Expression of extracellular matrix metalloproteinase inducer and matrix metalloproteinase-2 and -9 in horses with chronic airway inflammation

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OBJECTIVE
To examine whether expression of extracellular matrix metalloproteinase inducer (EMMPRIN) can be detected in equine lungs and whether it correlates with matrix metalloproteinase (MMP)-2 and -9 expression in bronchoalveolar lavage fluid (BALF) of horses with chronic inflammation of the lungs (ie, lower airway inflammation [LAI]).

ANIMALS
29 horses with signs of chronic respiratory tract disease, which were classified as the LAI (n = 17) and LAI with respiratory distress (RDLAI [12]) groups, and 15 control horses.

PROCEDURES
BALF, tracheal aspirate, and blood samples were obtained, and EMMPRIN expression was determined from BALF cells and RBCs by use of western blotting. Activities of MMP-2 and -9 were determined with zymography.

RESULTS
Expression of EMMPRIN protein was identified in BALF cells of all horses. Expression of EMMPRIN protein was highest for the RDLAI group and was correlated with MMP-2 and -9 protein expression, MMP-9 gelatinolytic activity, and airway neutrophilia.

CONCLUSIONS AND CLINICAL RELEVANCE
Results suggested that EMMPRIN was involved in the pathophysiologic processes of asthma in horses. However, additional studies of horses and other species are warranted to elucidate the regulation of EMMPRIN expression in asthmatic lungs. (Am J Vet Res 2017;78:1329–1337)

Chronic inflammation of the lungs (ie, LAI) is common in horses, especially in countries where horses are housed in stables for a large part of the year. Long-term exposure to organic dust and molds in stable air is considered to be the most important predisposing factor. Chronic LAI in horses is evident as 2 conditions (milder IAD and more severe RAO), which together are considered to represent the asthma syndrome of horses. Horses of all ages can be affected by IAD, but RAO is more commonly seen in older horses. Both disease conditions are considered noninfectious, and they have similar clinical signs of chronic cough, nasal discharge, and exercise intolerance leading to poor performance.

However, only horses with RAO have respiratory distress at rest related to bronchoconstriction, which is reversible with a reduction in organic dust inhalation and drug intervention. Both IAD and RAO are evident as neutrophilic inflammation of the lungs, although eosinophilic and mastocytic disease phenotypes have also been recognized for IAD. The pathophysiologic mechanisms of RAO are characterized by airway hyperresponsiveness and mucus accumulation, which are in addition to bronchoconstriction and inflammation; therefore, it closely resembles asthma in humans. Thus, horses with naturally occurring disease may be useful for research on asthma in humans.

Extracellular MMP inducer (also known as CD147, basigin, and neurothelin) is a pleiotropic 58-kDa transmembrane protein of the immunoglobulin superfamily. It is found in various cell types, including leukocytes, epithelial and endothelial cells, and fibroblasts. Also, EMMPRIN induces the expression and activity of MMPs, which are zinc-dependent proteolytic enzymes that degrade the extracellular matrix. The MMPs are secreted by WBCs and various epithelial cells during inflammation, and they are classified into several subgroups on the basis of their function. Because they hydrolyze gelatin, MMP-2 and

ABBREVIATIONS
BALF Bronchoalveolar lavage fluid
EMMPRIN Extracellular matrix metalloproteinase inducer
IAD Inflammatory airway disease
IQR Interquartile range (25th to 75th percentile)
LAI Lower airway inflammation
MMP Matrix metalloproteinase
PELF Pulmonary epithelial lining fluid
RAO Recurrent airway obstruction
RDLAI Lower airway inflammation with respiratory distress
TA Tracheal aspirate
TBST Tris-buffered saline solution with 0.1% Tween
-9 are termed gelatinases. In addition to its ability to induce MMPs, EMMPRIN also regulates several other biological functions, including spermatogenesis, lymphocyte responsiveness, and expression and function of monocarboxylate transporters.

During the past decade, EMMPRIN has been extensively studied in humans and rodents, and increased EMMPRIN expression has been found in inflammatory conditions of the lungs as well as in several types of cancer. Moreover, increased MMP activity has been reported for humans with airway inflammation and lung damage, including asthma and chronic obstructive pulmonary disease. Activity of gelatinolytic MMPs in respiratory secretions is increased in horses with RAO, in which MMP-9 is the main elevated gelatinolytic enzyme.

