*, et al.*, 2007). Studies examining prevalence of IAD have, despite various definitions of IAD in the studies, generally suggest it to be common in pleasure horses (Gerber*, et al.*, 2003a; Robinson*, et al.*, 2006) and National Hunt horses of all age (Allen, Tremaine & Franklin, 2006). In flatrace horses (Thoroughbred racehorses) the disease appears to decrease in frequency with increased age (Chapman *et al.*, 2000; Wood*, et al.*, 2005b). Further studies of prevalence of IAD according to the consensus IAD definition (Couetil*, et al.*, 2007) in racing Standardbreds is needed, especially in Nordic stabling and weather conditions. The pulmonary eosinophilia observed during summer measurement in the horses of study III was presumably related to other factors than stable environment. Because of their potential confounding effect on the ongoing stable environmental study (study IV) the statistical calculations in paper IV were carried out after removal of the data of the horses with pronounced pulmonary eosinophilia. Despite this, the remainder of the horses also had a small, but statistically significant increase of eosinophil

percentage in BALF during summer measurements. However, as the eosinophil changes were very small in relation to other cell types present in BAL cytology it is unlikely they played a measurable role in cytokine expression being analysed in evaluation of seasonal changes in stall environment (study IV). Inclusion of control samples from healthy trotters continually at pasture could greatly assist in refining interpretation of the contribution of stable air components to BAL cell profile and gene expression in this study. Unfortunately, due to a change of ownership of the racing stable the originally intended second summer sampling was not possible to obtain.

The observed eosinophilia during summer measurement in these horses provided a unique opportunity to analyse samples collected prior, during and after naturally occurring pulmonary eosinophilia in horses (paper III). As the pulmonary eosinophilia was unexpected and the study designed to test other hypotheses fecal floation was not part of the normal protocol. The time delay of repeated sampling of the most severely affected horses two months after observed eosinophilia was largely because the final interpretation of the BAL-cytology from that period was only completed at that later time. Thus, the repeated sampling was collected from three horses (most affected) just after the results arrived from laboratory, approximately 2 months after eosinophilia. Unfortunately the owner had dewormed all horses with pyrantel (Banminth®vet, Orion, Animal Health, Sollentuna, Sweden) about 5 days prior, due BAL findings. Regrettably, these animals were privately owned competition horses from "real life" where the experimental setup is difficult to control to due the demands and expectations of the trainers and owners. On the other hand this "real-life" clinical finding is clinically highly relevant as it would be very difficult to predictably repeat and conduct in the laboratory environment, where the nature, dose and timing of a possible challenge to the horses' immune systems that were reflected in transient pulmonary eosinophilia remain unknown.

Sampling methods

In the presented studies the BAL and endobronchial biopsy samples were chosen to investigate inflammatory markers. These methods represent different parts of the inflammatory process where bronchial epithelium samples allows investigation of structure, histology and the proximal part of airways whereas BAL samples cells and soluble factors from more peripheral airways.

Endobronchial biopsy sampling in humans is considered as an invasive yet safe method (Elston*, et al.*, 2004; Hattotuwa*, et al.*, 2002; Jeffery, Holgate & Wenzel, 2003; Payne*, et al.*, 2001; Saglani *et al.*, 2006; Saglani*, et al.*, 2003). This technique is not standardized in human medicine; biopsy sampling method and process for tissue handling differ between centres and different studies. The procedure is generally well tolerated even in patients with low FEV_1 (down to 30% of predicted value), but extra caution is required in patients with hyperreactive airways (e.g. selected asthma patients), as there is increased risk for acute bronchospasm (Djukanovic *et al.*, 1998; Djukanovic*, et al.*, 1991; Jeffery, Holgate & Wenzel, 2003). Studies in children have shown that bronchial biopsies

can provide information about early morphological changes in wheezing disorders or asthma (Payne*, et al.*, 2001; Saglani *et al.*, 2005; Saglani *et al.*, 2007). In man at least up to 10 biopsies can be obtained safely from asthmatics (Humbert *et al.*, 1996).,We sampled 6 biopsies at each bronchoscopy event, which ensures the minimum two for each analysis method to be used (Jeffery, Holgate & Wenzel, 2003; NHLBI, 1991).

Bronchial biopsy sampling was performed after sedation (se sampling methods) in a standing horse, In horses included to papers III and IV, topical anesthesia (mepivacain 2%, Carbocain®, AstraZeneca, Södertälje, Sweden) was administered at the carina and bifurcations before biopsy and BAL sampling, whereas in the studies for papers I and II local anesthesia was used. Despite this, no adverse reactions at biopsy sampling was noted in those studies. However, in some horses, especially post provocation, BAL induced uncontrollable cough and occasionally made recovery of fluid difficult. Based on these observations, and work we have done previously, (Riihimäki unpublished data) horses with RAO appear to react less to sampling with biopsy than routine endoscopy or BAL, which is consistent with experience in man (Elston*, et al.*, 2004; Jeffery, Holgate & Wenzel, 2003; Saglani*, et al.*, 2003). Biopsy sampling was performed proximally to distally direction and there were no incidents of excess bleeding. When biopsies were performed prior BAL (study III and IV), the biopsies were collected contralateral to lung to BAL to avoid small amounts of blood from biopsy sites contaminating BAL samples. When biopsy sampling was repeated the contralateral lung was also chosen in order to avoiding sampling artefacts induced by trauma of earlier biopsy locations (Robinson *et al.*, 1998). To date, we have collected bronchial biopsies from approximately 80 horses in 140 occasions (data not shown). When sampling was repeated, we observed no visual evidence of previous trauma or scarring at earlier biopsy sites. Transient fever and coughing in two horses were the only complication after this examination procedure. However, both BAL and biopsy sampling were performed in all horses and transient pyrexia is a common finding following performance of BAL. Transient nasal bleeding was observed in two horses which could be associated to endoscope procedure and bleeding from nasal cavity. Although endobronchial biopsy in our hands appeared to be a safe procedure it is prudent to have at ready access to reversal agents to α_2-adrenoreceptor agonists, atropine (for eventually local administration to sample localisation in case of bleeding) and inhalable bronchodilatators (Jeffery, Holgate & Wenzel, 2003), in the event of complications due to sedation or sampling from the airways.

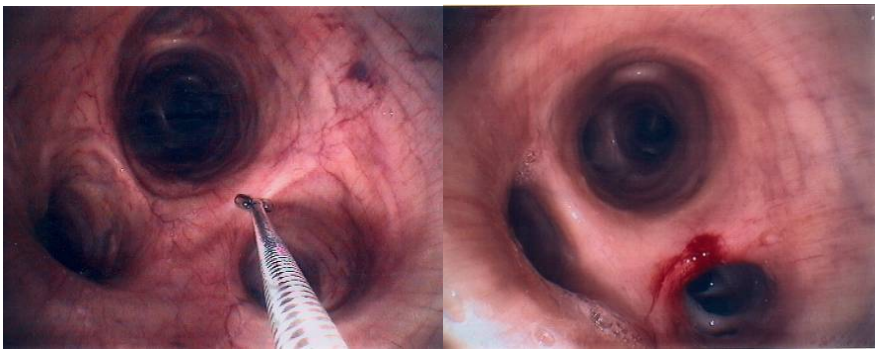

Fig. 8. The observed mucosal bleeding in horses during biopsy sampling was minimal. Photo: John Pringle, Miia Riihimäki.

In order to optimize tissue morphology each new biopsy instrument was used on maximum four horses (24 biopsies) as recommended in human medicine (Jeffery, Holgate & Wenzel, 2003; Robinson*, et al.*, 1998). All biopsies were collected by two veterinarians who were experienced with the collection technique. Rapid sample handling with less than one minute between biopsy to freezing in liquid nitrogen or fixation was used in order to avoid RNA degradation and induction of inflammation in the tissue due to mechanical effects of biopsy collection (Guy, 2002; Jeffery, Holgate & Wenzel, 2003).

