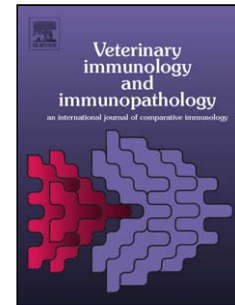


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Partial divergence of cytokine mRNA expression in bronchial
tissues compared to bronchoalveolar lavage cells in horses
with recurrent airway obstruction

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Keywords: equine, PCR, bronchial biopsy, BAL

24 Abstract

25 The aim of this study was to investigate mRNA levels of cytokines in bronchial epithelium in
26 horses with Recurrent Airway Obstruction (RAO) during acute crisis and remission.

27 Additionally, cytokine mRNA levels in endobronchial biopsies and bronchoalveolar lavage
28 cells were compared. Seven RAO horses were examined while in respiratory crisis following
29 provocation and again while in remission after two months on pasture, during which time six
30 healthy horses on pasture were also examined.

31

32 Quantitative real-time PCR was used to assess mRNA expression for cytokines IL-5, IL-6,
33 IL-8, IL-10, IL-17 and TGF- β 1 in endobronchial biopsies and bronchoalveolar lavage (BAL).
34 Expression of IL-8 mRNA was significantly upregulated during crisis in both endobronchial
35 biopsies and BAL cells ($p=0.036$), while there was similar trend for upregulation of IL-10
36 mRNA only in BAL cells that approached significance ($p=0.059$). Moreover, during crisis the
37 expression of IL-8 mRNA in BAL cells was positively correlated to relative IL-6 mRNA
38 expression ($r_s=0.971$, $p=0.001$) and bronchial epithelial expression of IL-10 and TGF- β 1
39 mRNA were positively correlated ($r_s=0.943$, $p=0.005$). In comparing the relationship of
40 mRNA expression in BAL to biopsy in individual RAO horses, there was a positive
41 correlation with IL-6 to IL-8 mRNA expression in BAL during respiratory crisis ($r_s=0.971$,
42 $p=0.001$) that also correlated positively with IL-8 expression in biopsies on pasture ($r_s=0.986$,
43 $p<0.0001$ for both).

44 Regarding RAO horses at pasture versus controls neither the cytokine mRNA levels in
45 endobronchial biopsy nor in BAL cells differed significantly. These results further support
46 previous findings that IL-8 mRNA in both BAL cells and bronchial epithelium is upregulated
47 in RAO horses during crisis. However, apart from IL-8, it appears that expression of other
48 cytokines, including IL-5, IL-6, IL-10, IL-17 and TGF- β 1 in bronchial epithelium does not

necessarily mirror cytokine expression in BAL cells in individual horses with RAO. Accordingly, examination of markers of inflammation in endobronchial tissue provides complementary but not necessarily identical information to that obtained in BAL cells. Given the potential for repeated sampling over time bronchial biopsy can serve an invaluable additional tool for investigation of time dependent changes in inflammatory process in this animal model of asthma.

1. Introduction

Recurrent Airway Obstruction (RAO) is a common clinical problem worldwide in horses that are housed indoors. Horses with RAO develop bronchospasm, neutrophil influx and mucus accumulation in airways in response to inhaled particles or substances (Robinson, 2001). Factors controlling neutrophilic accumulation in lungs in RAO horses have been studied predominately using samples collected by bronchoalveolar lavage (BAL), but other methods, such as bronchial brushing, tracheal lavage, exhaled breath condensate, and even lung tissue samples have also been of value (Ainsworth et al., 2003; Bureau et al., 2000a; Couetil et al., 2006; Gerber et al., 2003; Giguere et al., 2002; Lugo et al., 2006; Schatzmann et al., 1974; Wyse et al., 2005). The airway inflammation and obstruction of RAO has many similarities to airway alterations in asthma in man, where bronchial biopsy of airway epithelium has provided valued insight into tissue inflammatory mechanisms. A distinct advantage of endobronchial biopsy over bronchoalveolar lavage is that it can provide details of structural alterations to the airways, and, in some instances, an indication of severity or prognosis (Jeffery, 1998; Jeffery et al., 2003; Jeffery, 1996; Jeffery et al., 2000; Saetta et al., 1998). However the utility of bronchial biopsy for study of RAO in the horse has only recently been reported (Ainsworth et al., 2006).