In healthy horses, 2 distinct amounts of EMMPRIN expression have been identified in RBCs and muscle. In these tissues, the expression of EMMPRIN differs widely among individual animals because approximately 25% of horses express extremely little or none of this protein, whereas the remaining 75% express it in abundance. This bimodal expression affects lactate transport activity in RBCs; however apart from this effect, its physiologic importance is unknown. To our knowledge, constitutive (baseline expression in healthy tissue) or inducible (eg, inflammation-related EMMPRIN expression) expression and the relationship with MMP activation have not been studied in horses.

The primary objective of the study reported here was to examine expression of EMMPRIN in the lungs of horses. A second objective was to evaluate the relationship between EMMPRIN expression and activity of gelatinases MMP-2 and -9 in the BALF of horses with chronic LAI and control horses. We hypothesized that horses with chronic LAI would express EMMPRIN in abundance and have enhanced MMP expression and activity, compared with results for control horses.

Materials and Methods

Horses

A total of 44 client-owned horses (15 mares, 28 geldings, and 1 stallion) were used in this case-control study. A newspaper announcement was used to invite participation of client-owned horses in the study. Participating horses were selected on the basis of interviews with the owners and a convenient geographic location (southern Finland). The study was performed at a single university facility in southern Finland. Owners provided informed consent prior to participation of their horses in the study. The Finnish Animal Experiment Board approved the study.

Breeds included Standardbred (n = 11), coldblood horses (19), warmblood horses (4), and ponies (10). Signs of chronic (>6 months) or recurrent respiratory tract disease (including cough, nasal discharge, exercise intolerance, and an abdominal breathing pattern) were used as inclusion criteria for case horses. Horses with signs of chronic or recurrent respiratory tract disease without respiratory distress during physical examination at rest were classified as the LAI group (n = 17), and horses with respiratory distress during physical examination at rest in addition to signs of chronic or recurrent respiratory tract disease were classified as the RDLAI group (12). The control group consisted of 15 horses that had no history of chronic respiratory tract disease and no abnormal findings during physical examination. Control horses were matched to the clinically affected horses that lived on the same farm. Horses that had been administered medications during the month preceding the study were excluded.

Collection of samples

A blood sample (20 mL) was obtained from a jugular vein. Part of each sample was placed in an EDTA-containing tube, and the rest was placed in a clot tube. Samples were used for immediate (<1 hour after they were obtained) hematologic analysis. The EDTA-containing blood samples were screened (leukocyte count and plasma fibrinogen concentration determined by use of a heat precipitation method) to exclude horses with infectious disease. The remainder of the blood was centrifuged (1,500 g for 10 minutes), and plasma and RBCs were separated. Plasma, RBCs, and serum were stored at −80°C until further use.

Horses were restrained in stocks and sedated by IV administration of detomidine and butorphanol tartrate. A 220-cm-long, 11-mm-diameter video endoscope was used for transendoscopic collection of TA, as described elsewhere. Immediately after TA collection was completed, bronchoalveolar lavage was performed, as described elsewhere. A solution of 1% mepivacaine (40 mL for horses and 20 mL for Shetland Ponies) was administered to the carina and right mainstem bronchi to decrease coughing during the procedure. Sterile saline (0.9% NaCl) solution (360 mL for horses and 240 mL for Shetland Ponies; aliquots of 60 mL/injection) at ambient temperature was used for collection of BALF samples. Syringes containing recovered BALF were immediately placed on ice; samples were processed within 30 minutes after collection.

Cytologic analysis of TA and BALF

Slide preparations for differential cell counts were made by centrifugation of undiluted TA samples and subsequent smear of the cell pellet. The BALF aliquots for a horse were pooled, and the undiluted sample was cytocentrifuged. All slides were stained with May-Grünwald-Giemsa stain. An experienced investigator (MMR) who was not aware of the source of the samples performed differential counts of inflammatory cells by counting 300 cells on both TA and BALF slides. Results for each cell type were expressed as a percentage of the total number of cells. Reference values for BALF and TA cell types were obtained from the American College of Veterinary Internal Medicine.
revised consensus statement and another published report, with a neutrophil count of >5% in BALF and >20% in TA considered abnormal. The BALF cells and supernatant were separated by centrifugation (1,500 X g for 10 minutes at 4°C) and then stored at −80°C until further use.