Some of the limitations to endobronchial biopsy sampling include small sample size (usually 1-2 mm) and sampling from bronchi rather than bronchioles (Haahtela, Laitinen & Laitinen, 1993; Jeffery, Holgate & Wenzel, 2003; Robinson*, et al.*, 1998). There is a risk that small tissue volumes from bronchi might not be representative inflammation in lower respiratory tract (Hirota, Ellis & Inman, 2006; Holgate, 2000). The biopsies collected in these equine studies were sufficient in size for analysis methods used in papers included to thesis; RT-PCR (papers I and II), morphological analyses (paper III) and IHC (paper II). In humans even smaller biopsies obtained from young children are reported to have satisfactory quality for histological assessment (Saglani*, et al.*, 2003). The biopsy samples collected for PCR were consistently from the first branch off the main bronchus from either the left or right side, whereas the rest of the samples were collected from segmental and subsegmental carinae. Thus, a key question regarding RAO in horses is whether the inflammatory process restricted to bronchioles, or if it is present, and mechanistically similar in the larger airways (bronchi) of the horse. Interestingly, in detailed ultrastructural morphological studies the inflammatory process in RAO horses, while most severe in the small airways (Kaup*, et al.*, 1990), is also present to at least a degree in the larger airways (Kaup, Drommer & Deegen, 1990). While the observed morphological changes were generally focal and non-specific, they had also partial correlation with clinical score of RAO in observed horses (Kaup, Drommer & Deegen, 1990). Nyman et al. (Nyman*, et al.*, 1991) also showed a functional correlation between morphological changes (bronchial epithelial hyperplasia in RAO horses) in lung biopsies and pulmonary gas exchange. In recent work Ainsworth et al. (Ainsworth*, et al.*, 2006) showed that environmental challenge of RAO horses

increased gene expression and immunoreactivity of neutrophil chemotactic cytokine IL-8 in bronchial epithelial cells, indicating an inflammatory response. In human asthma the inflammatory process in asthmatics seems to be present both in large and small airways (Carroll, Cooke & James, 1997; Haahtela, Laitinen & Laitinen, 1993). Recent evidence utilizing newly developed molecular biologic marker have show that altered airway epithelial processes in RAO can also be detected bronchial epithelial cells (Berndt *et al.*, 2007; Bureau*, et al.*, 2000b). Experience from bronchial biopsy performed for this thesis work have demonstrated that it is technically readily accomplished, has low risk for complications, and appears to inflicts little discomfort for the animal. Most importantly, adoption of bronchial biopsy provides a complementary mode to study mechanistic processes of airway inflammation in the horse.

BAL was performed similarly in all studies but, due to differences in routine in these two research centres there were minor divergences in BAL sampling and processing for cytological examination (see chapter material and methods). The total volume used in lavage can affect the results in cytology (Sweeney*, et al.*, 1992). However in our studies the lavage volumes were very similar (6 x 60 ml = 360ml (studies I and II) versus or 3 x 100 ml = 300ml (studies III and IV) and likely of minimal influence on comparability of BAL cytology between studies. Moreover, studies III and IV did not focus on RAO where the neutrophil component of BAL is an accepted and important criteria of disease. As BAL sampling itself can cause neutrophil influx to the lung (Sweeney *et al.*, 1994) biopsy sampling can be used as alternative method in time dependent studies with early and repeated sample collection.

Environmental measurements in study III and IV were collected during the daytime routines in the investigated stable. As these measurements took place only on individual days, over 4-7 hours, we considered that data presentation was more meaningful as mean/median and ranges since standard deviations from so few measurements are of less value to the reader. Due to technical problems with pump failure on some occasions the data presented on a certain day can be based on only two samples.

Air sampling was performed with filter pumps with specific filters (SKC Inc., Eighty Four, PA, USA, see further material and methods) with were placed in the breathing zone approximately 1-1.5 m above ground level at three points in the stable corridor. The equipment for respirable dust was attached to the personnel's clothing in the breathing zone. Some disadvantages in sampling with filters are the long sampling time, in our study with a flow of 2 L/min for 4-7 hours, loss of real-time information and finally risk for sampling contamination (Clements & Pirie, 2007a). The real-time monitoring system for dust has recently been validated for equine stable environmental analysis (Clements & Pirie, 2007a; Rosenthal, Gruntman & Couetil, 2006). It is able to detect short term peaks in dust (both total dust and inorganic dust) levels e.g. in correlation with stabling routines. However classical filter based pumps provide an opportunity to sample dust for further analysis such as differentiation of organic dust from nonorganic dust, microbiological analysis and analysis of endotoxin and allergens (Art, McGorum

& Lekeux, 2002; Clements & Pirie, 2007a). Thus in later studies we have used real-time continuous particle monitoring system together with filter based system for measuring dust concentrations. As the initial aim of the study was also to examine the relationship between exposure to stable environment and inflammation markers in the respiratory tracts of the personnel working in stables, the air sampling was performed in stable corridor and the respirable dust was collected in the breathing zone of the personnel. As the sampling performed during day time routines as cleaning stable, the values obtained can potentially be substantially higher than 24 hour average (Crichlow, Yoshida & Wallace, 1980; Webster*, et al.*, 1987; Woods*, et al.*, 1993). Collection of both the total and respirable dust in the background and from breathing zones of horses would have been desirable as higher values are expected in breathing zone when compared to stationary measurements. Moreover, these values are more representative for the total respiratory challenge to which the horse is exposed (Woods*, et al.*, 1993).

Processing and Analyses

The quantitative real-time PCR was selected to due its sensitivity to measure gene expression. Amplification efficiencies were calculated from raw fluorescence data by using the DART-PCR software (Peirson, Butler & Foster, 2003) or by serial dilutions of RNA. The real-time PCR reverse transcription was performed with either one-step (study II and IV) or two-step reactions (study I and II). In the one-step reaction the reverse transcription reaction is done in the same tube as for PCR. It was chosen for study III and IV due economical reasons, time aspects and lower contamination risk. Additionally there was ample sample material in these studies (Wacker & Godard, 2005). In studies I and II, a two-step reaction was chosen due a limited amount of BALF and tissue material, and in order to increase the sensitivity. In the two step RT-PCR, gene specific oligos were used for cDNA synthesis, cDNA samples were then used for PCR reactions for selected cytokines (Wacker & Godard, 2005). GAPDH was selected as house- keeping gene since expression of GAPDH was constant relative to β-actin expression, and its expression levels were closer to the target genes than were β-actin's. In humans GAPDH expression varies between tissues types (Barber *et al.*, 2005). However, in horses it has been validated for endobronchial tissue and BAL cells (Ainsworth*, et al.*, 2006). Amplification efficiencies were calculated from raw fluorescence data by using the DART-PCR software (Peirson, Butler & Foster, 2003). The PCR reactions for each cytokine were run in triplicate and repeated samples from same animal were analyzed on the same plate in order to minimize the interassay variance. In all studies the comparative C_T ($2^{-\Delta\Delta C_T}$) method was used for calculating relative cytokine mRNA expression (Livak & Schmittgen, 2001; Wong & Medrano, 2005; Yuan *et al.*, 2006). The ΔC_T value is the difference between the C_T value for the target gene and housekeeping gene and the $\Delta\Delta C_T$ value is the difference between mean ΔC_T values for samples compared against each other. The type of cells responsible for production of different cytokine mRNA expression in BAL and biopsies needs to be further investigated, as in our studies the total mRNA was analysed from all cells irrespective of cell type (Kleiber*, et al.*, 2005). Moreover, the mRNA expression is considered as a surrogate marker for protein expression, but actual levels of protein produced may be affected by

several post- and co translational effects and may not necessary directly mirror the mRNA levels measured (Mehra, Lee & Hatzimanikatis, 2003).

