Lipidomic analysis of surfactant and plasma from horses with asthma and age-matched healthy horses

Undine Christmann, DVM, PhD; Allen E. Page, DVM, PhD; David W. Horohov, PhD; Amanda A. Adams, PhD; Seth E. Chapman, DVM, MS; Courtney L. Hancock, DVM; Audrey L. Emer, DVM; Jesse R. Poovey, DVM; Casey Hagg, DVM; Saira M. Ortega Morales, MSc; Ashley R. Duncan, BSc; Joey Morgan; Laurent L. Couetil, DVM, PhD; Peter Morresey, MVM, BVSc; Paul L. Wood, PhD

1Lincoln Memorial University, College of Veterinary Medicine, Harrogate, TN
2Gluck Equine Research Center, University of Kentucky, Lexington, KY
3Rood and Riddle Equine Hospital, Lexington, KY
4Department of Veterinary Clinical Sciences, Purdue University College of Veterinary Medicine, West Lafayette, IN

*Corresponding author: Dr. Christmann (undine.christmann@lmunet.edu)
https://doi.org/10.2460/ajvr.21.11.0179

OBJECTIVE
To perform lipidomic analysis of surfactant and plasma from asthmatic and healthy horses.

ANIMALS
30 horses with clinical signs of asthma and 30 age-matched control horses.

PROCEDURES
Detailed history, physical examination, CBC, and bronchoalveolar lavage fluid (BALF) cytologies were obtained. Asthmatic horses were grouped based on their BALF inflammatory profile: severe equine asthma (SEA), mild equine asthma with neutrophilic airway inflammation (MEA-N), or mild equine asthma with eosinophilic airway inflammation (MEA-E). Each asthma group was assigned its own age-matched control group. Lipidomic analysis was completed on surfactant and plasma. Surfactant protein D (SP-D) concentrations were measured in serum and BALF.

RESULTS
SEA surfactant was characterized by a phospholipid deficit and altered composition (increased ceramides, decreased phosphatidylglycerol, and increased cyclic phosphatidic acid [cPA]). In comparison, MEA-N surfactant only had a decrease in select phosphatidylglycerol species and increased cPA levels. The plasma lipidomic profile was significantly different in all asthma groups compared to controls. Specifically, all groups had increased plasma phytoceramide. SEA horses had increased plasma cPA and diacylglycerol whereas MEA-N horses only had increased cPA. MEA-E horses had increases in select ceramides and dihydrocermides. Only SEA horses had significantly increased serum SP-D concentrations.

CLINICAL RELEVANCE
The most significant surfactant alterations were present in SEA (altered phospholipid content and composition); only mild changes were observed in MEA-N horses. The plasma lipidomic profile was significantly altered in all groups of asthmatic horses and differed among groups. Data from a larger population of asthmatic horses are needed to assess implications for diagnosis, prognosis, and treatment.

The diagnosis of equine asthma (EA) relies on history, clinical signs, and detection of airway inflammation on bronchoalveolar lavage fluid (BALF) cytology. EA affects between 12% and 80% of horses, depending on the population sampled. It impairs performance, leads to significant economic losses, and decreases the well-being of affected horses. In mild equine asthma (MEA), clinical signs consist of coughing, with or without decreased performance. In contrast, severe equine asthma (SEA) is associated with more pronounced signs, including increased respiratory effort at rest. Clinical signs are triggered and exacerbated by exposure to organic dusts and allergens.

In a quest to find systemic markers for EA, a number of studies have investigated potential systemic inflammatory markers. In recent years, serum surfactant protein D (SP-D) has been proposed as a potential biomarker for MEA. Combining SP-D with other inflammatory markers such as haptoglobin can further enhance the distinction between healthy horses and those with MEA. Causes for increased serum SP-D in MEA and their relationship to local surfactant alterations remain to be determined. Pulmonary surfactant lipid alterations have been described in horses with SEA and consist of a surfactant deficit (decreased phospholipid content).
in conjunction with alterations in lipid markers (cyclic phosphatidic acid [cPA] and diacylglycerol [DG]).1,10

In human asthma, some of the surfactant alterations described include altered phospholipid content, modified composition, variations in surfactant proteins A and D, and increased surface tension.15-15 Elevated serum SP-D concentrations are seen in severe human asthma and may be linked to impaired airway epithelial integrity.16 The lipidomic approach has been used recently to analyze BALF, surfactant, and plasma from asthmatic patients to gain a better understanding of lipid metabolism, to identify possible biomarkers, and to establish specific asthma profiles.17-20 This type of analysis makes it possible to look at a large array of lipids within 8 categories that each contain a spectrum of molecular species with variable chain length, degree of saturation, and type of bonds. Lipid composition influences physical (eg, surface tension) and other biological (eg, immunomodulation) functions.21,22 This article uses shorthand notation where each lipid is identified by (1) class abbreviation, (2) number of carbon atoms and double bonds, and (3) amount of hydroxylation (m = mono-, d = di-, and t = tri-hydroxy bonds). For example, Cer d18:1/16:0 is the annotation used for a ceramide with a common sphingoid backbone (d18:1 = 18 carbons in length with 1 double bond and 2 -OH bonds) and a fatty acid (16:0 = 16 carbons in length with no double bonds).

