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*Original article*

# Evaluation of serum cytokine levels in recurrent airway obstruction

**A. Niedźwiedź[1], H. Borowicz[1], K. Kubiak[1], J. Nicpoń[2], P. Skrzypczak[2], Z. Jaworski[3], M. Cegielski[4], J. Nicpoń[1]**

[1] Department of Internal Diseases with Clinic for Horses, Dogs and Cats, Faculty of Veterinary Medicine, Wrocław University of Environmental and Life Sciences. Pl. Grunwaldzki 47, 50-366 Wrocław, Poland
[2] Department of Surgery, Faculty of Veterinary Medicine, Wrocław University of Environmental and Life Sciences Pl. Grunwaldzki 47, 50-366 Wrocław, Poland
[3] Department of Horse Breeding and Riding, University of Warmia and Mazury, Prawocheńskiego 2, 10-720 Olsztyn, Poland
[4] Department of Histology and Embryology, Medical University of Wroclaw, Chalubinskiego 6a, 50-368 Wroclaw, Poland

## Abstract

Recurrent airway obstruction (RAO) represents a serious health problem and is traditionally classified as an allergic disease, where contact with an antigen can induce clinical airway inflammation, bronchial hyper-responsiveness and reversible airway obstruction. Previous studies have demonstrated the presence of the Th2 response in the lungs of human patients with asthma and horses with heaves. These cells are involved in the production of cytokines which regulate the synthesis of immunoglobulins. 40 horses were evaluated: 30 horses with RAO and 10 healthy animals. The expression levels of interferon-alpha 1 (IFN-$\alpha_1$), interferon-gamma (IFN-$\gamma$), interleukin-1$\beta$, (IL-1$\beta$), IL-2, IL-4, IL-13 and tumor necrosis factor alpha (TNF-$\alpha$) were measured in the serum obtained from control and RAO-susceptible horses during crisis. In all the patients, serum cytokine levels were detected. Serum median IL-13 and IFN-$\gamma$ levels were significantly higher in RAO-affected horses than in the healthy group ($p < 0.001$). The serum median IFN-$\alpha_1$, IL-1$\beta$, IL-2, IL-4, and TNF-$\alpha$ levels were similar in both groups. These results indicate a low variability of the levels of cytokines and a high frequency of their detection in serum samples from horses with RAO. Immune mechanisms involved in equine RAO are more complex than those defined by a simple Th1/Th2 dichotomy.

**Key words**: heaves, horse, immune system, interleukins

## Introduction

Recurrent airway obstruction (RAO), formerly known as heaves, is an allergic respiratory condition that develops in aged horses following exposure to mouldy hay and straw. This is manifested by airway hyperreactivity, inflammation, bronchoconstriction, and neutrophil influx into the airways (Niedźwiedź 2014, Couëtil et al. 2016). The chronic, untreated disease may lead to the remodeling of the airway wall.

Correspondence to: A. Niedźwiedź, e-mail: artur.niedzwiedz@up.wroc.pl

Clinical remission of this disease is induced by reducing the exposure of the animal to the antigen or by administering corticosteroids, such as dexamethasone or beclomethasone, and the use of bronchodilators (Leguillette 2003, Gerber et al. 2015).

This condition shares many characteristic features with human asthma. It has been suggested that expression of RAO is not only influenced by environmental factors (mouldy hay) but also by a genetic predisposition (Gerber et al. 2015). Moreover, it a similar immunological background is suspected, suggesting that some pathophysiological mechanisms, both in RAO and human asthma, are common (Pacholewska et al. 2015).

Previous studies performed on horses in acute crisis reported an increased IL-8 and interferon (IFN)-γ gene expression in BAL cells, without simultaneous increase in the expression of the Th2 cytokines, such as IL-4 and IL-13 (Ainsworth et al. 2003). In other studies, authors found the expression of IL1β, IL8, TLR4, TNFα, TGFβ1 and NFkβ transcripts to be significantly up-regulated in RAO-affected horses compared to healthy horses (Padoan et al. 2013). However, other studies reported a modified Type 2 cytokine response characterized by the production of IL-4 and IL-13 mRNA, but not IL-5 mRNA in BAL cells (Horohov et al. 2005).