74

75 Resident airway epithelial cells play an important role in inflammation through interaction
 76 with inflammatory cells, acting as a source of various inflammatory cytokines and mediators,
 77 expression of adhesion molecules for recruitment of inflammatory cells, and contributing to
 78 airway remodelling (Djukanovic et al., 1990; Holgate, 2000; Jeffery et al., 2000; Knight and
 79 Holgate, 2003; Rennard et al., 1995; Takizawa, 1998). Bronchial biopsies are used in human
 80 medicine to study airway inflammation in asthma, COPD and even to response to various air
 81 pollutants (Behndig et al., 2006). A disadvantage with this technique is the small size of tissue
 82 material (usually 1-2mm) and that sampling is performed at the subcarinae rather than more
 83 peripheral airways (Jeffery et al., 2003). However with current molecular biological
 84 techniques the sample size is adequate for selected analyses. However, it remains unclear
 85 whether inflammatory changes in larger airways can reflect the inflammatory events in small
 86 airways (Gamble et al., 2006; Gerber et al., 2003; Hirota et al., 2006). Closer examination of
 87 inflammatory changes within bronchial tissues in horses with RAO has recently been
 88 conducted (Ainsworth et al., 2006) but, as most of our current understanding of immunologic
 89 mechanisms of RAO has come from BAL, comparison of indices of inflammation in
 90 bronchial tissues to those in BAL cells is needed.

91

92 In this study we hypothesized that selected cytokines, IL-5, IL-6, IL-8, IL-10, IL-17,
 93 transforming growth factor β 1 (TGF- β 1), related to initiation and regulation of inflammation
 94 and neutrophil chemotaxis (Ainsworth et al., 2006; Debrue et al., 2005; Desjardins et al.,
 95 2004; Franchini et al., 2000; Franchini et al., 1998; Giguere et al., 2002; Laan et al., 2006a;
 96 Sagara et al., 2002), would be upregulated in horses with RAO in acute crisis compared
 97 remission, and also in comparison to healthy control subjects. As well, we examined whether

expression of the above selected cytokines within bronchial tissues correlated to those in BAL cells from individual horses.

2. Material and methods

2.1 Horses

Seven horses with a history of clinical signs of RAO when exposed to moldy hay (Mean weight $431 \pm$ SD 90 kg, age 19.7 ± 6.6 years) and six healthy controls (Mean weight $475 \pm$ SD 33 kg, age 16.8 ± 7.9 years) were included in the study. The RAO horses were examined once respiratory crisis occurred after provocation with mouldy hay, straw bedding and poor stable ventilation, and secondly after remission when horses had been two months on pasture, during which six healthy pasture mates were also examined. When all RAO horses responded to provocation with mouldy hay with clinical signs of respiratory exacerbation (blinded observer clinical score higher than 5, according to Rush et al., 1998) and pulmonary function measurements that indicated typical signs of dyspnea with maximum change in pleural pressure ($\Delta P_{pl_{max}}$) more than 1.5kPa (Art et al., 2006) the sample collection was performed. In our horses, these conditions occurred after two days challenge.. This study was approved by the institutional animal care and use committee, University of Liège, Belgium.

2.2 Pulmonary function tests

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 Fleish pneumotachograph (Model NR4; Gould Electronics, Eichstetten, Germany). The

maximum change in transpulmonary pressure ($\Delta P_{pl_{max}}$), pulmonary resistance (R_L) and dynamic compliance (C_{dyn}) were obtained from a lung function computer (Haemodynamics Respiratory System; ACEC, Namur, Belgium). Each measurement period was 2min on average, and average values of 10 representative breaths were selected for analysis.

