

## Serum Surfactant Protein D and Haptoglobin as Potential Biomarkers for Inflammatory Airway Disease in Horses

M. Bullone\*, M. de Lagarde\*, A. Vargas, and J.-P. Lavoie

**Background:** The identification of serum biomarkers of lung inflammation would facilitate the diagnosis of inflammatory airway disease (IAD) in horses.

**Hypothesis:** Horses with IAD have higher serum concentrations of markers of inflammation compared to controls.

**Animals:** Twelve horses with IAD and 10 control horses.

**Methods:** This was a prospective case-control study. Blood and BALF were collected from horses with IAD and controls. Serum concentration of surfactant protein D (SP-D), haptoglobin, serum amyloid A (SAA) and of the soluble form of triggering receptor expressed on myeloid cells 1 (sTREM-1) was measured using commercial ELISA tests.

**Results:** Horses with IAD had higher serum concentration (log-transformed values) of SP-D (mean  $\pm$  SD:  $1.773 \pm 0.51$ ), haptoglobin ( $6.657 \pm 0.202$ ) and SAA ( $0.128 \pm 0.396$ ) compared to controls ( $0.942 \pm 0.226$ ,  $6.38 \pm 0.22$ ,  $-0.398 \pm 0.319$ , respectively;  $P < .01$  for all). Furthermore, the concentrations of SP-D and haptoglobin combined allowed differentiating the 2 groups (IAD:  $8.43 \pm 0.564$ , controls:  $7.322 \pm 0.249$ ,  $P < .0001$ ) with a sensitivity and specificity of 100% when a cut-off of 7.70 (log value) was employed.

**Conclusions and Clinical Importance:** Surfactant protein D and haptoglobin serum concentrations could be a diagnostic aid in IAD. Further studies are necessary to establish the specificity of our findings before they can be applied in everyday practice.

**Key words:** Acute phase proteins; Biomarker; Lung; sTREM-1.

Inflammatory Airway Disease (IAD) includes clinical entities characterized by cough, decreased performance and delayed recovery after exercise, with lower airway inflammation detected at bronchoalveolar lavage fluid (BALF) cytology.<sup>1</sup> As the bronchoalveolar lavage is a procedure not routinely performed in the field, IAD remains underdiagnosed. The identification of serum biomarkers for lung inflammation would facilitate disease recognition.

Biomarkers are “substances, structures or processes that can be measured in the body or its products which influence or predict the incidence of an outcome or disease”.<sup>2</sup> Biomarkers of lung inflammation have been studied, but few are pathognomonic for a given condition or specific for single organs. Both specific (surfactant protein D, SP-D) and nonspecific (acute phase proteins) inflammatory biomarkers are elevated in blood of horses suffering from lower airway inflammatory diseases (heaves and IAD). In heaves, circulating

### Abbreviations:

BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
ELISA	enzyme-linked immunosorbent assay
IAD	inflammatory airway disease
SAA	serum amyloid A
SP-D	surfactant protein D
TREM-1	triggering receptor expressed on myeloid cells 1
sTREM-1	soluble form of triggering receptor expressed on myeloid cells 1

haptoglobin concentrations are increased compared to controls, with no overlapping measures between groups.<sup>3</sup> There is a significant increase in serum SP-D concentrations in horses with IAD.<sup>4</sup>

We hypothesized that the protein expression of 4 biomarkers of pulmonary (SP-D) and nonspecific inflammation haptoglobin, serum amyloid A, SAA and the soluble form of triggering receptor expressed on myeloid cells 1, sTREM-1, is higher in the serum of horses with IAD. We aimed at investigating the concentrations of these biomarkers in the serum of horses with IAD and controls, and at studying whether their distribution pattern is associated with the inflammatory phenotype detected at BALF cytology.

### Material and Methods

#### Animals

Horses with IAD were selected among the cases referred to the equine hospital of the Université de Montréal for respiratory problems between September 2011 and 2013. The study was performed in accordance with the guidelines of the Canadian Council on Animal Care and the protocol approved by the Ethics Committee of the Université de Montréal (#Rech-1647). Horses with IAD were included in the study if they presented clinical signs of cough, nasal discharge or increased breathing effort during/after exercise, together with increased neutrophil (>5%), mast cell (>2%) or

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Submitted April 1, 2015; Revised June 8, 2015; Accepted July 22, 2015.