GMA embedding technique of biopsies gives an opportunity to investigate the interrelated structural components and IHC analysis of protein expression in addition to mRNA levels analyzed by PCR. In order to inhibit the proteolytic enzymes present in the mucosal tissue, samples for GMA embedding were immediately fixed in cold (-20°C) acetone with protease inhibitors. This embedding technique generates an immunophenotypic profile close to frozen sections, but providies morphologically superior quality than either paraffin or frozen sections (Britten, Howarth & Roche, 1993; Jeffery, Holgate & Wenzel, 2003; Saetta*, et al.*, 1998). Additionally, the GMA embedding technique allows very thin sections 1-2 µm, whereas usually 8 µm sections are used in frozen sections and 4-6 µm in paraffin embedded tissue (Britten, Howarth & Roche, 1993). While this has an importance in studies with small tissue samples, more importantly it allows cutting extremely thin sections to provide serial sections and differential analysis of the same cell (*e.g.* two distinct epitopes within one cell) (Britten, Howarth & Roche, 1993). The GMA technique does have some limitations which include the time consuming embedding and need for expensive instruments for cutting and quantification of immunostaining (Saetta*, et al.*, 1998). Additionally the plastic resin embedded biopsies are unsuitable for application of *in situ* hybridization techniques (Jeffery, Holgate & Wenzel, 2003).

In study II we conducted PCR technique alongside IHC analysis of protein expression in order to provide an alternative view of the immunological mechanisms of inflammation in the bronchial epithelium in RAO horses. The visualization of immunostaining and quantification was done at the respiratory research laboratory at Umeå University by an immunohistochemist with extensive experience in IHC on bronchial biopsies (Pourazar, 2006). For IHC staining the only biopsies used were those where the morphology of the epithelial and submucosal tissue were optimally conserved. When planning the study, the choice of equine antibodies was limited. At present there are several equine specific antibodies available for the investigated cytokines. Serial dilutions of the antibodies were tested against equine submandibular biopsies, in order to define optimal staining intensity with minimal background staining.

Regarding environmental analyses, (1→3)-β-D-glucan method is not fully validated. However, the laboratory which performed the analyses in study IV used the most optimal combination of sample handling, including alkaline extraction and the limulus amebocyte lysate, LAL-method (Douwes, 2005). The endotoxin analysis based on Limulus test detects biologically active endotoxin, but can be somewhat imprecise and nonspecific. As differences in sampling procedures, extraction and analysis methods can affect the result of analysis there is need for further standardization of endotoxin assessment (Liebers *et al.*, 2007; Pomorska *et al.*, 2007; Reynolds *et al.*, 2002).

In each of the studies the number of horses examined was low and therefore the data were treated non-parametrically. When the fold increase of investigated

cytokine expression in real-time PCR analysis was statistically significant, but in biological terms small, the results were treated with strong caution, due to the extreme sensitivity of the real-time PCR, inherent biological variation and low number of animal examined (Peirson & Butler, 2007).

Discussion of main results

Experimental inhalation provocation of RAO horses (study I and II)

Clinical response to inhalation provocation

The results from the summarized clinical examination and pulmonary function testing in studies I and II confirmed that RAO horses developed appropriately severe respiratory embarrassment and inflammation in response to provocation with the moldy hay and dusty stable environment. Following provocation RAO horses had significantly higher clinical scores than when on pasture (p=0.022). Whereas classical pulmonary function tests in RAO studies lack sensitivity (Couetil*, et al.*, 2001) the RAO horses in this work showed significantly impaired pulmonary function with significant increases in maximum change in transpulmonary pressure (ΔPpl_{max}) and pulmonary resistance (R_L) (p = 0.022 for both). Dynamic compliance (Cdyn) was also decreased, but due to a single outlier, did not reach statistical significance (p=0.272, without the outlier p=0.036). Additionally, there was no difference in clinical signs between RAO horses and controls on pasture (p=0.1002). However, while lung function improved in the RAO group; it still remained significantly impaired when compared to healthy controls (R_L, p= 0.003, ΔPpl_{max}, p=0.015).

Cytological and cytokine changes as response to inhalation provocation

The BAL cytology profile of neutrophilia further supported the clinical and pulmonary function findings suggestive of inflammatory response to inhalation provocation in RAO horses. The total cell count, neutrophil percentage and total number of neutrophils in BALF were significantly higher (p value for all= 0.022) after provocation than on pasture in RAO horses. During remission at pasture BALF neutrophil percentage did not differ from pastured controls. However, the absolute number of neutrophils in BALF (p= 0.038) lung function remained different between RAO horses after remission and controls (R_L, p= 0.003, ΔPpl_{max}, p=0.015). This is not unexpected as the lung function impairment and BALF neutrophilia can persist in RAO horses despite remission on pasture or low-dust environment (Miskovic, Couetil & Thompson, 2007; Tesarowski, Viel & McDonell, 1996; Tremblay*, et al.*, 1993). Interestingly, in study I there was also a trend *(p*=0.086) for increased IL-8 mRNA expression in biopsies from RAO horses in remission compared to healthy controls at pasture. Art et al (Art*, et al.*, 2006) have also shown an significantly increased myeloperoxidase concentration in BALF from same RAO horses during remission, both findings suggesting residual s influence of the initial neutrophilic inflammatory reaction and potential irreversible airway remodelling.

In spite of clear and consistent clinical signs of respiratory crisis and neutrophilia in BALF, the neutrophil chemotactic cytokine IL-8 was the only cytokine that showed statistically enhanced mRNA expression in both BALF and biopsies in RAO horses during in crisis (study I). One horse with greatly elevated

IL-8 expression in both BALF and biopsies following challenge was a clear outlier. However this horse did not differ from other studied horses in clinical score, in BALF findings or pulmonary mechanics.

Fig. 9 a.

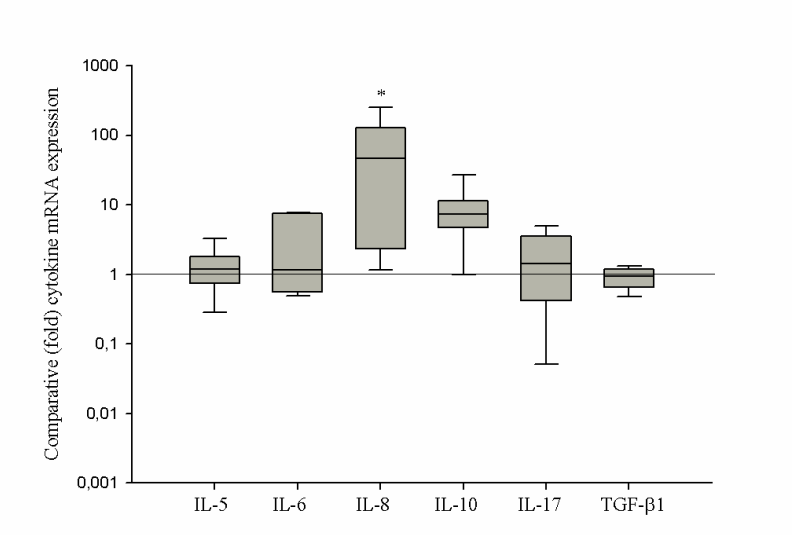

Fig. 9 b.