2.3 Bronchoalveolar lavage

Upper and lower airway endoscopy (Pentax, Breda, The Netherlands, 170cm x 12.9 mm) with guided bronchial biopsy and BAL were performed in all horses after sedation with romifidine (0.01 mg/kg, iv, Sedivet; Boehringer Ingelheim, Ingelheim, Germany) and butorphanol tartrate (0.02 mg/kg IV, Torbugesic; Fort Dodge, Wyeth, Madison, New Jersey, USA). The BAL was performed with 6x 60 ml sterile isotonic saline solution (at 37°C). Fluid recovered was placed on ice, samples were pooled and an aliquot collected for cytology. BAL samples total leukocyte counts were first determined using a manual cell counter (THOMA cell; VWR International, Fontenay-sous-Bois, France). 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, and total neutrophils calculated from the product of cell count per volume of BAL fluid recovered and cell differential.

2.4 Endobronchial biopsies

Six biopsies (approximately 1-2 mm in diameter) were collected via endoscopy from the bronchial epithelium from subsegmental and segmental subcarinae from one lung with biopsy

forceps (reusable, 240cm, Pentax, KW3433S, Pentax Corporation, Tokyo, Japan). The first two biopsies in each horse were taken at the first generation bronchus and immediately frozen in liquid nitrogen and stored at -70°C until analysis for cytokine mRNA. The remaining four biopsies were immediately placed in chilled acetone containing protease inhibitors and processed for immunostaining studies (data not shown). Biopsies were taken from either the left or the right lung of individual horses and BAL was performed endoscopically to ensure that lavage was performed on the lung contralateral to biopsy.

2.5 Quantitative real-time PCR

BAL samples were centrifuged at 1000 x g for 10 minutes and the resulting cell pellets and BAL-fluid were kept frozen (-80°C). Isolation of total RNA from BAL cells was performed with TrizolTM (Sigma-Aldrich Corp. AB, Stockholm, Sweden) according to the manufacturer's instructions, dissolved in DMPC-H₂O and stored at -80°C until analysis. cDNA was synthesized from 500 ng total RNA using gene specific primers and Superscript III reverse transcriptase (Invitrogen). Relative quantification of equine cytokine mRNA expression was performed by quantitative real-time PCR (RT-PCR) using a 7300 Real Time System (Applied Biosystem). Primer and probe sequences are listed in Table 1. The real time PCR reactions were run for 45 cycles of: 95°C 15 s and 60 °C 1 min. The reactions were run with Platinum Taq polymerase and reaction conditions were optimised for each primer set. All samples were run in triplicate and non template controls were included in each run. The RNA levels of the target genes were normalized against GAPDH mRNA levels and the comparative $C_T(2^{-\Delta\Delta C_T})$ method was used for calculating relative cytokine mRNA expression (Livak and Schmittgen, 2001). The PCR efficiencies, as determined by assaying serial dilutions of RNA, were approximately equal for the target genes and the housekeeping genes.

2.6 Statistical analyses

Statistical comparisons were performed using a commercially available statistical software program (MINITAB). Data were analyzed using nonparametric statistics because of low animal numbers. Descriptive statistics from each sampling period were expressed as median $\pm$ SD. The Wilcoxon signed rank test used to assess for differences between provocation and remission in RAO-horses, using a two tailed test (BAL cytology, clinical score, pulmonary function). This test was also used for relative cytokine expression where the theoretic median value 1 was used (theoretically no difference between provocation and remission or $\Delta\Delta C_T=0$, $2^{-\Delta\Delta C_T}=2^0=1$). 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 valued using the Mann Whitney test. Spearman rank order correlation was used to evaluate the correlation in cytokine expression in BAL and biopsies in RAO horses and for correlation between cytokine expression and cytological findings. A p value < 0.05 was selected as the level of significance. Results from the real-time PCR analysis for one horse were unsatisfactory due to extremely low number of cells in BAL and this horse was thus excluded from statistical analysis regarding cytokine mRNA expression in BAL cells.

Results:

2.1 Clinical examination and pulmonary function test

The clinical score of RAO horses on pasture was significantly lower than after provocation ($p=0.02$), and did not differ from the pastured control horses. The RAO horses showed a significant deterioration in pulmonary function as a response to provocation with mouldy hay (table 2) which included significant increases in maximum change in pleural pressure, $\Delta P_{pl_{max}}$, and total pulmonary resistance and R_L (p value for all = 0.022). Dynamic compliance, C_{dyn} was also decreased but due to a single outlier (horse nr 1) did not reach statistical significance ($p=0.272$, without outlier $p=0.036$). There was no statistical difference in age and body weight between principal and control animals.