The mechanism by which airway inflammation develops in RAO affected horses appears to be multi-factorial and dynamic. Hence, more information is required to better define the sequence of events that result in the development of the airway inflammation (Couëtil et al. 2016). The purpose of this study was to determine whether alterations in the peripheral blood cytokine profile are apparent within 48 h following a natural challenge exposure.

## Materials and Methods

This study was conducted with the approval of the 2nd Local Ethics Committee responsible for Animal Experimentation in Wroclaw (resolution No. 65/2014).

### Horses

The study population consisted of 40 horses and included 1 male, 7 castrated males (geldings) and 32 mares. 30 horses had chronic naturally-induced RAO and 10 were healthy controls. The animals were divided into two groups – group I was the study group (n=30) consisting of RAO affected-horses, while group II was the control group, (n=10) containing healthy horses with no evidence of respiratory tract disease. The total study population included 26 Polish half bred horses and 14 Polish Konik horses. The age of the horses ranged from 4 to 19 years. The Polish Konik horses were owned by the Polish Academy of Sciences Research Station for Ecological Agriculture and Preservation of Animal Breeding in Popielno, while the remaining horses were privately owned.

### Experimental protocol

Prior to the study, all the horses were kept on a pasture or in a stable with wood shavings as bedding (Allspan, Allspan GmbH, Karlsruhe, Germany). They were fed with a complete horse feed (EMH Heu Cubes, Eggersmann, Rinteln, Germany) with an addition of muesli (EMH Krauter Müsli, Eggersmann, Rinteln, Germany). An acute crisis of RAO was induced by placing the horses in a poorly ventilated stable, bedding them on straw and feeding them hay with a visible mold growth for 48 hours prior to the examination. The response to the environmental exposure was confirmed based on a clinical RAO score and additional testing, which included an endoscopic examination, BALF cytology, arterial blood gas analysis, and venous blood sampling (Wysocka and Kluciński 2015). Venous blood was obtained to rule out evidence of pulmonary infection, based on a blood count and acute phase protein concentration. A score of less than 10% of neutrophils in BALF was required for healthy horses, while > 50% neutrophils in a differential cell count following the environmental exposure was required to define RAO-affected horses as being in crisis (Cian et al. 2015).

### Endoscopic examination and bronchoalveolar lavage fluid collection and cytology

Endoscopy of the airways and bronchoalveolar lavage was performed on horses sedated with 0.01 mg/kg of detomidine (Domosedan, Orion Corporation, Espoo, Finland) and 0.01 mg/kg of butorphanol (Morphasol, aniMedica GmBH, Senden-Bösensell, Germany). A 1.8 m long endoscope was passed through the nasal passage into the trachea (Karl Storz GmbH, Tuttlingen, Germany). Changes in the airways were graded by two clinicians using a modified RAO staging scale, previously described by Tilley et al. (2012). Bronchoalveolar lavage was performed by instilling 300-400 ml of sterile saline (0.9% NaCl) at body temperature through the endoscope working channel into the bronchus using successive 60 ml boluses. BALF (bronchoalveolar lavage fluid) was

Table 1. Results of clinical assessment, BAL fluid cytology, blood gas analysis and acute phase proteins in healthy horses and RAO-affected horses. Values are expressed as median and 25th and 75th percentiles.

| | Control | | RAO-affected | |
|---|---|---|---|---|
| | median | 25th and 75th percentiles | median | 25th and 75th percentiles |
| Clinical score[a] | 1 | 0-2 | 6 | 5-6 |
| $pO_2$ (mmHg)[a] | 99 | 92.25-106 | 85.5 | 81-90.25 |
| $pCO_2$ (mmHg)[b] | 44.5 | 43-45.75 | 44 | 42-45.25 |
| BALF Neutrophils (%)[a] | 5.1 | 4.3-5.6 | 70.8 | 66.33-79.5 |
| BALF Lymphocytes (%)[a] | 35.5 | 33.85-37.83 | 15.9 | 10.18-19.18 |
| BALF Macrophages (%)[a] | 59.1 | 57.15-60.67 | 11.25 | 7.675-14.18 |
| BALF Eosinophils (%)[b] | 0.4 | 0.3-0.475 | 0 | 0-0 |
| BALF Mast cells (%)[b] | 0.15 | 0.025-0.3 | 0 | 0-0 |
| Fibrinogen (g/l)[b] | 1.83 | 1.612-2.228 | 2.39 | 1.805-4.532 |
| Serum Amyloid A (µg/ml)[b] | 15.1 | 4.492-24.81 | 20.95 | 10.02-38.58 |