**Processing of RBCs and BALF**

Thawed RBCs were placed in sodium phosphate buffer (5 mmol/L; pH 8.0), and membranes were washed and separated by centrifugation (4 centrifugations, each of which was 48,000 X g for 15 minutes at 4°C). The BALF cells were lysed with nonionic surfactant-buffer solution (50mM Tris HCl, 138mM NaCl, 5mM EDTA, 0.5% nonionic surfactant, and 10% glycerol; pH 7.5) supplemented with protease inhibitory cocktail and endonuclease; lysed cells were centrifuged (800 X g for 5 minutes), and supernatant was collected. Undiluted BALF supernatant was used for analysis. Processed samples were stored at −80°C until analysis.

**Western blotting of RBCs and BALF**

Antibody against the C-terminus of equine EMMPRIN was generated in rabbits and purified with affinity chromatography (sequence GHHVNDKD-KNVQRQNAS; GenBank accession No. ABQ53583.1). Antibodies against human MMP-2 (polyclonal antibody) and mouse MMP-9 (polyclonal goat antibody) were used to detect MMPs. Cross-reaction between MMP-2 and MMP-9 antibodies was evaluated by use of MMP-2 and MMP-9 recombinant proteins (0.4 µg/dot) as sham antigens for each other. For all antibodies, a blot without the first antibody was evaluated, and no bands were detected. Samples of RBC membrane proteins (10 µg/lane), BALF cells (8 µg/lane), and BALF supernatant (18 µL/lane) in loading buffer were separated on 4% to 12% SDS-polyacrylamide gels and subsequently blotted onto nitrocellulose filters. A molecular weight standard was included in every blot. Equal loading was confirmed with β-actin. Blots were blocked by incubation with 10% dry milk in TBST; filters then were incubated overnight at 4°C with primary antibodies (EMMPRIN for RBC membranes and BALF cells; MMP-2 and MMP-9 for BALF supernatant) diluted in blocking buffer. After incubation was completed, filters were washed in TBST (15 minutes followed by two 5-minute washes). Filters then were incubated with horseradish peroxidase-conjugated antirabbit antibody in TBST supplemented with 2.5% dry milk for 1 hour at room temperature (21°C). Washes were repeated as described previously. A chemiluminescence reagent was used to develop visible bands. Filters were imaged with a luminescent image analyzer by use of incremental exposure times, and proteins were semiquantified by use of an image data analyzer. For RBCs and BALF cells, each gel contained 2 control samples (5 and 15 µg/lane) obtained from RBC membranes of a healthy horse. For BALF supernatants, pooled BALF obtained from 9 horses, which included healthy and affected horses, was used for control samples (3 and 18 µL/lane). Intensities of bands for the study samples were measured in relation to intensities of the control samples.

**Measurement of MMP-2 and -9 activities in BALF**

Samples of BALF supernatant (45 µL/lane; 2 parts of sample to 1 part of loading buffer) were loaded onto an 11% SDS-polyacrylamide gel. Gels were also loaded with a molecular weight standard and 2.5 µL of diluted (1:200) MMP-9 and -2 human recombinant proteins as positive control samples. Zymography was performed at room temperature, and zymograms were incubated in 2.5% nonionic surfactant for 1 hour and in developing buffer (50mM Tris, 5mM CaCl₂, and 0.02% detergent) for 17 hours at 37°C. Gels then were stained, washed, and scanned for quantification of enzyme activities. Densitometric results were measured with an image analysis system by use of the area calculation. For each band, the final result was assessed by comparison with the band for pro-MMP-2 (positive control standard) on each gel. Zymograms were analyzed for activity of pro-MMP-2, pro-MMP-9, active MMP-2, and active MMP-9.

**Measurement of urea concentration for estimation of marker dilutions in BALF**

Urea concentrations in serum and BALF were determined with a kinetic enzymatic method by use of a clinical chemistry analyzer and commercial reagent. Concentrated reagent solution was used to maintain final reagent concentrations in BALF samples. The proportion of PELF for the total amount of recovered BALF was calculated by use of the following equation: PELF = (BALF urea concentration/serum urea concentration) x 100. Results for supernatants were divided by the calculated PELF percentage.