2.2 BAL cytology

The total cell count, percentage of neutrophils and total number of neutrophils in BAL were statistically higher ($p=0.022$) after provocation in RAO horses compared to samples taken during remission on pasture (table 2) whereas once the RAO horses were in remission after 2 months at pasture BAL neutrophil percentage did not differ from pastured controls.

2.3 Real-time PCR on BAL and biopsies

The differences in cytokine mRNA expression during crisis and under remission on pasture are shown in figure 1a and 1b. In the RAO horses the median value for IL-8 mRNA expression in BAL was 47-fold greater during crisis ($p=0.036$) (fig 1a) and in biopsies the IL-8 expression was 4-fold increased ($p=0.052$) (fig 1b) and reached statistical significance after removing one outlier (horse nr 3) ($p=0.036$). There was also a positive correlation between IL-6 and IL-8 mRNA expression in BAL in the horses during respiratory crisis ($r_s=0.971$, $p=0.001$) (figure 2).

During crisis there was a trend for upregulation in IL-10 mRNA levels in BAL cells with median 7 fold increase ($p=0.059$) and epithelial expression of IL-10 and TGF- β 1 mRNA under this period were positively correlated ($r_s=0.943$, $p=0.005$).

Comparison of biopsy to BAL cytokine expression in individual horses demonstrated enhanced expression of IL-8 mRNA in both BAL and biopsies in crisis. Interestingly the IL-8 and IL-6 mRNA expression in BAL during respiratory crisis in individual horses correlated positively with IL-8 mRNA expression in biopsies during remission on pasture ($r_s=0.986$, $p<0.0001$) for both). There was also a significantly positive correlation between BAL and biopsies for difference in IL-17 mRNA expression during crisis and remission ($r_s=0.943$, $p=0.005$). However for all other comparisons of biopsy to BAL cell findings there was poor to no correlation.

In comparing RAO horses at pasture versus control horses there was a trend for increased IL-8 expression in endobronchial biopsies in RAO subjects ($p=0.086$) whereas for all other comparisons there was no difference in cytokine expression in either endobronchial biopsies or BAL cells. The morphological quality of biopsies was satisfactory and found suitable for immunohistochemical analysis (Riihimäki et al, submitted). No adverse reactions to biopsy sampling was noted.

Discussion

The most prominent finding regarding cytokine expression was an increased expression of neutrophil chemotactic protein IL-8 mRNA in endobronchial biopsies and in BAL cells in RAO horses during crisis, which supports and extends the findings of a number of earlier studies (Ainsworth et al., 2003; Ainsworth et al., 2006; Franchini et al., 2000; Franchini et al., 1998; Laan et al., 2006a). Ainsworth et al (Ainsworth et al., 2006) recently showed an

increase of IL-8 mRNA expression, not only in BAL cells but also increased IL-8 immunoreactivity in endobronchial biopsies in RAO horses after challenge. In another recent study (Berndt et al., 2006), where samples were collected by endobronchial brushing, the IL-8 mRNA expression from epithelial cells was increased after exposure to stable dust in both RAO and control horses, but there was no significant difference of expression between these groups (Berndt et al., 2006).

In our study there was correlation between IL-6 mRNA and IL-8 mRNA expression in BAL during crisis that also correlated with IL-8mRNA expression in bronchial tissue during remission ($r_s=0.986$, $p<0.0001$). Thus those horses with the highest mRNA levels of these cytokines in BAL during crisis also had the highest bronchial tissue IL-8 mRNA expression during remission at pasture. Moreover, there was a trend ($p=0.086$) for increased IL-8 mRNA expression in biopsies from RAO horses in remission compared to healthy controls at pasture. Ainsworth et al (Ainsworth et al., 2006) showed that samples from RAO horses obtained prior to exposure to moldy hay were more likely to express IL-8 in endobronchial biopsies, but not in BAL compared to controls. As in our findings the difference in their study did not reach statistical significance. In relation to other aspects of the pulmonary neutrophilia of RAO Art et al (Art et al., 2006) have shown significantly increased myeloperoxidase concentration in BAL fluid two months post exposure to moldy hay from the RAO horses used in this study, providing evidence of persistence of an inflammatory reaction well beyond initial crisis even when BALF neutrophilia has resolved. Thus, in our study neutrophil chemotactic IL-8-mRNA was elevated in both BAL and biopsies during respiratory crisis associated with neutrophil influx to the lung, and in individual horses there was a strong correlation between expression of the pluripotent inflammatory cytokines IL-6mRNA and IL-8mRNA. This indicates a similar mechanism of inflammatory response both in the bronchial epithelium from larger airways and in bronchoalveolar cells.