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DOI: 10.1111/jvim.13602

eosinophil (>1%) percentage at the BALF cytology.<sup>1</sup> Control horses were either selected among horses referred to the hospital for nonrespiratory elective procedures and did not have signs of diseases or inflammation detected at BALF cytology ( $n = 5$ ), or they were owned by the Université de Montréal ( $n = 5$ ). Exclusion criteria for both groups of horses were the presence of systemic inflammatory or infectious processes based on clinical examination or results. Racehorses in training/racing were excluded, as were horses with a history of increased respiratory effort at rest, or any drug administration within the 15 days before admission. All the client-owned horses were transported to the hospital on the day of examination. The horses owned by the Université de Montréal were not trailered before sampling. All horses underwent complete clinical and endoscopic examination of the respiratory system. All owners signed an informed consent form to store samples for research purposes.

### **Bronchoalveolar Lavage**

Bronchoalveolar lavage procedure was performed as previously described.<sup>5</sup> Cytopreparations from unfiltered BALF<sup>a</sup> with Protocol Hema 3<sup>b</sup> for differential counting of 400 cells.

### **Blood Collection**

Blood was collected from the jugular veins by means of 18G needles into sterile tubes. Ninety millilitres of blood were collected from each horse. Within 2 hours from collection, plasma and serum were harvested from the sample and 1.5 mL aliquots were stored at -80°C until used. Serum samples were used for ELISA.

### **Protein Expression Studies**

Surfactant protein D, SAA, haptoglobin and sTREM-1 were quantified in serum samples using commercially available ELISA kits. The cross-reactivity of the antibody used in the Surfactant Protein D human Elisa kit<sup>c</sup> with the equine SP-D was validated by the Western blot technique. The Phase Serum Amyloid A Multispecies Elisa,<sup>d</sup> the Equine Haptoglobin Elisa,<sup>d</sup> and horse TREM-1 Elisa<sup>e</sup> were previously validated by the manufacturer and used accordingly to the instructions. Plates were washed using an automatic plate washer<sup>f</sup> and absorbance was obtained using a microplate reader.<sup>g</sup> The assay standard curves ranged from 1.56 to 100 ng/mL for SP-D (serum dilution 1:10), 9.375 to 600 ng/mL for haptoglobin (serum dilution 1:32000), 1.25–20 ng/mL for SAA (serum dilution 1:2000) and 62.5 pg/mL–2000 pg/mL for sTREM.

When SAA concentrations exceeded the assay quantification range, there were attributed the value 3.30 µg/mL (higher limit of the linear part of the curve).

### **Statistics**

Statistical analyses were performed with Prism 6.<sup>h</sup> All data were analysed after Log<sub>10</sub> transformation to reduce intergroup variability. Normal distribution of data within each group was assessed with Kolmogorov-Smirnov tests. Student t-tests or one-way ANOVA with Tukey's post-tests were used to compare the variables studied in different groups. Pearson tests were used for detecting correlations between serum biomarker concentrations and other variables assessed in serum or BALF in horses with IAD.

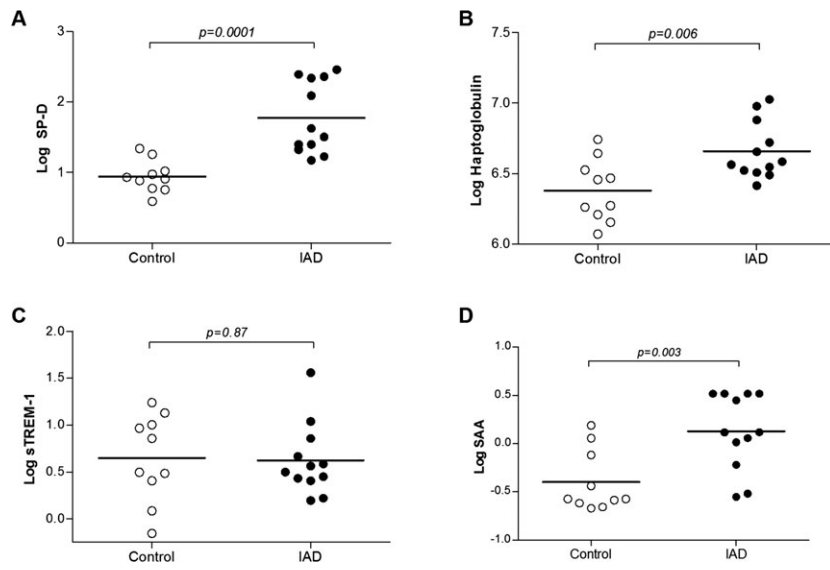
### **Results**

Twelve horses with IAD and 10 controls were included. The IAD group consisted of 4 Warmbloods, 5