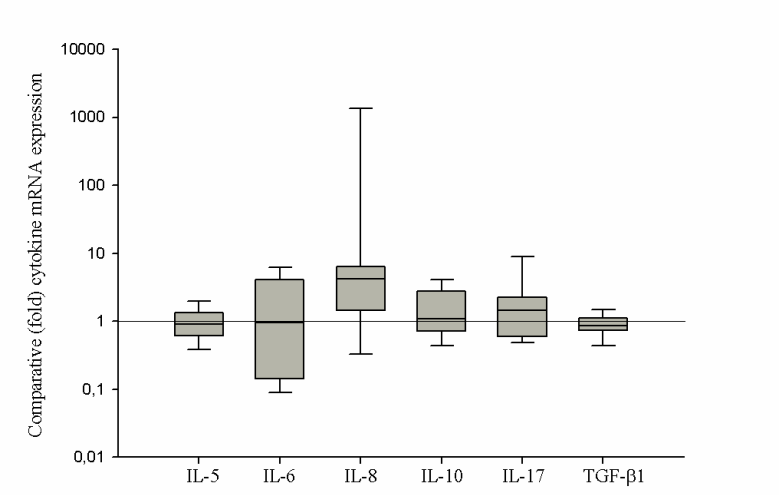

Fig. 9. The comparative (fold) expression of cytokine mRNA expression BAL (a) and in endobronchial biopsies (b) during respiratory crisis compared to remission. The results are shown with interquartile range with the line in bars representing the median value. The horizontal line represents theoretical median value 1 (theoretically no difference between provocation and remission or $\Delta\Delta C_T=0$, $2^{\Delta\Delta CT}=2^0=1$). *= statistical difference in comparative (fold) expression of cytokine mRNA.

Consistent with earlier studies we also found an increased expression of IL-8 mRNA in BAL cells in RAO horses after provocation (Ainsworth*, et al.*, 2003; Ainsworth*, et al.*, 2006; Franchini*, et al.*, 2000; Giguere*, et al.*, 2002). As well, we also detected this in the expression of IL-8 mRNA in endobronchial tissues, confirming recent findings by Ainsworth (Ainsworth*, et al.*, 2006).

Regarding mRNA for other cytokines, in study II the slight (1.1 fold) decrease in TNF-α mRNA in bronchial tissue during crisis was not likely of clinical or biological relevance. In examining possible connections between various cytokine alterations with others, and their proposed immunologic relationship one interesting correlation was observed. In study I IL-6 mRNA and IL-8 mRNA expression in BAL during crisis correlated with IL-8 mRNA expression in bronchial tissue during remission (rs=0.986, p=0.00). Thus the same horses that had the highest mRNA levels of these cytokines in BAL during crisis had the highest bronchial tissue IL-8 mRNA expression during remission at pasture. However, the total IL-6 mRNA was not increased in the RAO horses after challenge in this study, possibly related to differences in sampling times.

Consistent with earlier finding in horses (Desjardins *et al.*, 2004) the TGF-β1 mRNA expression was not increased during respiratory crisis. Nor was there any difference detected in TGF-β1 expression between RAO-susceptible horses and controls on pasture. However, there was a positive correlation with IL-10 mRNA and TGF-β1 mRNA expression in biopsies (rs=0.943, p=0.005). In human bronchial epithelial cells it has been shown that IL-10 partly regulates TGF-β1 expression (Fueki *et al.*, 2007; Nakao *et al.*, 2002). Thus this observed in our study correlation may indicate that IL-10 has an immunoregulatory role in equine bronchial epithelium.

The protein expression of investigated cytokines measured by IHC in study II, did not show any statistically significant changes in challenged RAO horses compared remission on pasture or when in RAO horses and controls on pasture were compared. In comparing the protein expression of investigated cytokines measured by IHC in study II, did not show any statistically significant change in challenged RAO horses compared remission on pasture or when in RAO horses and controls on pasture were compared. In comparing real-time PCR analysis to results of IHC the correlation was generally poor, where only IL-10 mRNA and protein levels in RAO horses on pasture were significantly correlated (rs= 0.893, p= 0.007). The expression of investigated cytokines in RAO horses at pasture did not differ from controls.

Significance of the results

Several factors have influence on measured cytokine expression and have to be considered when results of provocation studies are analyzed. The effects of sampling time in correlation to provocation, study design and analysis methods are discussed in detail earlier under chapter “Aspects on material and methods and in the papers following. Generally, the study design is one of the major concerns

since time-dependent expression of inflammatory mediators in horse airways can occur within hours or days after provocation (Ainsworth, *et al.*, 2003; Ainsworth, *et al.*, 2006; Davis *et al.*, 2005; Davis *et al.*, 2007; Debrue, *et al.*, 2005; Laan, *et al.*, 2006). As discussed earlier, our samples were collected when RAO horses had developed clinical signs of impaired pulmonary function, (ΔPpl_{max} more than 1.5kPa), rather than at a predetermined time after provocation. Thus we could have missed early, or delayed signals. Another key aspect is the mode of provocation and individual components of challenge agent may influence the dynamics of cytokine regulation (Giguere, *et al.*, 2002; Laan, *et al.*, 2006). In our study the provocation was performed with moldy hay but the exact hygienic content was not evaluated.

In papers I and II the relationship between inflammation markers was determined between BAL cells and biopsy results. The correlation was generally poor. This is not surprising and can have several explanations; both related for time dependent aspects in study design and used sampling analysis technique as discussed earlier under chapter "Material and methods". Generally the cytokine expression was lower in biopsies than in BAL cells, probably related to differences in cell population in samples examined. BALF is generally sampled from more peripheral airways and includes cells flushed from epithelium or cells from airway lumen, whereas biopsies contain airway epithelium, connective tissue, blood vessels, smooth muscle tissue and mucous glands (Fabbri, *et al.*, 1998; Jeffery, Laitinen & Venge, 2000; Maestrelli *et al.*, 1995). Endobronchial biopsies are also limited in sampling size (Hirota, Ellis & Inman, 2006; Holgate, 2000; Maestrelli, *et al.*, 1995) and might not be representative for airways as whole (see under chapter "Material and methods - Processing and analyses").

The correlation of the results between real-time PCR analyses of endobronchial biopsies and ICH for the protein product was also generally poor (study II). One reason for this may be that levels of protein produced from the quantified mRNA levels in the cytoplasm are affected by several post- and co translational effects and do not necessary mirror the measured mRNA expression (Mehra, Lee & Hatzimanikatis, 2003). Additionally the IHC analysis was performed on airway epithelium, whereas the biopsies analyzed with PCR included subepithelial tissue, containing blood vessels, smooth muscle and other nonepithelial structures. Moreover, while, six biopsies were obtained from each horse, the first two were from the first generation bronchus and examined by PCR. The remaining four biopsies were obtained progressively more distally in the lung and from these morphologically representative biopsies were then selected for further analysis by IHC. Thus, there was some difference in sampling site for biopsies obtained for PCR versus IHC analysis.

These studies not only further support the role of IL-8 in neutrophil recruitment in RAO horses, but also provide more detailed insight of the role of other cytokines in airway epithelium in neutrophil influx to lungs secondary to environmental challenge in RAO- horses. Moreover, these studies combining quantitative real-time PCR and IHC methods on bronchial biopsy tissues can provide a new complementary research tool to study the role of mediators and structural elements in bronchial tissue associated with the inflammatory process in

RAO-horses. Additionally, in order to understand the inflammatory response in airways as whole, this sampling technique can be complementary to BAL or bronchial brushing sampling. Further studies with repeated sampling over time in controlled inhalation provocation studies and even in naturally occurring RAO cases can capture serial events not possible to closely study using BAL.

Transient pulmonary eosinophilia, study III

The transient pulmonary eosinophilia detected during the summer measurements in the group of Standardbreds which were part of stable environmental study presented in paper IV, review of data from sampling before, during and after the eosinophilic event provided a unique opportunity to evaluate clinical, clinicopathologic and immunologic events associated with equine pulmonary eosinophilia.