270

271 The pluripotent cytokine IL-6, with both pro-inflammatory and anti-inflammatory properties
 272 (Opal and DePalo, 2000; Xing et al., 1998) is induced by a variety of different substances,
 273 such as endotoxin (Giguere et al., 2002; Morris et al., 1992) and cold weather exercise (Davis
 274 et al., 2005; Davis et al., 2007). In the present study however IL-6 mRNA expression in BAL
 275 was not significantly increased in the RAO horses during crisis despite cytologic evidence of
 276 pulmonary inflammation, which is consistent with an earlier study also involving exposure to
 277 mouldy hay (Giguere et al., 2002). Laan et al (Laan et al., 2006a) found increased IL-6
 278 expression in BAL, both in controls and RAO-horses, but in another study failed to detect
 279 increase in IL-6 expression in BAL (Laan et al., 2006b). Thus aspects of sampling time with
 280 respect to provocation and type inhaled challenge should be considered when analysing the
 281 results.

282

283 This study also showed a median of 7-fold greater expression of IL-10 in BAL cells that
 284 approached significance ($p=0.059$) after provocation with “natural challenge” in RAO horses.
 285 Due to its immunoregulatory effects IL-10 may have a key role to play in the inflammatory
 286 process associated with RAO. Earlier studies in equine species have shown that inhalation
 287 challenge with lipopolysaccharide (LPS) elevates IL-10 mRNA expression in both BAL
 288 (isolated alveolar macrophages) (Laan et al., 2006a) and peripheral blood cells (van den
 289 Hoven et al., 2006) in RAO horses. However inhalation challenge with other agents such as
 290 hay dust suspension (HDS) (Laan et al., 2006a) or provocation with *Aspergillus fumigatus*
 291 antigen (Laan et al., 2005) did not induce this upregulation in BAL macrophages. The kinetics
 292 of cell traffic is probably not only related to the type of provocation, such as provocation with
 293 inhalation challenge (Laan et al., 2006a; Pirie et al., 2002a; Pirie et al., 2002b; Pirie et al.,
 294 2002c) or cold air (Davis et al., 2005; Davis et al., 2007) but also by the time course of the

challenge versus sampling (Ainsworth et al., 2003; Ainsworth et al., 2006; Debrue et al., 2005; Laan et al., 2006a). In this study the provocation mimicked natural conditions, using mouldy hay together with poor indoor stable environment, in contrast to inhalation challenge. The time course from exposure to sampling in our study was based on development of clinical signs of disease in the studied animals rather than assigned time points for sampling post provocation. Both strategies assigned time point for sampling and sample collection based on clinical signs influence the results and the optimal way to investigate inflammatory response in airways is repeated sampling over time. Sampling at a precise time point post challenge can capture early events in gene regulation if ideally timed. In contrast, as there is individual variation in development of disease, sampling once clinical signs are sufficiently developed ensure gene expression upregulation has had time to occur. However, a disadvantage to the use of clinical parameters as start point for sampling is that early events may not have been captured since development of clinical abnormalities may lag after key gene upregulation initiating the inflammatory cascade. Whereas BAL has some limitations as it might affect the results when repeated, bronchial biopsies can be collected repeated over time, and in future work the time component may be more closely examined using serial bronchial biopsy. Unfortunately, samples from control horses after provocation for comparison of cytokine expression in bronchial tissue and BAL cells were unavailable. Earlier studies have, however, failed to detect increased expression of this immunomodulatory cytokine in healthy controls after provocation (Laan et al., 2006a). Further, in horses with pulmonary eosinophilia (Riihimäki, et al, 2007, in press) and seasonally related airway responses to stable air (Riihimäki, et al, 2007, submitted) we have not observed any difference in IL-10 expression in BAL cells.