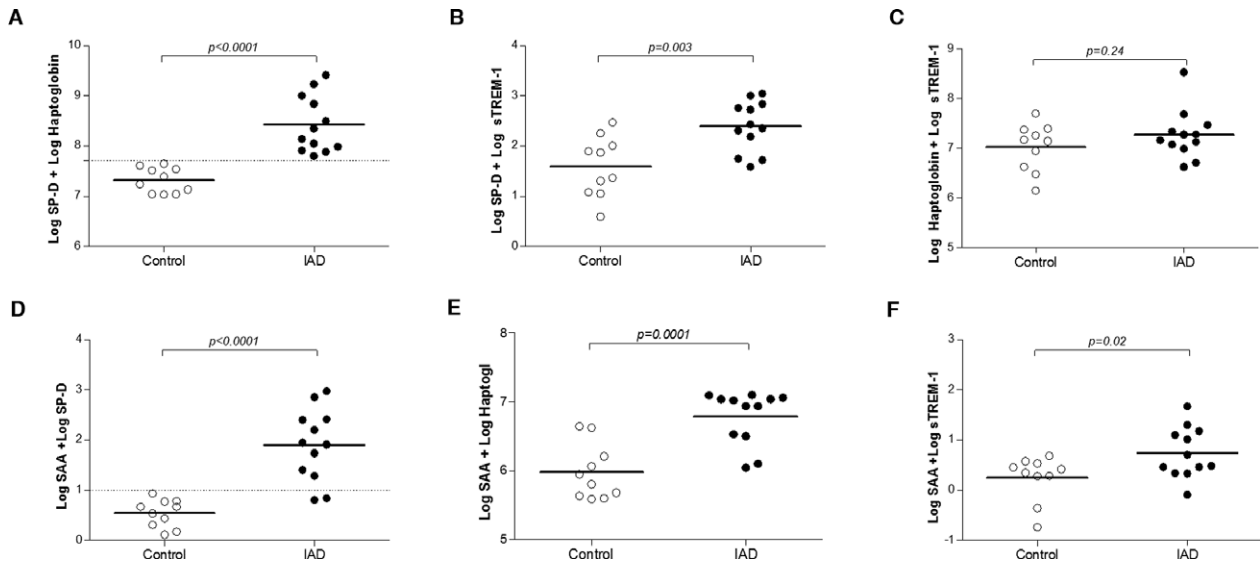
Quarter-Horses and associated breeds, and 3 Draft horses. Their average age was 6 years old (range 2–14). There were 4 females, 6 geldings and 2 males. Horses were performing light to moderate work. Disease duration was less than 2 years for most of them except one (4-year duration). They were fed hay while being stabled (8/12) or kept in paddock/pastures (4/12). The control group consisted of 2 Draft horses, 1 Haflinger, 2 Quarter-horses and 5 Standardbreds that were part of the University's herd. Their average age was 6 years old (range 2–9); there were 5 females, 4 geldings and 1 male. All control horses were performing light work, and were stabled and fed hay.

All IAD horses had increased BALF neutrophil percentage (range 6–53%), 7 horses also had BALF mastocytosis (range 3–9%), 1 horse had BALF eosinophilia (3%) and 1 horse had both BALF mastocytosis (3%) and eosinophilia (3%). Control horses had normal BALF neutrophil (range 1–3.5%) and mast cell percentage (<2%). Blood neutrophil ( $P = .3$ ), leucocyte ( $P = .4$ ), and fibrinogen ( $P = .6$ ) concentrations were similar between the 2 groups of horses.