[a] Differences statistically significant ($p<0.05$); [b] NS

Table 2. Results peripheral blood cytokine levels in healthy horses and RAO-affected horses. Values are expressed as median and 25th and 75th percentiles.

| | Control | | RAO-affected | |
|---|---|---|---|---|
| | median | 25th and 75th percentiles | median | 25th and 75th percentiles |
| IFN-$\alpha_1$ [pg/ml][b] | 18.10 | 15.10-19.3 | 14.30 | 13.15-21.05 |
| IL-2 [pg/ml][b] | 20.30 | 19.80-24.25 | 25.6 | 21.15-36.1 |
| IL-4 [pg/ml][b] | 34.0 | 32.65-57.5 | 37.45 | 30.6-97.7 |
| TNF-α [pg/ml][b] | 15.90 | 11.60-17.05 | 10.05 | 8.45-14.95 |
| IFN-γ [pg/ml][a] | 36.4 | 34.70-38.95 | 78.9 | 44.13-122.18 |
| IL-13 [pg/ml][a] | 10.20 | 8.70-16.15 | 65.55 | 39.42-142.0 |
| IL-1β [pg/ml][b] | 72.70 | 65.95-85.60 | 90.5 | 72.9-107.05 |

[a] Differences statistically significant ($p<0.05$); [b] NS

then re-aspirated through gentle suction using a 60 ml syringe until no further fluid was obtained. The amount of recovered fluid was recorded. The BALF for each individual horse was pooled in a sterile specimen cup, placed on ice and processed within 2h after collection. In order to carry out the cytologic examination of the BALF, a 10 mL aliquot was centrifuged at 300g for 10 minutes using a centrifuge (Beckman Coulter Allegra x-22; Beckman Coulter Inc., CA, Brea, USA), and the smear of the sediment was stained with Wright's stain. A 400- leukocyte differential count was performed; epithelial cells were not included in the differential count (Fernandez et al. 2013).

## Arterial blood gas analysis

Arterial blood was collected anaerobically into heparinized syringes through an arterial puncture of the facial artery using an 18G butterfly needle. The blood was immediately analyzed for the partial pressure of oxygen and carbon dioxide ($PaO_2$ and $PaCO_2$) with the use of an OPTI CCA-TS (OPTI Medical Systems, Inc., Roswell, GA, USA) blood gas analyzer. Horses were identified as healthy if their $PaO_2 \geq 90$ mmHg, while those with a $PaO2 \leq 85$ mmHg were considered to have a relapse of RAO (Stopyra et al. 2012).

## Cytokine assays

Cytokine concentrations in the serum samples were measured using an enzyme-linked immunosorbent assay (ELISA) using commercially available IFN-$\alpha_1$, interferon-gamma (IFN-γ), IL-1β, IL-2, IL-4, IL-13 and TNF-α (R&D Systems, Inc., Minneapolis, MN, USA) Quantikine sets for horses according to the manufacturer's instructions. Both intra- and inter-assay coefficients of variation were below 10%. Because of the log-normal distribution of the studied parameters, the geometric mean of concentrations (GMTs) accompanied by 95% confidence intervals (CI) was calculated for each cytokine.

### Statistical analysis

The data normality was assessed using the Shapiro-Wilk test and graphical assessment (histogram and Q-Q plot). Nonparametric statistics were used because of the lack of data normality or an ordinal nature of data. Data were described using the median and quartile range. A comparison of quantitative variables between groups was carried out using the Kruskall-Wallis test. Analysis was performed using R for Windows (version 3.2.1).