319 Of the other cytokines examined TGF- β 1 was also included in our evaluation as it is
 320 considered to be a multifunctional cytokine, with immunoregulatory properties (Nakao et al.,
 321 2000), associated with airway remodeling in asthma (Nakao et al., 2002; Sagara et al., 2002).
 322 Consistent with earlier finding in horses (Desjardins et al., 2004) the TGF- β 1 mRNA
 323 expression was not increased during respiratory crisis, nor was there a difference in TGF- β 1
 324 expression between RAO horses and controls on pasture. However, the positive correlation of
 325 IL-10 mRNA to TGF- β 1 mRNA expression in biopsies may have biological implications,
 326 since, as shown in human bronchial epithelial cells, IL-10 partially regulates TGF- β 1
 327 expression (Fueki et al., 2007; Nakao et al., 2002), and thus our findings may point to an
 328 immunoregulatory role for IL-10 even in equine bronchial epithelium.
 329 The correlation of the difference in IL-17 mRNA (also known as IL-17A) expression between
 330 BAL and biopsies in crisis and remission in the RAO horses may have been of immunologic
 331 relevance. In humans IL-17 is associated with neutrophilic inflammation by initiating the
 332 activation of NF- κ B, which initiates activation of other proinflammatory cytokines such as
 333 IL-8. IL-17 is also involved in activation of TNF- α (Jones and Chan, 2002; Jovanovic et al.,
 334 1998; Linden and Adachi, 2002; Linden et al., 2000). Interestingly an activation of NF- κ B in
 335 epithelial cells, collected by bronchial brushing, has been shown in RAO horses compared to
 336 controls (Bureau et al., 2000a; Bureau et al., 2000b; Sandersen et al., 2001). Interleukin-17
 337 mRNA in BAL has been shown to be up regulated in RAO horses after long term exposure
 338 (14-35 days) to moldy hay (Ainsworth et al., 2006; Debrue et al., 2005). In our study the
 339 samples were collected after two to eight days provocation which may have been too early to
 340 detect a rise in IL-17 mRNA. While the IL-17 mRNA expression did not correlate with the
 341 pulmonary neutrophilia in either of these studies, Debrue et al, showed a correlation of IL-17
 342 mRNA levels in BAL with lung function parameters (Debrue et al., 2005). Time-dependent
 343 studies (Ainsworth et al., 2006) in cytokine mRNA expression showed that neutrophil influx

in BAL and significantly elevated IL-8 gene expression in BAL and biopsies occurred prior to a significant increase in IL-17 mRNA expression in BAL in RAO horses compared to controls during provocation studies, suggesting that IL-17 was not a likely initiating stimulus for IL-8 production in RAO horses.

Generally the levels of cytokine expression were lower in biopsies than in BAL cells, probably related to differences in cell population in samples examined. In humans there is poor correlation between biopsies' cellular findings in endobronchial biopsies and BAL (Jeffery, 1996) and thus direct correlation of cytokine regulation between bronchial epithelium versus bronchoalveolar lavage cells was not expected.

Conclusion

In conclusion, this study adds further support that IL-8 mRNA in both BAL cells and bronchial tissues is upregulated in RAO horses in acute crisis. Moreover, our findings 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. This study also demonstrates that, despite the small amount of tissue obtained, endobronchial biopsies are valuable complement to traditional BAL or bronchial brushing sampling. This sampling technique gives also a possibility to readily collect tissues for expanded analysis techniques, such as immunohistochemistry, PCR on bronchial tissue, and for morphological evaluation. Further studies with repeated sampling over time in controlled inhalation provocation studies and even in naturally occurring RAO cases can provide far greater information on the role of the endobronchial tissue in development and progression of RAO in horses.

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531 **Table 1.** Primers and probes designed from nucleotide sequence for this study. ^a from Ainsworth et al. 2003, ^b forward and reverse primers; from
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 533 177-185.