The concentrations of SP-D ( $P = .0001$ ), haptoglobin ( $P = .006$ ) and SAA ( $P = .003$ ), but not sTREM-1 ( $P = .87$ ), were significantly increased in the sera of horses with IAD (Fig 1). Data overlap between the 2 groups was minimal for serum SP-D concentrations. Raw data ranged from 3.88 to 288 ng/mL for SP-D (min-max: 3.88–21.98 in controls and 14.8–288 in IAD), from  $1.18 \times 10^6$  to  $10.6 \times 10^6$  ng/mL for haptoglobin ( $1.18 \times 10^6$ – $5.52 \times 10^6$  in controls and  $2.59 \times 10^6$ – $10.6 \times 10^6$  in IAD), from 0.69 to 36.14 ng/mL for sTREM-1 (0.69–17.34 in controls and 1.56–36.14 in IAD) and from 0.21 to 3.30 µg/mL for SAA (0.21–1.55 in controls and 0.28–3.30 in IAD). Serum samples from 4 horses with IAD had SAA concentrations exceeding the assay quantification range. When combined, the sum of the log-transformed values of SP-D and haptoglobin ( $P < .0001$ ), of SP-D and sTREM-1 ( $P = .003$ ), of SAA and SP-D ( $P < .0001$ ), of SAA and haptoglobin ( $P = .0001$ ) and of SAA and sTREM-1 ( $P = .025$ ), were significantly higher in the sera of horses with IAD compared to controls. The sum of the concentrations of haptoglobin and sTREM-1 was not different between both groups ( $P = .24$ ) (Fig 2C). No overlap was observed between the 2 groups when the sum of SP-D and haptoglobin concentrations was studied (Fig 2A). A cut-off point of 7.70 (Log-transformed value) allowed complete distinction of horses with IAD from controls. A cut-off value of 0.80 for the sum of SAA and SP-D (Fig 2D) permitted differentiation of horses with IAD from controls (sensitivity = 100%, specificity = 90%). In horses with IAD, SAA concentration was significantly correlated with neutrophil percentage in BALF ( $r = 0.56$ ,  $P = .05$ ). The two horses with BALF eosinophil percentage >1% had the highest serum concentrations of SP-D. Haptoglobin values were negatively correlated with serum neutrophil concentrations ( $r = -0.69$ ,  $P = .01$ ), neutrophil percentage in BALF ( $r = -0.75$ ,  $P = .005$ ) and with serum leucocyte concentrations ( $r = -0.61$ ,  $P = .04$ ). Haptoglobin



**Fig 1.** Serum concentration of SP-D (A), haptoglobin (B), sTREM-1 (C) and SAA (D) in horses with IAD and controls. Values were log transformed for analysis.



**Fig 2.** Serum concentration of combined SP-D and haptoglobin (A), SP-D and sTREM-1 (B), haptoglobin and sTREM-1 (C), SAA and SP-D (D), SAA and haptoglobin (E), and sTREM-1, and SAA (F) in horses with IAD and controls. Values were log transformed for analysis.

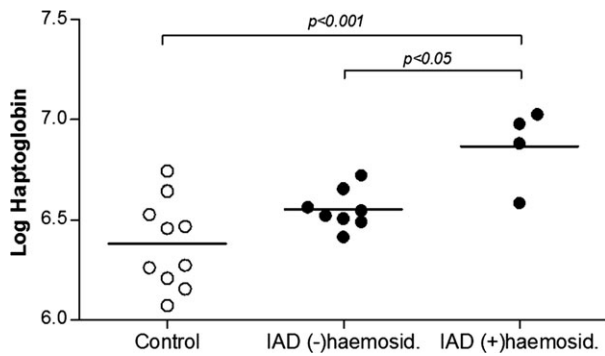
concentrations were significantly affected by the presence of haemosiderophages ( $P = .0009$ ) in BALF. Horses with haemosiderophages detected at BALF cytology (there were no haemosiderophages found in the BALF of the control group (Fig 3) had higher values of haptoglobin concentration in the serum compared to the IAD-affected horses with no haemosiderophages and to the controls ( $P < .05$  and  $P < .001$  respectively, Fig 3).

### Discussion

Inflammatory Airway Disease is highly prevalent in the equine population. However, diagnosis remains a

challenge, as clinical signs are not pathognomonic. The implementation of biomarkers specific for lung inflammation could facilitate its diagnosis. Our results demonstrate that the serum concentrations of SP-D, haptoglobin and SAA are increased in horses with IAD compared to controls, moreover when combined, the serum concentrations of SP-D and haptoglobin allow differentiating horses with IAD from healthy horses with no overlap between groups.

Surfactant protein D is a collectin mainly synthesized in the lungs by alveolar type II cells. It plays an important role in protecting the lungs against various inflammatory processes, allergies and infections. In our study, and in agreement with a previous report,<sup>4</sup> horses with



**Fig 3.** Effect of the presence of BALF haemosiderophages on serum haptoglobin concentrations. Haptoglobin levels  $>6 \times 10^6$  ng/mL (log-transformed values  $\geq 6.8$ ) were associated with the presence of haemosiderophages in the BALF.

IAD had higher serum concentrations of SP-D. Despite SP-D being also expressed in the synovial fluid, diseases of these organ systems tend to lower circulating SP-D levels in man,<sup>6</sup> whereas increases in serum SP-D concentrations have been associated to lung pathologies.<sup>4</sup> Whether serum SP-D concentration also decreases in horses with joint diseases has not been studied to date, and would require investigation.