**Statistical analysis**

A Shapiro-Wilk test and Q-Q plots were used to assess normality of the data. For almost all variables, the data were not normally distributed; therefore, nonparametric tests were used. Differences among groups were analyzed by use of a Kruskal-Wallis 1-way ANOVA. When significant differences were detected, pairwise comparisons between groups were conducted with exact Wilcoxon-Mann-Whitney tests and multiple comparison adjustment via a Bonferroni correction. Interpretations of results for pairwise comparisons were based solely on adjusted P values. Spearman rank correlation coefficients were determined for 15 variables. Because of the exploratory nature, no multiplicity adjustment methods were used for the correlations. Values were considered significant at P < 0.05. Statistical analyses were conducted by use of statistical programs.
Results
Horses were from 28 farms. The 29 horses with signs of chronic or recurrent respiratory tract disease had a history of cough (n = 26), nasal discharge (18), exercise intolerance (10), and occasionally an abdominal breathing pattern (17). There were 17 horses classified as LAI and 12 as RDLAI. Of the 15 control horses, 13 resided on the same farms and were managed in a similar manner as affected horses. There were 3 ponies in the LAI group, 3 ponies in the RDLAI group, and 4 ponies in the control group. No horses were excluded on the basis of evidence of systemic disease.

Median age of LAI horses was 16 years (IQR, 10 to 19 years; range, 5 to 26 years), RDLAI horses was 16 years (IQR, 14 to 18 years; range, 8 to 22 years), and control horses was 6 years (IQR, 5 to 9 years; range, 3 to 19 years). Significant differences were detected among groups for neutrophil percentages in TA (P < 0.001) and BALF (P = 0.001). The RDLAI horses had a higher neutrophil percentage in both TA and BALF, compared with percentages for the control and LAI horses (Figure 1). There was no significant difference among groups for percentages of eosinophils in BALF (P = 0.57) or mast cells in BALF (P = 0.88) or the percentage of eosinophils in TA (P = 0.51). Mast cells were not detected in TA samples. Results of cytologic examination for BALF and TA for cell types other than neutrophils were summarized (Table 1).

Results of EMMPRIN western blotting were available for RBCs of 44 horses and BALF cells of 36 horses (11 LAI, 12 RDLAI, and 13 control horses). The EMMPRIN protein was identified as an approximately 50-kDa band in RBC and BALF cell membranes. As described elsewhere,21,22 horses of the present study could be categorized into 2 distinct groups on the basis of EMMPRIN protein expression for RBCs. An intense EMMPRIN band could be detected for 36 horses, which were considered to have high expression, whereas 8 horses had an extremely faint or absent

Figure 1.—Box-and-whisker plots for neutrophil percentage of total cells in TA (A) and BALF (B) obtained from 15 control horses, 17 horses with LAI, and 12 horses with RDLAI. Each box represents the IQR (ie, 25th to 75th percentiles), the horizontal line in each box represents the median, the whiskers represent the range, and black circles represent outliers. Significant differences between groups are indicated.

Table 1.—Median (IQR) values for cytologic examination results of TA and BALF and EMMPRIN protein expression in RBCs for 15 control horses, 17 horses with LAI, and 12 horses with RDLAI.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Variable</th>
<th>Control</th>
<th>LAI</th>
<th>RDLAI</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA</td>
<td>Eosinophil (%)</td>
<td>0 (0–0.4)</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td></td>
<td>Lymphocyte (%)</td>
<td>2.4 (1.0–3.5)</td>
<td>3.7 (1.7–5.4)*</td>
<td>1.0 (0.3–1.5)*</td>
</tr>
<tr>
<td></td>
<td>Macrophage (%)</td>
<td>50.4 (30.2–59.7)</td>
<td>43.4 (14.4–55.0)†</td>
<td>5.0 (2.7–8.7)‡</td>
</tr>
<tr>
<td>BALF</td>
<td>Eosinophil (%)</td>
<td>0 (0–0.4)</td>
<td>0 (0–0.4)</td>
<td>0.2 (0–1.0)</td>
</tr>
<tr>
<td></td>
<td>Mast cell (%)</td>
<td>3.0 (1.2–3.6)</td>
<td>2.7 (1.0–3.4)</td>
<td>1.2 (1–3.4)</td>
</tr>
<tr>
<td></td>
<td>Lymphocyte (%)</td>
<td>52.0 (45.2–57.5)</td>
<td>61.7 (53.0–65.4)§</td>
<td>45.4 (32.2–54.0)§</td>
</tr>
<tr>
<td></td>
<td>Macrophage (%)</td>
<td>40.4 (36.9–45.4)</td>
<td>30.7 (21.7–35.0)</td>
<td></td>
</tr>
<tr>
<td>RBCs</td>
<td>EMMPRIN protein (arbitrary units)</td>
<td>8.2 (1.1–13.7)</td>
<td>7.8 (4.3–11.2)</td>
<td>12.7 (3.2–19.7)</td>
</tr>
</tbody>
</table>