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<i>Gene</i>	<i>Forward primer 5' to 3'</i>	<i>Reverse primer 5' to 3'</i>	<i>Probe 5' to 3'</i>
GAPDH^a	AAG TGG ATA TTG TCG CCA TCA AT	AAC TTG CCA TGG GTG GAA TC	TGA CCT CAA CTA CAT GGT CTA CAT GTT TCA
IL-5	GAT GGG AAC CTG ATG ATT CCT ACT ^b	TCC CCT TGG ACA GTT TGA TTC T ^b	AA TCA CCA ACT CTG CAT TGA AGA AGT CTT TCA GGG A
IL-6	AGC AAG TGT GAA AAC AGC AAG	CAT CAG GCA GGT CTC CTG AT	CTG GCA GAA AAC AAC CTG AAT CTT CCA
IL-8	TTC CAA GCT GGC TGT TGC T	TGA TAC AAC CGC AGC TTC ACA	TT GGC CGT CTT CCT GCT TTC TGC A
IL-10	TTC AGC AGG GTG AAG ACT TTC	CTT GGC AAC CCA GGT AAC CCT TA	TGT TGA ACG GGT CCC TGC TGG AG
IL-17	ACT GTG AAG GCG GGA ATA GTA A	TCG TTT TCC GGT TAA GGA CG	AC AAG AAC TTC CCT CAG AAT GTG AAG ATC AA
TGF-β1	CCG GAG AGA GCG CTG AGA	ATA GAT TTC GTT TTC CTT TTC	CTACTACGCCAAGGAGGTCACCCGC

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Table 2. BAL neutrophil percentage and pulmonary function parameters in RAO horses during crisis versus remission and in controls on pasture.

	RAO in crisis	RAO in remission	Controls pasture
Neutrophil %	48.00 ± 13.31	11.00 ± 10.66*	9.60 ± 6.82
$\Delta P_{pl_{max}}$ (cmH ₂ O)	41.80 ± 17.27	9.70 ± 1.67* [†]	5.89 ± 1.87
R _L (cmH ₂ O/L/s)	2.96 ± 0.76	0.08 ± 0.02* [†]	0.44 ± 0.12
C _{dyn} (L/cmH ₂ O)	0.21 ± 1.16	1.34 ± 0.49 [†]	1.54 ± 0.47

$\Delta P_{pl_{max}}$, maximal change in pleural pressure; R_L, total pulmonary resistance; C_{dyn} dynamic compliance.

Data is expressed as median ± SD. *Significantly different values from provocation ($p < 0.05$). [†]Significantly different values from control horses at pasture ($p < 0.05$).

Legends to the figures

Figures 1a and 1b.

The comparative (fold) expression of cytokine mRNA expression in BAL cells (1a) and in endobronchial biopsies (1b) during respiratory crisis compared to remission. Expression of genes of interest was normalized to GAPDH mRNA level. The results are shown with interquartile ranges and the horizontal lines within the bars representing medians. The value 1 on the y axis represents the theoretical point where crisis and remission are identical ($\Delta\Delta C_T = 0$. $2^{\Delta\Delta C_T} = 2^0 = 1$). *= significant difference ($p < 0.05$) in comparative (fold) expression of cytokine mRNA.

Figure 2.

Correlation of IL-6 to IL-8mRNA expression in BAL cells from RAO horses during respiratory crisis ($r_s = 0.971$, $p = 0.001$, Spearman Ranked).