Clinical response to pulmonary eosinophilia

Despite having collected very detailed clinical respiratory examination we observed no clinical signs suggestive of pulmonary inflammation (such as increased tracheal mucous, elevated respiratory rate, increased lung sounds in auscultation) in association with the pulmonary eosinophilia. The only clinically detectable alteration during the examination was a brownish coloration of the BAL fluid in two of the five horses during eosinophilia. However, while unaware of the BAL results the trainer had observed exercise intolerance in the three horses with highest BAL eosinophil numbers and removed these horses from active training for several months. According to racing statistics the observed exercise intolerance was temporary, as even the horses in this study with most profound pulmonary eosinophilia had similar or better performance capacity after recovery from eosinophilia (official racing statistics, race winnings prior to September 2004 to 7th august 2006, from Swedish Trotting Association). However, as racing performance is dependent on many factors, it is difficult to interpret the contribution of solitary components when only three horses are being examined.

The clinical history in our horses was similar to the only other report of young standardbred racing horses with pulmonary eosinophilia (Hare & Viel, 1998). In contrast to our findings Hare and Viel (Hare & Viel, 1998) were able to detect clinical signs of respiratory disease, significantly higher peripheral eosinophil counts in principal horses and a two-fold increase in total BAL cell counts in their horses with eosinophilia. It is possible that the difference between our studies was largely sampling time in the course of disease rather than different underlying cause for pulmonary eosinophilia. The most common cause of peripheral blood eosinophilia in humans is considered to be parasitic infections (Coffer et al., 2003). While peripheral blood eosinophilia can be present in some parasitic infections, eosinophil accumulation and degranulation occur at the site of parasite invasion or migration or in local tissue fluids e.g. CFS or BAL (Chitkara & Krishna, 2006; Coffer*, et al.*, 2003).

All fecal samples following the period of the pulmonary eosinophilia showed presence of strongyle-type eggs (qualitative method). Additionally, *Anoplocephala* sp and *Parascaris equorum* eggs were present in pooled fecal samples from the entire group, but were not present in individual fecal samples from the three horses with highest eosinophilia in BAL. Further, viable roundworms were observed in feces from several of the horses in this group following subsequent deworming with pyrantel. All fecal samples were negative for *Dictyocaulus* spp as analysed by the Baermann funnel technique. Fecal flotation from individual samples one month prior to the final sampling occasion showed a reduced parasite load (quantitative method), with strongyle–type eggs generally less than 50 epg. *P. equorum* eggs detectable in only one horse and there were no detectable *Anoplocephala* sp eggs at that assessment.

Inflammation markers in horse airways

The pulmonary eosinophilia observed during Sept 04 was marked at a mean BAL eosinophil of 9.0%±4.0 SE, whereas reported normal BAL eosinophil numbers are scant to absent (<0.1%) (Couetil*, et al.*, 2007; Robinson, 2003). Remarkably the total cell numbers and amount of BAL fluid recovered did not statistically differ between samplings, nor was there any significant difference in percentage or absolute cell number of other cell types in our samples. Cytological examination of these samples showed no free erythrocytes nor were there hemosiderophages. We had however expected to find more subtle changes in cytokine regulation during this period. Thus it was surprising that mRNA expression of the eosinophil related cytokines IL-4 or IL-5 were not measurably altered, (figure 10a, 10b) despite evidence of such marked influx of eosinophils to the lung. While cytokines such as IL-4 and IL-5 have key roles in eosinophil kinetics (Coffman*, et al.*, 1989; Culley *et al.*, 2002; Johansson*, et al.*, 2004; Rennick*, et al.*, 1990; Tomaki *et al.*, 2000), their changes are not of diagnostic value for a specific agent or allergen.

Fig. 10 a.

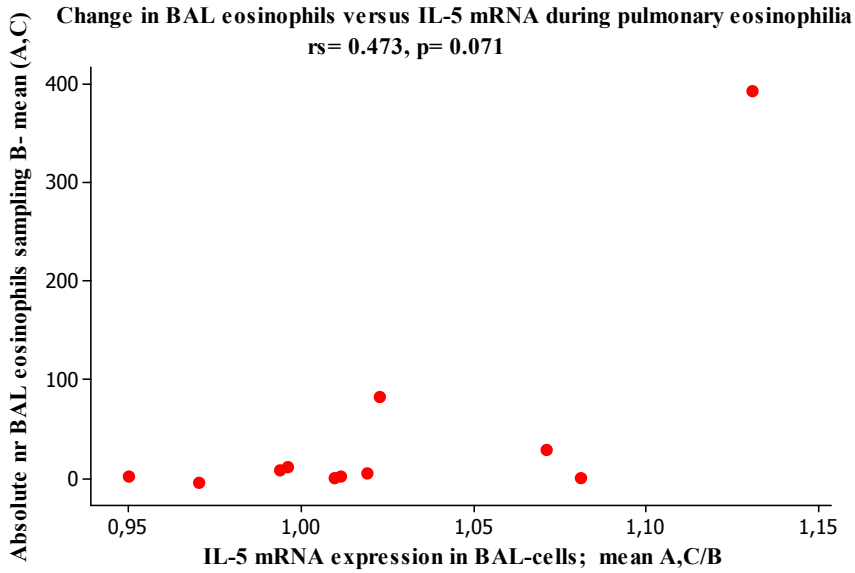

Fig. 10 b.

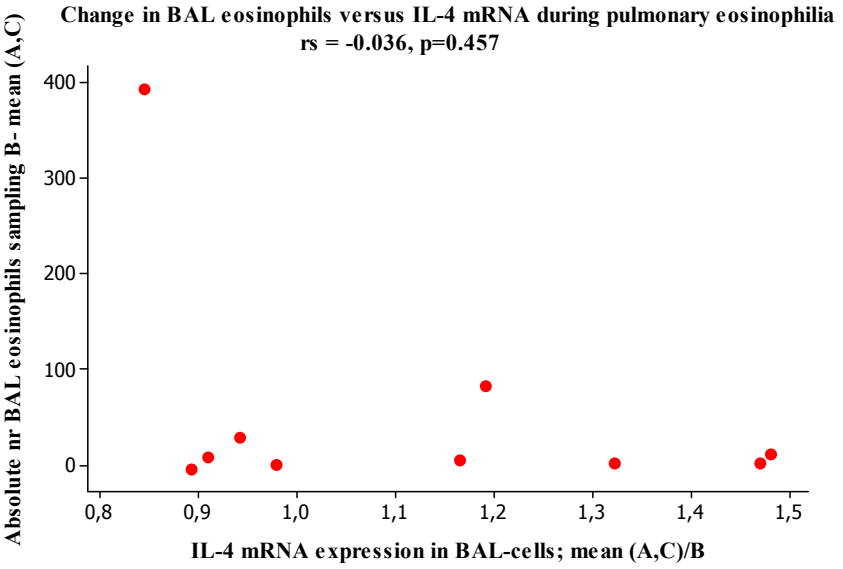

Fig. 10. The ratio of expression of IL-5 (a) IL-4 (b) and in BAL-cells and difference in total eosinophil numbers did not correlate in studied group of horses.
A= winter 04, B= summer 05, C= winter 05.