*†‡§||Within a row, values differ significantly (*P = 0.002, †P = 0.006, ‡P < 0.001, §P = 0.011, and ||P = 0.016) from the value for the control group.
band and were considered to have low expression. In contrast, all samples had low amounts of EMMPRIN expression for BALF cells, and the bimodal expression of RBCs could not be identified for BALF cells. No correlation was found in EMMPRIN protein expression between RBCs and BALF cells. However, when expression in BALF cells was compared among groups, there were significant \( P = 0.014 \) differences. The RDLAI group had the highest expression of EMMPRIN protein (Figure 2).

Figure 2—Box-and-whisker plots of western blot results (arbitrary units) for the expression of EMMPRIN in BALF cells (A), the total immunoreactivity for MMP-2 in BALF supernatant (B), and the total immunoreactivity for MMP-9 in BALF supernatant (C) among 15 control horses, 17 horses with LAI, and 12 horses with RDLAI. Notice that the scale on the y-axis differs among panels. See Figure 1 for key.

Figure 3—Photographs of representative western blot membranes depicting the expression of MMP-2 (A) and MMP-9 (B) in BALF supernatant and results of gelatin zymography of MMP-9 in BALF supernatant (C) for control horses, horses with LAI, and horses with RDLAI. Bands consisted of complex, pro, and active forms of MMPs and degradation products. Bands at approximately 92 kDa are representative of pro–MMP-9, and bands of lower molecular weight are representative of active MMP-9, as confirmed by evaluation with a protein marker and human MMP-2 and MMP-9 (data not shown).

Figure 4—Box-and-whisker plots of gelatin zymography results (arbitrary units) for the activity of pro–MMP-9 (A) and active MMP-9 (B) in BALF supernatant among 15 control horses, 17 horses with LAI, and 12 horses with RDLAI. See Figure 1 for key.
Western blots for MMP-2 and MMP-9 in BALF supernatant consisted of complex, pro, and active forms and degradation products (Figure 3). All bands were not detected in all horses. Total immunoreactivity (all visible bands) was calculated for MMP-2 and MMP-9 for comparisons among groups. The Kruskal-Wallis test revealed significant \( (P = 0.004) \) differences in total immunoreactivity for both MMP-2 and MMP-9 among groups. Total immunoreactivities for MMP-2 and MMP-9 were higher for RDLAI horses than for control horses (Figure 2). Furthermore, LAI horses had higher total immunoreactivity for MMP-2 and MMP-9 than did control horses.

Gelatinolytic activity of MMP-9 was observed as complex, pro, and active forms of the enzyme (Figure 3). All forms were not detected in all horses. The Kruskal-Wallis test revealed significant \( (P < 0.001) \) differences in gelatinolytic activity for both pro–MMP-9 and active MMP-9. The BALF of RDLAI horses had higher activity of pro–MMP-9, compared with results for control and LAI horses, and LAI horses had higher activity than did control horses (Figure 4). Furthermore, the activity of active MMP-9 was also higher in RDLAI horses, compared with results for control and LAI horses. There was no difference in the activity of active MMP-9 between LAI and control horses.

Expression of EMMPRIN protein was correlated with number of airway neutrophils, MMP protein expression, and MMP-9 activity. Significant correlations were detected (Table 2).