## Results

The results of the BALF analysis, clinical assessment and arterial blood gasometry are shown in Table 1. The median BALF recovery was 53.7% in the control horses and 42.5% in RAO-affected horses. Calculations, using the Kruskall-Wallis test, demonstrated a significant effect of a dusty environment on the percentage of BALF cell populations in horses with RAO compared to control animals.

The summarized results of the levels of serum cytokines are shown in Table 2. A hay/straw challenge had no statistically significant effect on the levels of IFN-$\alpha_1$, IL-1β, IL-2, IL-4 and TNF-α. There were significant inter-group differences in the levels of IFN-γ and IL-13.

The median level of IL-13 in the control horses was 10.2 pg/ml (8.7 and 16.15), and there was a statistical increase in the level of IL-13 in the RAO-affected horses (Fig. 1). The median level of IL-13 in the study group was 65.55 pg/ml (39.42 and 142.0) ($p< 0.0001$).

The median (1st and 3rd quartile) level of IFN-γ in the control horses was 36.4 pg/ml (34.7 and 38.95) (Fig. 2). In RAO-affected horses, the median IFN-γ level was statistically different, and was 78.9 (44.13 and 122.18), respectively ($p< 0.0001$).

## Discussion

A hay and straw challenge in horses prompts airway obstruction, airway neutrophilia and an increased respiratory effort in RAO-affected horses, whereas there was no impact on these variables in healthy horses. Although the results obtained from horses with RAO are consistent with our findings and the previous observations of other researchers, a hay/straw exposure had no significant effect on the BALF cytology in the control horses (Pirie et al. 2001, Niedzwiedz and Jaworski 2014, Niedzwiedz et al. 2014). Our previous studies revealed changes in the BALF percentage of healthy horses after exposure to aeroallergens. The difference may primarily arise from different sizes of research groups, and the time of year in which the studies were performed.

In recent years, research on the pathophysiology of recurrent airway obstruction focused on the assessment of mRNA expression profiles for inflammatory mediators mainly in the bronchoalveolar lavage fluid. Researchers (Gigumre et al. 2002, Laan et al. 2006) have already investigated the mRNA expression of IL-1β, IL-6, IL-8, IL-13, IL-17, TNFα, INFγ, TGF-1β and TLR4. However, little is known about the systemic alterations in the cytokine profile in the serum of horses with symptomatic RAO. Therefore, we aimed to determine the levels of cytokines in the serum of RAO-affected and control horses after 48 h of natural antigen exposure .

We found that affected horses exhibited increased serum levels of IL-13 and IFN-γ in the serum compared with healthy control horses, whereas there was no difference in levels of IFN-$\alpha_1$, IL-1β, IL-2, IL-4, and TNF-α between groups.

It has been hypothesized that $CD4^+$ T cells which produce a T-helper 2 cell (Th2) pattern of cytokines, including IL-4, IL-5, and IL-13, play a pivotal role in the pathogenesis of RAO in horses (Lavoie et al. 2001, Cordeau et al. 2004, Horohov 2005). It is not surprising that the affected horses had elevated IL-13 in the serum as this cytokine plays a central role in regulating the production of IgE (Wills-Karp et al. 1998). These results are consistent with other reports indicating that allergen-specific IgE antibodies play a central role in equine RAO (Horohov et al. 2005). On the other hand, our studies are significantly different from the study of Anisworth et al. (2003), Kleiber et al. (2005) and Padoan et al. (2013), who found no differences between mRNA expression of IL-13 in the cells of the bronchoalveolar lavage fluid and BALF. The difference in the expression of IL-13 may result from a different phase of the disease. Our study, as well as the study by Horohov et al. (2005), investigated horses in an acute stage of the disease, rather than in the chronic phase investigated by other researchers.