Serum amyloid A is a major acute phase protein reported to range from  $<0.5$  to  $20 \mu\text{g/mL}$ . Serum SAA levels are low in healthy individuals, and increase from 10 to 1000 times within a few hours from tissue injury, to reach peak values within 24–28 h.<sup>7</sup> Serum amyloid A has immunoregulatory functions, linked to the development of chronic diseases. In this study, and in agreement with findings in heaves,<sup>3</sup> serum SAA concentrations had a 3.5-fold increase in horses with IAD when compared to controls. As SAA increases are proportional to the extent of tissue damage,<sup>7</sup> it indicates that horses with IAD sustain lung injuries of similar magnitude to those found in heaves. Serum amyloid A increases after transportation.<sup>8</sup> In our study, horses with IAD and 5 of the control horses underwent transportation. The SAA values of control horses, whether transported or not, were not significantly different (Mann–Whitney *t*-test,  $P = .8$ ), suggesting that trailer-likely affected our results.

Circulating haptoglobin concentrations increase 1–10 times in the 12–24 h after an injury, and peaks 72 h later.<sup>7</sup> Haptoglobin is mainly produced to scavenge haemoglobin. In our study, haptoglobin was increased on average 2-fold in horses with IAD, whereas its serum concentration has been reported to increase 5–10-fold after inflammation and infection in horses.<sup>7</sup> Serum haptoglobin was increased in 3/4 horses with IAD presenting haemosiderophages in their BALF, whereas horses with IAD with no haemosiderophages have a concentration of serum haptoglobin similar to the controls. These findings suggest that the specificity of serum haptoglobin as biomarkers for IAD should be ascertained in the presence of exercise-induced pulmonary haemorrhage. It has been hypothesized that peripheral blood and airway neutrophil degranulation may constitute a source of hap-

toglobin in horses with inflammatory lung diseases such as heaves.<sup>3</sup> The negative correlations we observed between haptoglobin serum concentrations and both blood and BALF neutrophils would not support this hypothesis, and was unexpected. Haemosiderophages may have a less potent neutrophil chemo-attractive activity than other inflammatory cells, but more studies are needed to corroborate this assumption.

Comparing results from this study to a previous report,<sup>3</sup> horses with IAD have higher haptoglobin values than those of heave-affected horses, which would support the hypothesis of disease progression from IAD to heaves. However, a study evaluating both diseases in the same conditions is needed to confirm that hypothesis.

The TREM-1 is a membrane receptor expressed on blood neutrophils and monocytes, and also on alveolar macrophages, which has a role in lung inflammatory response against infections by amplifying the TLR-associated response.<sup>9</sup> As IAD is clinically comparable to mild to moderate human asthma, not surprisingly sTREM-1 serum concentrations were similar between horses with IAD and controls.

## Conclusions

Measuring several inflammation-related blood proteins increases their sensitivity as blood biomarkers for the diagnosis of IAD. The combination of an organ-specific biomarker (SP-D) and of an acute phase protein (haptoglobin) yielded the best diagnostic results. However, the effect of other causes of reduced performances in horses, whether affecting the lungs or other organs on SP-D is still unknown, even though our group recently reported no difference in SAA and haptoglobin concentrations in blood of racehorses with and without IAD.<sup>10</sup> Also, as healthy horses were studied as controls, the effect of other pulmonary conditions on acute phase proteins could represent a potential source of bias (decreasing sensitivity and specificity of the test). Prospective studies investigating these aspects are needed before these biomarkers can be employed as a diagnostic tool in the field.

## Acknowledgments

This work was supported by the Natural Sciences and Engineering Research Council of Canada (RGPIN-2014-06-198), Le Fonds du Centenaire and by an unrestricted grant from the Pfizer Clinical Research Fund.

*Conflict of Interest Declaration:* Authors disclose no conflict of interest.

*Off-label Antimicrobial Declaration:* Authors declare no off-label use of antimicrobials.

## Footnotes

<sup>a</sup> Cytospin, Rottorfix Hettish, Beverly, MA.

<sup>b</sup> Fisher Scientific, Ottawa, ON, Canada.

<sup>c</sup> Biovendor, Ashville, NC.

<sup>d</sup> Cederlane, Burlington, ON, Canada.

<sup>e</sup> MyBioScience, SanDiego, CA.

<sup>f</sup> ELX50 Auto Strip washer; Biotek-Instruments Inc., Winooski, VT.

<sup>g</sup> Power Wave X340; Biotek-Instruments Inc.

<sup>h</sup> GraphPad Software Inc., La Jolla, CA.

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