Figure 1.a

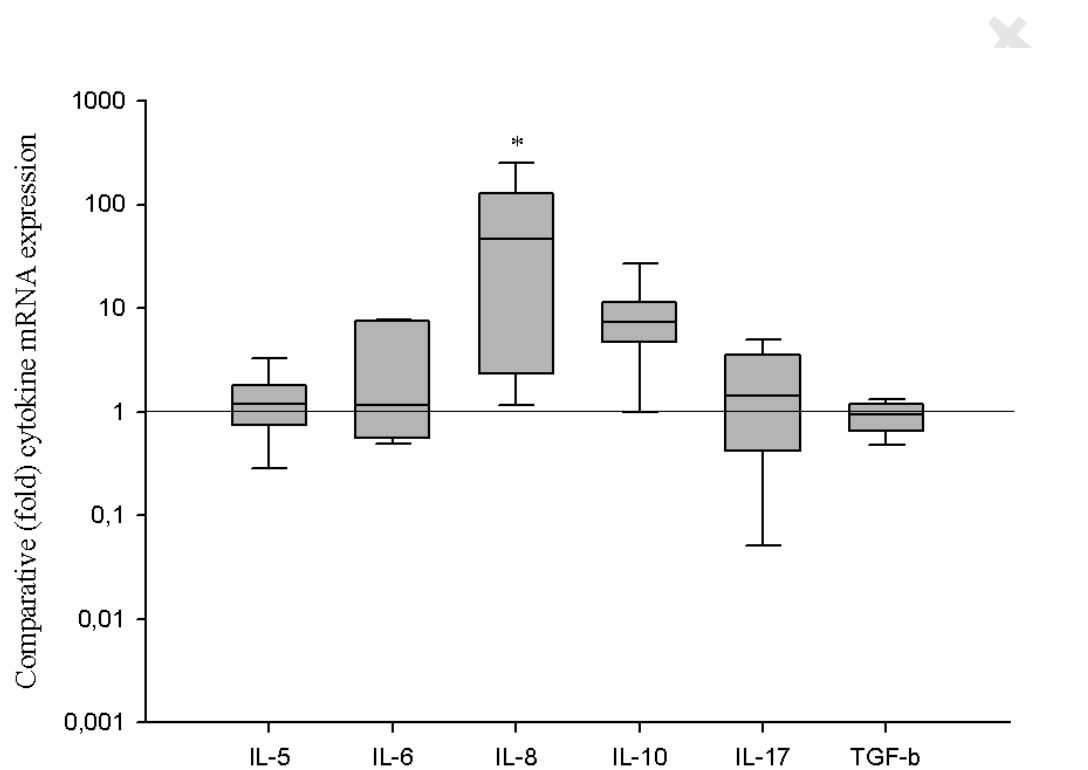


Figure 1b.

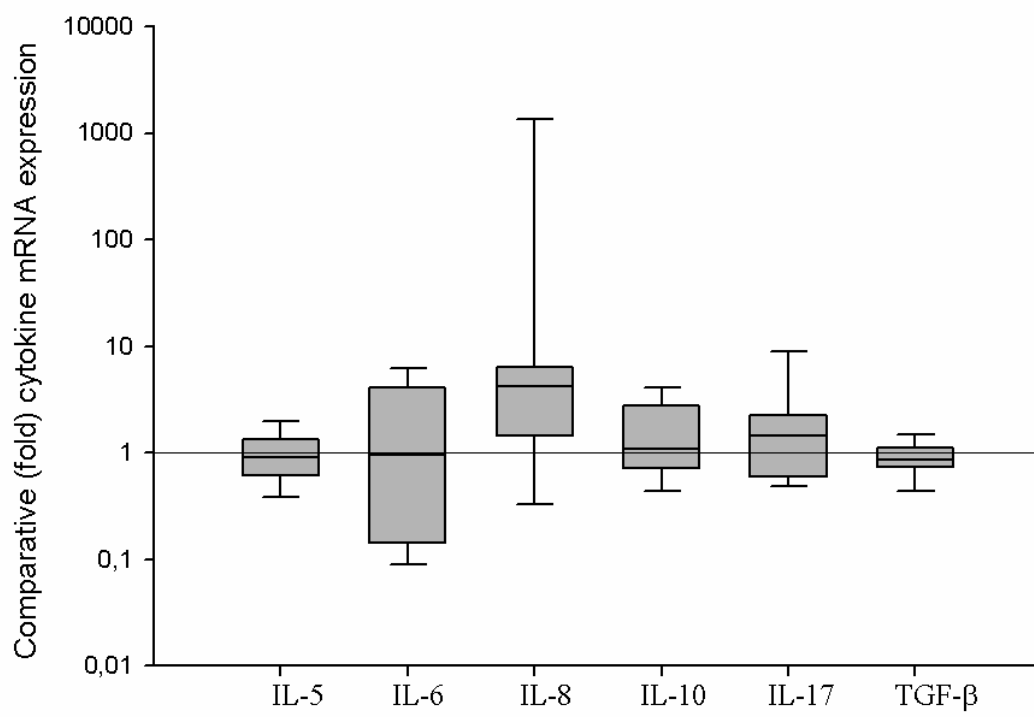


Figure 2