The other inflammatory mediator which was upregulated in horses with RAO was IFN-γ. The synthesis of this factor was also upregulated in the airway secretions obtained from humans with asthma and horses with heaves (Franchini et al. 1998, Ainsworth et al. 2003, Tsoumakidou et al. 2004). It has been hypothesized that a higher level of IFN-γ may be a marker of an ongoing inflammatory process which is more pronounced in patients with a chronic form of the disease. However, the horses included in our research remained in remission prior to the antigen

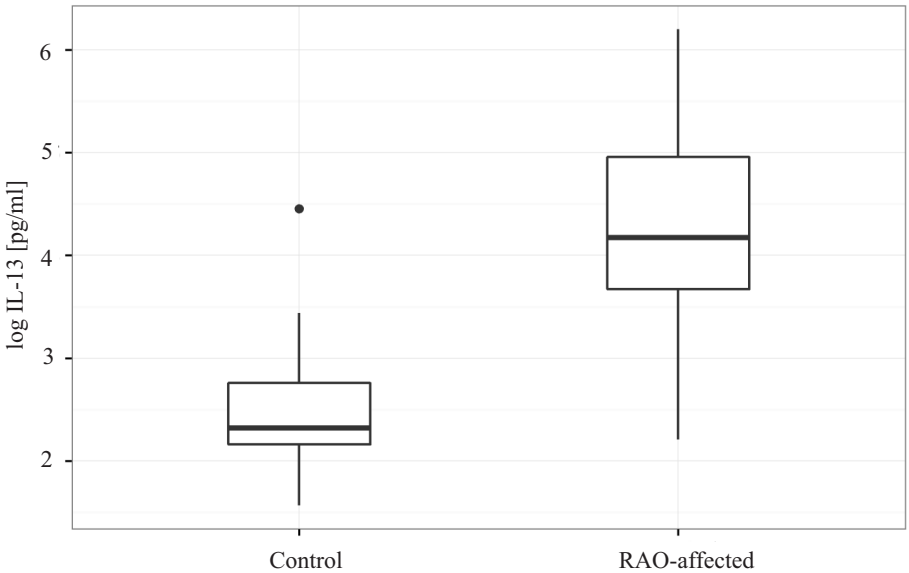


Fig. 1. Box and whiskers plot of the median serum IL-13 levels in RAO-affected horses and controls. The line in the center of each box represents the median, top and bottom boundaries of the box represent the 25th and 75th percentile and the whiskers indicate maximum and minimum values.

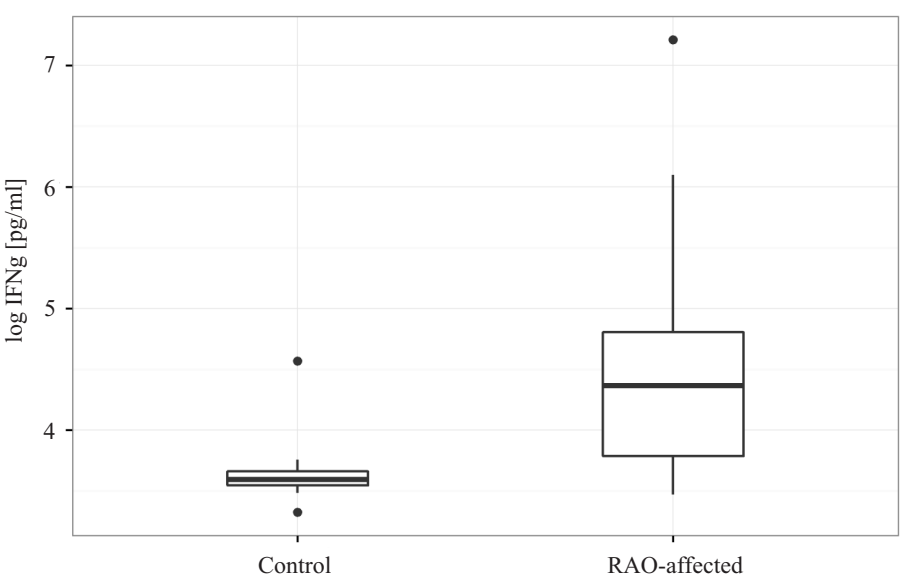


Fig. 2. Box and whiskers plot of the median serum IFN-γ levels in RAO-affected horses and controls. The line in the center of each box represents the median, top and bottom boundaries of the box represent the 25th and 75th percentile and the whiskers indicate maximum and minimum values.