**Discussion**

In the study reported here, expression of EMMPRIN and its relationship to MMPs in equine lungs were characterized. In the present study, expression of EMMPRIN, MMP-2, and MMP-9 protein and MMP-9 gelatinolytic activity were significantly higher in the BALF of horses with chronic neutrophilic RDLAI, compared with results for control horses. In humans, EMMPRIN expression increases in the lungs of smokers and patients with chronic obstructive pulmonary disease and interstitial pneumonia, and EMMPRIN expression is known to regulate MMP-9 expression.\(^{10,14,16}\) Moreover, in human airways, mechanical strain increases EMMPRIN expression, which leads to increased MMP-dependent proliferation of smooth muscle cells, a structural change that appears in asthmatic airways of both humans and horses.\(^{7,25}\) The horses of the study reported here had naturally occurring asthma syndrome and thus may serve to provide information about asthma in other species. To our knowledge, EMMPRIN expression has not previously been examined in asthmatic lungs of any animal species with naturally occurring disease. However, anti-EMMPRIN antibody significantly reduced the inflammatory response in asthmatic lungs in mice\(^{15}\); therefore, EMMPRIN is probably also important in the pathophysiologic processes of asthma in horses and other species.

In the present study, the 2 distinct amounts for constitutive expression of EMMPRIN protein observed in RBCs could not be detected in BALF cells. However, the RDLAI horses had higher EMMPRIN protein expression in BALF, compared with results for control horses. Therefore, individual constitutive EMMPRIN expression in RBCs does not translate to expression in BALF cells, and the expression of EMMPRIN in lungs is related to the severity of disease, rather than to the ability of individual horses to express the protein. These findings suggested that the regulation of constitutive and inducible EMMPRIN expression was tissue specific.

The correlation of EMMPRIN mRNA expression and protein expression or enzyme activity was not

**Table 2**—Significant correlations detected by use of the Spearman correlation coefficient for control horses, horses with LAI, and horses with RDLAI.

<table>
<thead>
<tr>
<th>Variable 1</th>
<th>Variable 2</th>
<th>( P ) value*</th>
<th>( \rho )</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALF EMMPRIN protein expression</td>
<td>BALF neutrophil percentage</td>
<td>0.047</td>
<td>0.333</td>
</tr>
<tr>
<td></td>
<td>TA neutrophil percentage</td>
<td>0.018</td>
<td>0.391</td>
</tr>
<tr>
<td></td>
<td>Total immunoreactivity of MMP-2</td>
<td>&lt; 0.001</td>
<td>0.554</td>
</tr>
<tr>
<td></td>
<td>Total immunoreactivity of MMP-9</td>
<td>0.006</td>
<td>0.450</td>
</tr>
<tr>
<td></td>
<td>Activity of pro–MMP-9</td>
<td>0.010</td>
<td>0.424</td>
</tr>
<tr>
<td></td>
<td>Activity of active MMP-9</td>
<td>0.016</td>
<td>0.397</td>
</tr>
<tr>
<td>BALF neutrophil percentage</td>
<td>Activity of pro–MMP-9</td>
<td>&lt; 0.001</td>
<td>0.794</td>
</tr>
<tr>
<td></td>
<td>Activity of active MMP-9</td>
<td>&lt; 0.001</td>
<td>0.558</td>
</tr>
<tr>
<td></td>
<td>TA neutrophil percentage</td>
<td>&lt; 0.001</td>
<td>0.762</td>
</tr>
<tr>
<td>TA neutrophil percentage</td>
<td>Activity of pro–MMP-9</td>
<td>&lt; 0.001</td>
<td>0.770</td>
</tr>
<tr>
<td></td>
<td>Activity of active MMP-9</td>
<td>&lt; 0.001</td>
<td>0.560</td>
</tr>
<tr>
<td>Age</td>
<td>TA neutrophil percentage</td>
<td>0.019</td>
<td>0.352</td>
</tr>
<tr>
<td></td>
<td>Total immunoreactivity of MMP-9</td>
<td>0.022</td>
<td>0.346</td>
</tr>
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</table>

*Unadjusted \( P \) value; values were considered significant at \( P < 0.05 \).
evaluated in the present study. Previously, investigators determined that the expression of EMMPRIN is regulated at the transcriptional level in horses, which is similar to regulation in humans. Furthermore, it has been suggested for humans that post-translational regulation also occurs, and this regulation appears to be tissue specific. The regulatory network of EMMPRIN seems to be highly complex and influences transcription, translation, and membrane expression on cell surfaces involving (among other things) growth factors, specificity protein 1, microRNAs, monocarboxylate transporters, and cyclophilin, which were not investigated in the present study. Furthermore, EMMPRIN is regulated by various factors related to inflammation, including tumor necrosis factor-α and interleukins. Interestingly, EMMPRIN may also self-regulate its own expression. Thus, because of their complexity, the EMMPRIN regulation pathways warrant further research.