Eosinophil accumulation in lungs is seen in humans due asthma, helmintic infections, anaphylactic reactions or group of heterogeneous lung diseases causing pulmonary eosinophilia. While the etiology of this eosinophilia remains uncertain, fecal examination was indicated in order to rule out an infectious cause with parasitic larval migration. All fecal samples from first sampling showed the inadequate parasite control routines in the examined stable; with strongyle-type eggs, *Anoplocephala sp* and *Parascaris equorum* eggs present in samples. At the second sampling fecal flotation from individual samples showed a reduced parasite load. Pulmonary migration of *Parascaris equorum* is probably most likely the cause in sport horses. While *P. equorum* has a prepatent period of 72-110 days, pulmonary migration occurs as early as 7-14 days after ingestion of infective larvae (Austin S, 1990; Clayton & Duncan, 1977; Darien B, 1994; MacKay & Urquhart, 1979; Srihakim & Swerczek, 1978). Thus, negative fecal flotation results from some horses two months after pulmonary eosinophilia, can not rule out the possibility of present parascaris infection during the eosinophilia episode. Local immunity in lungs and liver can also prevent further larval migration to small intestine (Clayton, 1986) and partly explain lack of positive fecal samples. Despite regular antihelmintic treatments with ivermectin in these horses, the presence of *P. equorum* in pooled fecal samples is not unexpected, as resistance of *P. equorum* to macrocylic lactones has been reported has been reported from several groups (Boersema, Eysker & Nas, 2002; Hearn & Peregrine, 2003; Lyons, Tolliver & Collins, 2006; Stoneham & Coles, 2006). Pulmonary eosinophilia in horses can also be due to *Dictyocaulus arnfieldi* infection (George *et al.*, 1981; MacKay & Urquhart, 1979) or, as in experimental infections in foals, *Strongylus vulgaris* larvae migration (Turk & Klei, 1984). Thus negative fecal flotation results from some horses two months after pulmonary eosinophilia, can not rule out the possibility of present parascaris infection during the eosinophilia episode. Local immunity in lungs and liver can prevent further larval migration to small intestine (Clayton, 1986) and partly explain the results from fecal sampling. Hematology showed no significant changes in blood eosinophilis, however in humans with Ascaris infection blood eosinophilia is not persistent but dependent of infection stage (Chitkara & Krishna, 2006). The lack no clear pathological changes in endobronchial biopsies or lung biopsies, can possibly be due to sampling time or, as shown in mice with reinfection (Culley*, et al.*, 2002) due to preexisting local lung immunity. As shown in parasitic infection in mice (Volkmann *et al.*, 2003) expression of IL-4 and IL-5 may have been linked to different developmental stages of the parasites. Our serendipitous sampling in a clinical setting would not have captured a solitary developmental stage if parasites were indeed involved. Therefore, the mild clinical signs, with pulmonary eosinophilia in the absence of a similarly strong systemic eosinophilia might be partially explained by the number of infective larvae being few and that the horses were all fully adult (Austin S, 1990; Clayton & Duncan, 1977).

Lungworm infestation was also considered in the further investigation into a potential parasitic link. While all fecal samples were negative for *Dictyocaulus* spp, *D. arnfieldi* is more unlikely to be a common cause for eosinophilia in BAL in racehorses, partly due widespread use of ivermectin products and partly because of limited contact to donkeys. The stable under study was adjacent to a dairy

stable and stable paddocks frequented by roe deer *(Capreolus capreolus*). However there is no evidence of *Dictyocaulus* spp being transmissible from wild ruminants to cattle (Divina *et al.*, 2002), and thus it even less likely that wildlife could have been a source of aberrant parasite spread to our group of horses. Whereas *D. arnfieldi* infection in the horse may be nonpatent (Bailey, 1992) and thus negative on Baerman testing, it can be excluded as cause for eosinophilia in BAL in these horses as they were regularly dewormed with ivermectin and had no contact with donkeys. In summary, while some of the results from this study implicate a parasitic cause of pulmonary eosinophilia, the etiology of observed eosinophilic influx to the lung remains uncertain in these horses.

Significance of the results

In paper III a review of data from sampling before, during and after the eosinophilic event enabled us to evaluate clinical, clinicopathologic and immunologic events associated with this pulmonary eosinophilia. The lack of a clear positive correlation of elevated pulmonary eosinophils to expression of IL 4 or IL-5 could be related to sampling time as there are probably time-dependent changes in cytokine expression during eosinophilic influx to the lung. Our results show that, from clinicians' point of view, lung eosinophilia in horses can be transient, cause temporary exercise intolerance and abate without specific treatment. Our results provide valuable and complementary information in studies of equine inflammatory airway diseases where the contribution of the eosinophil is only scantly described.

Natural inhalation provocation with common stable environment during different seasons in healthy horses (study IV)

Hygienic measurements in the stable environment

Results from stable air quality indices observed during these three periods were well within guidelines for "acceptable" air quality" (table 2) for humans (The Swedish Work Environment Authority, AFS 2005:17) and for horses (Regulations on farm animal housing DFS 2004:17).

Table 2. Results from hygienic measurements in stable

	Winter 04	Summer 04	Winter 05	Unit	
Total dust	1.07	0.88 (0.67-1.27)	0.82 (0.68-0.96)	mg/m^3	Mean
Respirable dust	1.21 (0.8-1.6)	0.27 (0.24-0.31)	0.48 (0.45-0.51)	mg/m^3	Mean
Endotoxin	4.6 (1.9-6.9)	15 (9-16)	Nd	ng/m^3	Median
(1→3)-β-D-glucan	85 (24-121)	21 (19-27)	Nd	ng/m^3	Median
Temperature					
in stable	3	15	6	^oC	Mean
outdoors	-5	15	-5	^oC	Mean
Relative humidity					
in stable	67	55	43	RH%	Mean
outdoors	69	nd	43	RH%	Mean

The values of total and respirable dust obtained in this study were relatively high compared with earlier studies (Clements & Pirie, 2007a; Crichlow, Yoshida & Wallace, 1980; McGorum, Ellison & Cullen, 1998; Rosenthal, Gruntman & Couetil, 2006; Woods*, et al.*, 1993). When results from different stable environment studies are compared other factors need to be considered, such as the location of the sampling equipment (respiratory zone of the horses or in the stable corridor), type of ventilation, air humidity, stabling routines and time of day for sample collection as well as type of sampling devices and methods of analysis. In our study the sample collection was performed during normal daily stable routines, during feeding and stable cleaning, which together with differences with ventilation and straw quality can partly explain the relatively high particle load (Crichlow, Yoshida & Wallace, 1980; Webster*, et al.*, 1987) in comparison to studies with stables having the same type of feeding and bedding material (Clarke, 1987; Clements & Pirie, 2007a; Clements & Pirie, 2007b; Crichlow, Yoshida & Wallace, 1980; Woods*, et al.*, 1993; Zeitler, 1985). Since the majority of horses in our study were in paddocks during these activities, horses were probably generally exposed to lower dust levels during afternoon–night time stabling. During winter

stabling period the horses spent more time in the stable and the natural ventilation was less effective. Not surprisingly, the particle load was higher during the winter stabling periods than during summer (Rosenthal, Gruntman & Couetil, 2006). Given that the stable we examined fed haylage and had straw for bedding the environment can be considered moderately dusty (Clements & Pirie, 2007a; Vandenput*, et al.*, 1997; Woods*, et al.*, 1993). In this pilot study the respirable dust collected in the breathing zone of the personnel combined with stationary measurements was below upper limits for humans. Collection of both the total and respirable dust in the background and from breathing zones of horses would have been desirable as higher values are expected in breathing zone compared to stationary measurements (Woods*, et al.*, 1993).

The outdoor humidity varied considerably on a day-to day basis during winter conditions in Sweden. Cold air temperature can alter gene expression of chemokines in the horse (Davis*, et al.*, 2005; Davis*, et al.*, 2007; Robinson*, et al.*, 2006) and there are epidemiological studies pointing to the potential influence of climatic factors on the prevalence of RAO/SPAOPD and asthma (Costa *et al.*, 2006; Priftis *et al.*, 2006; Ward & Couetil, 2005). One explanation for increased particles in the air can also be exposure to hay and straw with poor hygienic quality after long term storage during winter time. Endotoxin present in the stable environment is a likely contributing factor to neutrophilic airway inflammation in horses (Pirie *et al.*, 2003; Pirie*, et al.*, 2001; Pirie, Dixon & McGorum, 2003). Endotoxin concentrations in horse stables varies greatly (McGorum, Ellison & Cullen, 1998), possibly related to stable routines, and hygiene as well as sampling and analysis methods used (Jacobs, 1997). However, at present there are no official guidelines regarding threshold values for either endotoxin or (1→3)-β-D-glucan in horse stables. In this study airborne endotoxin levels were lower than those reported by McGorum (McGorum, Ellison & Cullen, 1998) and indeed lower in the one winter sampling when compared to summer. When measuring endotoxin values the time aspect should be included to the analysis since there is a possible cumulative role for long term exposure for endotoxin levels (Vogelzang *et al.*, 1998). Due to loss of one of the winter seasons' (1→3)-β-D-glucan analysis, interpretation of the role of this airborne particle regarding season or airway inflammation remains unclear.