exposure, which was determined based on their medical history and clinical examination. Following the hay/straw challenge, they went into an acute crisis and remained in crisis at the time of sampling. The higher serum levels of IFN-γ in the RAO-affected horses, with simultaneous lower levels of IL-4, apparently do not support the hypothesis that Th2 lymphocytes are upregulated and Th1 lymphocytes suppressed in an equine model of asthma (Ainsworth et al. 2003). We hypothesize that the Th1/Th2-imbalance may be associated with the development of an IgE-mediated inflammation. Once RAO is present, other factors may be involved in the modulation of the severity of lower airway hypersensitivity and inflammation. Furthermore, it should be considered that serum cytokine levels may not specifically represent the secretion of Th1 or Th2 lymphocytes by the peripheral blood (ten Hacken et al. 1998).

There was no detectable alteration in the levels of the other assessed cytokines, which are involved in the

pathophysiology of RAO, according to the literature. The most surprising finding was the lack of changes in the levels of IL-4, which is a key cytokine in the development of airway inflammation. However, some reports suggest that an increased IL-4 cytokine response is not necessarily a reflection of asthma activity in humans (Moore et al. 2001).

Also, the lack of difference in the levels of pro-inflammatory cytokines such as IFN-$\alpha_1$, IL-1β, IL-2, and TNF-α is in contrast with the findings reported by Laan et al. (2006), and Padoan et al. (2013), who suggested their secondary role in disease development and immune response activation in horses with RAO.

The results of our research indicate that IFN-γ and IL-13 play a dominant role in the inflammatory process and in the exacerbation of the equine recurrent airway obstruction. Although the experimental design, sample size, and laboratory techniques may result in inconsistencies between studies, the differences may indicate that the immune mechanisms involved in equine RAO are more complex than those defined by a simple Th1/Th2 dichotomy.

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## References

Ainsworth DM, Grünig G, Matychak MB, Young J, Wagner B, Erb HN, Antczak DF (**2003**) Recurrent airway obstruction (RAO) in horses is characterized by IFN-gamma and IL-8 production in bronchoalveolar lavage cells. Vet Immunol Immunopathol 96: 83-91.

Cian F, Monti P, Durham A (**2015**) Cytology of the lower respiratory tract in horses: An updated review. Equine Vet Educ 27: 544-553.

Cordeau ME, Joubert P, Dewachi O, Hamid Q, Lavoie JP (**2004**) IL-4, IL-5 and IFN-gamma mRNA expression in pulmonary lymphocytes in equine heaves. Vet Immunol Immunopathol 97: 87-96.

Couëtil LL, Cardwell JM, Gerber V, Lavoie JP, Léguillette R, Richard EA (**2016**) Inflammatory Airway Disease of Horses-Revised Consensus Statement. J Vet Intern Med 30: 503-515.

Fernandez NJ, Hecker KG, Gilroy CV, Warren AL, Leguillette R (**2013**) Reliability of 400-cell and 5-field leukocyte differential counts for equine bronchoalveolar lavage fluid. Vet Clin Pathol 42: 92-98.

Franchini M, Gilli U, Akens MK, Fellenberg RV, Bracher V (**1998**) The role of neutrophil chemotactic cytokines in the pathogenesis of equine chronic obstructive pulmonary disease (COPD). Vet Immunol Immunopathol 66: 53-65.

Gerber V, Tessier C, Marti E (**2015**) Genetics of upper and lower airway diseases in the horse. Equine Vet J 47: 390-397.

Giguère S, Viel L, Lee E, MacKay RJ, Hernandez J, Franchini M (**2002**) Cytokine induction in pulmonary airways of horses with heaves and effect of therapy with inhaled fluticasone propionate. Vet Immunol Immunopathol 85: 147-158.

Horohov DW, Beadle RE, Mouch S, Pourciau SS (**2005**) Temporal regulation of cytokine mRNA expression in equine recurrent airway obstruction. Vet Immunol Immunopathol 108: 237-245.