It is believed that EMMPRIN is the main modulator of MMP activity from transcription to expression on cell surfaces. We confirmed the hypotheses that MMP protein expression and activity were higher in horses with chronic LAI than in control horses and that EMMPRIN protein expression in BALF cells correlated positively with both protein expression and gelatinolytic activity of MMPs. These results are in agreement with results for studies of humans regarding MMP9 in asthma and EMMPRIN and its relationship to MMP-9 in chronic obstructive pulmonary disease. However, to our knowledge, the relationship of EMMPRIN to MMPs has not been elucidated in patients of any species clinically affected with asthma. Similar to results of other studies, MMP-9 expression and gelatinolytic activity for the present study were increased in horses with respiratory distress, which indicated the RAO-exacerbation phase. In a recent study, increased MMP activity was also reported in horses with IAD and interstitial pneumopathies. The MMP gelatinolytic activity was significantly higher in LAI horses than in control horses, but the difference was small (Figure 4). However, MMP protein expression in LAI horses was approximately similar to that in RDLAI horses. Most horses in the LAI group had only mild lung inflammation at the time of sample collection, as indicated by results of cytologic examination of BALF and TA. MMP activation might explain differences in the amount of MMP protein expression and its gelatinolytic activity.

Results of cytologic examination of BALF and TA were as expected because horses in the RDLAI group had the highest neutrophil proportions. In horses, RAO is characterized by neutrophilic inflammation in the lungs, which typically involves periodic exacerbations of inflammation that result in respiratory distress. Similarly, IAD can be evident as a disease group that includes eosinophilic and mastocytic types of LAI, but we did not detect differences among the groups with regard to eosinophils or mast cells, which suggested that the LAI horses of the present study had neutrophilic types of disease, including IAD and RAO in remission. However, the staining method used for the present study might have resulted in an underestimation of the percentage of mast cells. Some horses in all groups, including the control group, had a mast cell percentage in BALF greater than the reference limit of 2%. This may have indicated low-degree mastocytic airway inflammation. However, the diagnostic importance for a particular percentage of mast cells in BALF has not been fully defined; a large range of 0.7% to 12.3% (median, 9%) has been reported for horses with no clinical evidence of respiratory tract disease. Moreover, the current expert consensus on IAD suggests that an alternative cutoff value of 5% might be more appropriate for most horse populations and sample collection techniques. Regardless of the cutoff values used, the importance of results for cytologic examination of BALF should always be interpreted in combination with the history and clinical signs of a horse. All of the control horses of the study reported here had no clinical signs of disease, as reported by the owners.

A correlation between gelatinolytic activity of MMP-9 in BALF and neutrophil accumulation in the airways was detected in the present study, which is similar to results of another study. This finding further emphasized the inflammatory nature of the asthma syndrome of horses and the role of neutrophils in MMP-9 stimulation. Similarly, in humans with severe asthma, MMP-9 protein is primarily derived from neutrophils. However, other inflammatory cell types may also contribute to MMP-9 expression. In human asthmatic bronchial tissue, increased MMP-9 expression has been detected in eosinophils. Interestingly, in the present study, expression of EMMPRIN protein in BALF cells also correlated positively with the amount of neutrophilic airway inflammation detected by use of cytologic examination of TA and BALF, which suggested that EMMPRIN was upregulated together with MMP-9 during LAI.

In accordance with results of other studies, control horses in the present study had minimal gelatinolytic activity in BALF. Furthermore, despite MMP-2 protein expression in BALF, no MMP-2 gelatinolytic activity was detected. It has been suggested that MMP-2 is expressed indigenously; thus, its activation in inflammation would rarely be observed. Hence, the role of MMP-2 as a constitutive enzyme might explain the findings of the present study.