Inflammation markers in horse airways

The median for neutrophil percentage in BAL during winter was more than double the level obtained in September (7.5 % versus 3.5%), witch after removing one outlier from the analysis significant reached significance (p=0.022). Neutrophil influx is not only observed in stabled horses but has also been previously reported as a response to exercise in cold air (Davis*, et al.*, 2007). The percentage and absolute number of cells in BAL, other than eosinophils, were statistically unaltered. Even when three horses with highest eosinophilia were excluded at study IV the eosinophil percentage in BAL was still elevated (p = 0,022). The respiratory rate was lower at September measurement compared to winter mean (p=0.021), likely related to examination of the horses in a higher ambient temperature at our clinic in contrast to the colder environment at the home stable,

to which they were accustomed.There was no significant difference over season in all other clinical parameters, including other vital signs, blood parameters, PaO_2 and subjectively graded appearance of tracheal mucus. Hematology revealed only minor variations of no clinical relevance.

Gene expression of IL-6 mRNA in BAL cells was significantly enhanced during winter periods compared to summer sampling with median winter values being 3.1 fold (p=0.014) increased in February 2004 over September 2004 and median 5.5 fold increase in March 2005 over September 2004 (p=0.022). When winter results were combined there was a 3.7- fold increased over September (p=0.014), (figure 11). The median IL-10 mRNA expression was 3.4 fold higher (p=0.036) during one winter sampling (March). Furthermore there was no correlation between cytokine expression and BAL cell cytology.

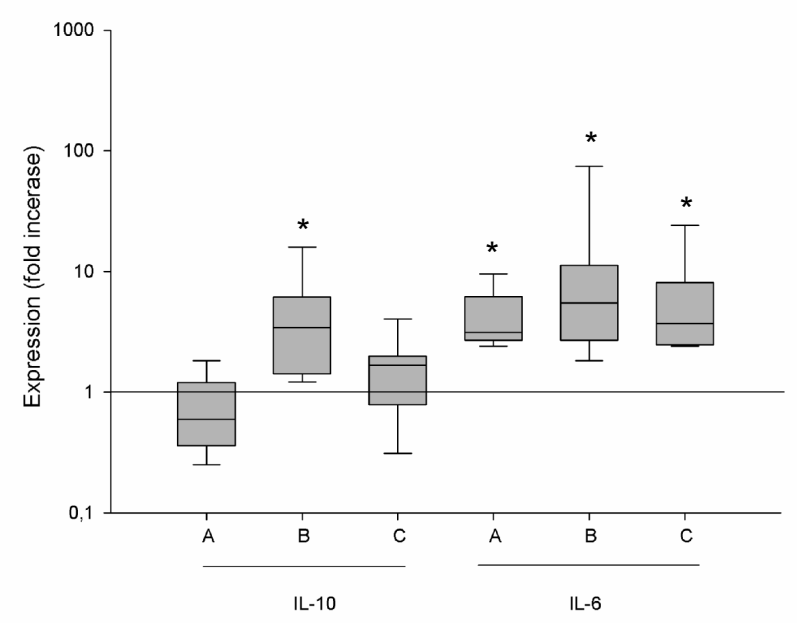

Figure 11. The relative (fold) increase of cytokine mRNA expression of IL-6 and IL-10 in horse BAL cells is shown with interquartile range at in different sampling occasions compared to September sampling. The horizontal line represents the median value. A= February versus September, B= March versus September, C= winter mean versus September. * Significant difference in median value from 1.

In this study the total IL-6 mRNA produced by all BAL cell types was measured from racing horses without history of underlying inflammatory disease. It is unclear whether the increased IL-6 mRNA in BAL during winter reflected stable environment or cold weather exercise. Increased IL-6 production can reflect differences in cumulative exposure to inhaled endotoxins during stabling period compared to after pasture, since summer conditions allow for far greater time

spent outdoors. On the other hand, increased IL-6 mRNA has also been shown in BAL cells after cold weather exercise (Davis*, et al.*, 2005; Davis*, et al.*, 2007), which may partly explain increased expression in our study during winter months. In these recent experimental inhalation studies with exercise in cold air, the selected healthy horses were kept in optimal stable environment, they were isolated from other horses in order to avoid infections and additionally trained prior experiment in summer temperatures. Thus these experimental studies, while valuable for time-related studies, don't mirror the everyday life of racehorses in northern climates during winter temperatures, where horses are kept in common and probably more dusty stable environments, exposed for different infectious agents and also trained in cold air conditions. Additionally the outdoor temperature is often below -5ºC used in these experimental studies. The biological importance of the small but significant elevation in BAL eosinophils in September remains uncertain as the contribution of eosinophils to the entire BAL cell population recovered was minimal, and the increase did not appear to affect IL-6 mRNA expression in BAL cells.

The altered expression of IL-10 in total BAL cell mRNA in this study was less consistent than mRNA for IL-6, with only March 2005 being significantly different by 3.8 fold over September 2004. Again it is uncertain if this increase was related to stable environment (Laan*, et al.*, 2006; van den Hoven *et al.*, 2006) or outdoor condition such as cold air (Davis*, et al.*, 2005; Davis*, et al.*, 2007) or changes in humidity. Up-regulation of mRNA to IL-10 in winter due to regular exercise at cold weather and long-term exposure to stable environment may be expected, especially when IL-6 mRNA expression indicated increased inflammatory response during winter period. On the other hand, from our summer measurements, even after exclusion of the horses with marked pulmonary eosinophilia, a trend for increased eosinophil percentage in BAL persisted in the remaining horses. Since IL-10 is also produced also by eosinophils, IL-10 mRNA expression might have been up-regulated due to parasite induced (Davidson *et al.*, 2005) or idiopathic eosinophilia during summer measurements, which may have interfered with our overall comparison of IL-10 mRNA expression between summer and winter months. Thus, further studies are needed to better define the immunomodulatory properties of IL-10 within equine airways.

In summary the eosinophil influx may have affected our conclusions regarding cytokine expression over time and season. Importantly though respirable particles were higher in both winter samplings when compared to summer, potentially contributing to the observed inflammatory response at winter measurements.

Significance of the results

In preparing this manuscript we were faced with the problems of how to interpret the numerous factors in management and environment of a conventionally managed racing stable that varied considerably and were beyond our ability to control. Our aim was to examine summer conditions versus winter, unfortunately at the only summer measurement the horses showed transient pulmonary eosinophilia probably related to other factors than stable environment. As our

initial research plan and hypotheses were based on the 12 horses but the three horses with obvious pulmonary eosinophilia were not included in the analyses. Additional summer measurement was not performed due change in ownership of the stable. It would also have been valuable to have data on race horses not being maintained indoors. However, in northern climates during winter shelter is required with bedding and hay/silage feeding. We therefore chose to sample the horses along with stable air during times that captured the presumed worst stable air quality (mid to late winter) and the optimal stable air quality (late summer/early autumn). The sampling was performed at each point during the stable conditions with the highest potential daily aerosol challenge: during feeding and changing of bedding. Whereas highly dusty and mould contaminated stable air has long been incriminated as inducing respiratory disease in horses (Clarke & Madelin, 1987) defining what is "acceptable" stable air quality and relating it to indices of respiratory health remains incompletely understood (Clements & Pirie, 2007b).