Kleiber C, McGorum BC, Horohov DW, Pirie RS, Zurbriggen A, Straub R (**2005**) Cytokine profiles of peripheral blood and airway CD4 and CD8 T lymphocytes in horses with recurrent airway obstruction. Vet Immunol Immunopathol 104: 91-97.

Laan TT, Bull S, Pirie R, Fink-Gremmels J (**2006**) The role of alveolar macrophages in the pathogenesis of recurrent airway obstruction in horses. J Vet Intern Med 20: 167-174.

Lavoie JP, Horohov DW (**2001**) Cytokine immunoregulatory elements in RAO. Equine Immunology. R&W Publications, Newmarket, pp. 73-82.

Leguillette R (**2003**) Recurrent airway obstruction--heaves. Vet Clin North Am Equine Pract 19: 63-86.

Moore WC, Hasday JD, Meltzer SS, Wisnewski PL, White B, Bleecker ER (**2001**) Subjects with mild and moderate asthma respond to segmental allergen challenge with similar, reproducible, allergen-specific inflammation. J Allergy Clin Immunol 108: 908-914.

Niedzwiedz A, Jaworski Z (**2014**) Oxidant-antioxidant status in the blood of horses with symptomatic recurrent airway obstruction (RAO). J Vet Intern Med 28: 1845-1852.

Niedzwiedz A, Jaworski Z, Tykalowski B, Smialek M (**2014**) Neutrophil and macrophage apoptosis in bronchoalveolar lavage fluid from healthy horses and horses with recurrent airway obstruction (RAO). BMC Vet Res 10: 29

Niedźwiedź A (**2014**) Equine recurrent airway obstruction. Mac Vet Rev 37: 115-120.

Pacholewska A, Jagannathan V, Drögemüller M, Klukowska-Rötzler J, Lanz S, Hamza E, Dermitzakis ET, Marti E, Leeb T, Gerber V (**2015**) Impaired cell cycle regulation in a natural equine model of asthma. PloS One 10: e0136103.

Padoan E, Ferraresso S, Pegolo S, Castagnaro M, Barnini C, Bargelloni L (**2013**) Real time RT-PCR analysis of inflammatory mediator expression in recurrent airway obstruction-affected horses. Vet Immunol Immunopathol 156: 190-199.

Pirie RS, Dixon, PM, Collie DD, McGorum BC (**2001**) Pulmonary and systemic effects of inhaled endotoxin in control and heaves horses. Equine Vet J 33: 311-318.

Stopyra A, Sobiech P, Wacławska-Matyjasik A (**2012**) Acid-base indicators in the venous and arterial blood of horses affected by recurrent airway obstruction (RAO). Pol J Vet Sci 15: 463-467.

ten Hacken NH, Oosterhoff Y, Kauffman HF, Guevarra L, Satoh T, Tollerud DJ, Postma DS (**1998**) Elevated serum interferon-gamma in atopic asthma correlates with increased airways responsiveness and circadian peak expiratory flow variation. Eur Respir J 11: 312-316.

Tilley P, Sales Luis JP, Branco Ferreira M (**2012**) Correlation and discriminant analysis between clinical, endoscopic, thoracic X-ray and bronchoalveolar lavage fluid cytology scores, for staging horses with recurrent airway obstruction (RAO). Res Vet Sci 93: 1006-1014.

Tsoumakidou M, Tzanakis N, Kyriakou D, Chrysofakis G, Siafakas NM (**2004**) Inflammatory cell profiles and T-lymphocyte subsets in chronic obstructive pulmonary disease and severe persistent asthma. Clin Exp Allergy 34: 234-240.

Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, Donaldson DD (**1998**) Interleukin-13: central mediator of allergic asthma. Science 282: 2258-2261.

Wysocka B, Kluciński W (**2015**) Cytological evaluation of tracheal aspirate and broncho-alveolar lavage fluid in comparison to endoscopic assessment of lower airways in horses with recurrent airways obstruction or inflammatory airway disease. Pol J Vet Sci 18: 587-597.