The present study had some limitations. Measurement of urea concentrations was used to correct for the effect of sample dilution, which remains a controversial challenge in analysis of BALF samples. However, in the present study, use of urea concentration as a dilution marker did not affect the results,
which indicated relatively similar dilution of PELF in the study samples. The study population consisted of client-owned horses that lived in various stable conditions, although management regarding the type of feed, bedding, and turnout time of cases and control horses on the same farm was similar. The study also included 10 ponies. It is possible that the inclusion of ponies increased data variation; however, the number of ponies was similar among all groups and thus was considered unlikely to have affected the results. The control horses were younger than were the horses with signs of chronic respiratory tract disease. This was because 13 of 15 control horses were selected from the same farms as the case horses to minimize the effect of environmental factors, which can have a marked influence on the severity of clinical signs and airway neutrophilia. A younger age was weakly associated with a lower neutrophil percentage in TA and total immunoreactivity of MMP-9. However, EMMPRIN expression was not affected by age. Some of the control horses had an increased mast cell percentage in BALF, which may have been interpreted as mastocytic airway inflammation despite the fact the horses had no clinical signs. This was a potential source of bias and may have affected the results, but the effect most likely would have decreased differences among the groups, rather than falsely increasing them.

In the study reported here, a history of signs of chronic respiratory tract disease and physical examination findings were used to categorize horses into groups. These horses represented true clinically affected animals with various severities of clinical signs and airway neutrophilia, which would be expected in patients with naturally occurring disease. A limitation of the study was the lack of respiratory function testing at our research setting, which precluded us from measuring the extent of breathing pressure changes in clinically affected horses. This would have enabled us to objectively assess the amount of respiratory distress. Respiratory distress at rest was used to categorize horses, and because only horses with RAO have respiratory distress while at rest, the RDLAI group most likely consisted of horses with RAO in the exacerbation phase. However, distinguishing more specifically between horses with IAD and RAO would have provided an opportunity to more precisely compare EMMPRIN and MMP expression between these diseases. This might have offered interesting insights into the pathophysiologic processes of these closely related diseases because there is still controversy regarding the possible precursor role of IAD in the development of RAO. However, this was not possible for the present study, and it will remain a subject for future studies.

For the present study, we concluded that the expression of EMMPRIN was increased in asthmatic lungs of horses. Expression of EMMPRIN was associated with protein expression of MMP-2 and MMP-9, gelatinolytic activity of MMP-9, and probably also with neutrophil accumulation in the airways during inflammation. However, the complexity of EMMPRIN regulation pathways, factors potentially affecting the induction of MMPs, and potential use of EMMPRIN as a marker of inflammation provide interesting aspects for further research of horses and other species.

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Footnotes

a. ADVIA 2120i, Siemens Healthcare Diagnostics Inc, Deerfield, Ill.
b. Pentax, Tokyo, Japan.
d. Triton-X 100, Sigma-Aldrich, St Louis, Mo.
e. Sigma-Aldrich, St Louis, Mo.
f. DNase1 +EN0521, Fermentas, Vilnius, Lithuania.
g. Sigma Genosys, Suffolk, England.
h. AF902, R&D Systems, Minneapolis, Minn.
i. AF909, R&D Systems, Minneapolis, Minn.
j. Bio-Rad Mini-Protein TGX gels, Bio-Rad, Hercules, Calif.
k. Protran, Perkin Elmer, Boston, Mass.
l. Kaleidoscope prestained SDS-PAGE standards, broad range, Bio-Rad, Hercules, Calif.
m. Supersignal West Dura, Pierce, Rockford, Ill.
n. LAS-3000 CCD-camera, Fujifilm Life Science, Düsseldorf, Germany.
o. AIDA, Raytest, Straubing, Germany.
p. Brij-55, Sigma-Aldrich, St Louis, Mo.
q. ScanJet 4c/T, Hewlett-Packard, Miami, Fla.
r. Alpha Ease FC stand alone, version 6.0.0.14, Alpha Innotech, San Leandro, Calif.
s. Kone Pro, Thermo Fisher Scientific, Vantaa, Finland.
t. UREA UV 250, bioMérieux SA, Marcy-l’Etoile, France.
w. IBM SPSS Statistics 24, SPSS Inc, Chicago, Ill.

References