Despite the complexity of study IV- we were able to detect a significant up regulation of innate immune response during both winter periods compared to summer for IL-6 mRNA (figure 11), which coincided with a trend for increased neutrophils in BALF. As we did not have specified data on exact time spent in the stables we could only speculate that increased exposure time during winter months may have contributed to upregulation of inflammation. While we also found significant alterations in mRNA IL-10 between summer and one winter, we preferred not to expand (further) on theoretical implications of this finding as the change was detected on only one of the two winter samplings.

Concluding remarks:

The findings in this thesis reveal:

- Biopsies from bronchial epithelium can be used as a complementary method to BAL in studies of inflammatory response in equine airways. The sampling technique is safe, easy to perform and provides good quality tissue material for biochemical and morphological studies. Importantly, the sampling can be repeated over short time intervals that can capture serial events not possible to study similarly using BAL.

- The mRNA for neutrophil chemotaxis cytokine, IL-8 is upregulated in respiratory crisis in RAO horses in both BAL cells and bronchial tissues.

- Quantitative real-time PCR and IHC methods on bronchial biopsy tissues can provide complementary research tool for examining temporal events associated with the inflammatory process in RAO-horses.

- While the etiology of transient pulmonary eosinophilia remains uncertain, it appears that eosinophil influx does not necessarily cause specific clinical signs apart from temporary exercise intolerance. Additionally, from a clinical point of view, the pulmonary eosinophilia in horses can abate without specific treatment other than deworming.

- Prolonged stabling period during winter along with exercise or exposure to cold air, can in combination, or individually initiate an inflammatory response in equine airways, with an increased of IL-6 mRNA in BAL cells that coincides with a trend for increased neutrophils in the BAL fluid.

Future research

The role of bronchial mucosa in immunological mechanism behind RAO should have the following focus:

- Examine temporal events with repeated sampling over time in controlled inhalation provocation studies in RAO horses.
- Expand the range of cytokines and molecular markers examined.
- In order to evaluate the inflammatory changes in bronchial tissue post provocation to conventional stable environment further investigations on inflammatory markers in bronchial epithelium from clinical cases and healthy controls from same stables are indicated.
- Assess the role of morphological changes in endobronchial biopsies in correlation to disease severity and progression in RAO horses.
- Investigate whether a potential human asthma susceptibility gene, a disintegrin and metalloproteinase (ADAM33) is expressed in equine bronchial epithelium and if so, whether the expression is increased or altered in relation to disease severity in horses with RAO

Assessment of new diagnostic techniques:

- Capitalisation of the recently published horse genome together with the technique of proteomics offers a highly promising new possibility to evaluate the protein expression in endobronchial tissue and BAL from time-dependent studies.

Investigations in pulmonary eosinophilia in horses:

- The role of eosinophil related cytokines, IL-4 and IL-5, on eosinophil influx to the lung in horses should be further evaluated with time dependent studies.
- Retrospective review of horses with eosinophilia in BAL is in order identify associated risk factors, such as age, breed, season, and management factors such as deworming, in relation to the frequency of pulmonary eosinophilia in the equine population.

To explore the role of stable environment in horse and human airways the following are suggested

- Evaluate the appropriate techniques and methods to examine qualitative differences in indoor stable air.

- Define thresholds of air quality in horse stables above which the inflammatory response in equine respiratory tract may be initiated.

- Explore how horse stable environment affects both horse and human airways, and what selected individual factors appear to be related to induction of an inflammatory response.

- Conduct further human studies investigating stable environments effects personnel's and grooms lung heath.

Populärvetenskaplig sammanfattning

Inflammatoriska luftvägssjukdomar hos häst är en av de vanligaste orsakerna till nedsatt prestation och långvariga hostproblem i alla länder där hästarna lever i stallmiljö. Hästar med astmaliknande symptom, s.k. Recurrent Airway Obstruction (RAO) och hästar med lindrigare grad av inflammatoriskt svar i luftvägarna, Inflammatory Airway Disease (IAD), har varit en målgrupp för intensiv forskning under de senaste decennierna. Traditionellt inom hästforskning har man undersökt olika komponenter i provmaterial som har samlats med lungsköljprov eller i samband med obduktion. Det finns ett stort behov av nya provtagnings- och analysmetoder inom forskningen för att förstå patofysiologin bakom inflammatoriska luftvägssjukdomar hos häst. Kunskapen om hur och varför inflammation utvecklas kan förhoppningsvis användas för att utveckla nya behandlingsstrategier och förebyggande åtgärder, och kan vara vägledande avseende möjligheterna att använda hästar som modelldjur för human astma. Vid forskning på astma- och KOLpatienter används inom humanmedicinen slemhinnebiopsier som insamlats med hjälp av bronkoskopi som en kompletterande provtagningsmetod till lungsköljprov. Denna provtagningsmetod, av så kallade bronkialbiopsier, är ett enkelt och säkert sätt att få vävnadsmaterial från levande individer vid upprepade tillfällen.

Målsättningen med detta avhandlingsarbete har varit att undersöka vilken roll luftvägsslemhinnan har i den inflammatoriska processen som orsakas av dammigt hö och dåligt stallmiljö. Ytterligare ett viktigt syfte i avhandlingen var att se hur vanlig stallmiljö påverkar hästens luftvägar. Vi har slutligen utvärderat om slemhinnebiopsier kan användas i forskningssyfte som komplement till lungsköljprov hos hästar.

I studierna I och II har vi undersökt slemhinnebiopsier avseende inflammationsmarkörer, samt jämfört resultaten med de analyserade lungsköljproverna. Den kliniska delen av dessa studier är genomförd i samarbete med University of Liège, i Belgien. Vi har undersökt väldefinierade RAO-hästar samt friska kontrollhästar. RAO hästarna undersöktes två gånger; först i samband med inhalationsprovokation med dammigt hö och halm i dåligt ventilerad stallmiljö och senare när hästarna var fria från luftvägssymptom och vistades på bete.

I studierna III och IV har vi undersökt hur stallmiljön i ett vanligt konventionellt svenskt stall påverkar hästarnas lunghälsa under olika årstider. Studierna utfördes på 12 privatägda varmblodiga travhästar i träning. Stallets luftkvalitet utvärderades vid två separata provtagningar vintertid samt en provtagning under sommarhalvåret. Samtidigt undersöktes hästarnas lunghälsa genom att tecken på inflammation samt förekomst av inflammationsmarkörer i luftvägarna registrerades.

Resultatet från studierna visar att slemhinnebiopsier insamlade med bronkoskopi kvalitetsmässigt kan användas för morfologiska, realtids-PCR och

immunohistokemiska analyser. Utöver detta ger vävnadsprover kompletterande information till lungsköljprov eftersom olika signalämnen i den komplexa inflammatoriska processen förändras kontinuerligt med tiden. Dessutom ger denna provtagningsteknik en unik möjlighet att upprepade gånger, enkelt och säkert, samla vävnadsmaterial från patienter. Resultatet från stallmiljöstudierna visar att även friska hästars luftvägar reagerar på stallmiljön eller på arbete i kall luft vintertid. Detta även om stallmiljön enligt mätningar anses ha en god kvalité. Vi har också visat att hästar kan ha en anmärkningsvärt kraftig inflammation i lungorna med en ansamling av så kallade eosinofila granulocyter, utan att ha några andra sjukdomssymptom än en av ägaren observerad nedsatt prestationsförmåga.

Sammanfattningsvis har denna avhandling visat att slemhinnebiopsier kan användas vid forskning på inflammation i hästens luftvägar. Resultaten indikerar också att vävnadsprov kompletterar analysresultat från lungsköljprov. Vidare visar avhandlingen att friska installade hästar har ett inflammatoriskt svar i luftvägarna under vintertid, sannolikt relaterat till stallmiljön i kombination med arbete i kall luft. Hästar kan också drabbas av en kraftig övergående eosinofil inflammation i lungan utan att visa andra kliniska symptom än en av ägaren observerad arbetsinsufficiens.

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The heart has more place and memory than my mind...

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