To our knowledge, the plasma lipidomic profile has not been investigated in asthmatic horses. Surfactant has been analyzed in horses with SEA, but has not been investigated in asthmatic horses. Surfactant and plasma profiles may exist depending on the predominant inflammatory cell type on BALF cytology. The purpose of our study was to evaluate lipidomic composition of surfactant and plasma from horses with MEA and SEA in comparison to age-matched healthy horses, and to assess SP-D in serum and BALF from the same horses.

Materials and Methods

Horses

Sample collection for this project occurred from June 2017 to January 2020. The study protocol was approved by the Lincoln Memorial University’s (LMU) and University of Kentucky’s (UK) Institutional Animal Care and Use Committee (LMU: IACUC No. 1705 Res.10 and No. 1915 Res., UK: IACUC No. 2017-2617). Horses with MEA or SEA and age-matched healthy control horses were recruited from the following sites: Rood and Riddle Equine Hospital (Lexington, KY), Purdue University (Lafayette, IN), the University of Kentucky Department of Veterinary Sciences (Lexington, KY), and LMU College of Veterinary Medicine (Ewing, VA). Each participating institution received specific forms to collect patient information, and was provided with a detailed protocol and the supplies needed for sample collection, processing, and storage.

Inclusion criteria for horses with MEA were as follows: a history of coughing and/or exercise intolerance; normal respiratory effort at rest; increased mucous upon tracheal endoscopy; an increased percentage of neutrophils (> 5%), eosinophils (> 1%), or mast cells (> 2%) on BALF cytology; CBC and fibrinogen within normal limits; and no treatment received within the past 15 days.1,2 MEA horses were further categorized into those with predominantly neutrophilic airway inflammation (MEA-N) and those with mostly eosinophilic airway inflammation (MEA-E). Inclusion criteria for horses with SEA consisted of a history of coughing and increased respiratory effort at rest, increased amounts of tracheal mucus on airway endoscopy, more than 20% neutrophils on BALF cytology, normal hematocrit, and no treatment received within the past 15 days.1,2 Healthy age-matched control horses for each group of asthmatic horses were selected from a pool of research and teaching horses. Control horses had no history of SEA or respiratory disease signs for at least 2 months, a normal physical examination, BALF cytology and hematocrit within normal limits, and no treatment administered within the past 15 days.2

Clinical evaluation and sample collection

A thorough history was collected for every case and a detailed physical examination was performed on each horse prior to sample collection. Horses were sedated with detomidine (Dormosedan, Zoetis) (0.01 mg/kg body weight) and butorphanol (Torbugesic, Zoetis) (0.01 mg/kg body weight) administered intravenously. Blood was collected from the jugular vein using a vacutainer system with plain and EDTA tubes. An endoscope was passed through the nasal passages and into the trachea to assess for the presence of mucus. BALF was collected by passing auffed BALF collection tube (Jorgensen Laboratories) through the nasal passages and into the trachea. A 0.4% lidocaine solution (approximately 60 mL) was infused to reduce coughing, the tube was then advanced until wedged, and the cuff was inflated with 6 mL air. Sterile saline (0.9% sodium chloride) solution (250 to 300 mL) was infused and removed manually by syringe. Recovered BALF was mixed, pooled, and placed in a sterile specimen cup on ice. BALF volume and quality were recorded, and samples were processed within 30 minutes after collection. BALF cell counts were analyzed using an automated cell counter, and a differential cell count of at least 400 cells was evaluated under immersion microscopy on a cytospin slide stained with a rapid Romanowsky stain. Cell-free BALF was obtained by centrifuging BALF at 400 x g for 10 minutes at 4°C
and aspirating the supernatant. Venous blood was left to clot for 60 minutes, and serum was isolated after centrifugation at 2,000 X g for 15 minutes. EDTA tubes were centrifuged at 2,000 X g for 15 minutes at 4°C, and plasma was removed by aspiration. Cell-free BALF, serum, and plasma were stored at −80°C until further analysis.

**Laboratory analysis**

**Surfactant isolation, extraction, and quantitation**

As previously reported, cell-free BALF was separated into crude surfactant pellets (CSPs) and supernatant by ultracentrifugation (40,000 X g for 1 hour at 4°C). The phospholipid content of CSPs was determined by organic extraction according to the Bligh and Dyer method, followed by measurement of the lipid phosphorus concentration using spectrophotometric analysis.

**Lipidomic analysis of plasma and crude surfactant pellets**

Plasma and CSPs were dried by centrifugal vacuum evaporation and dissolved in isopropanol:methanol:chloroform (4:2:1) containing 7.5 mM ammonium acetate. Stable isotope internal standards included [2H₃]docosahexaenoic acid (DHA), [2H₄]hexacosanoic acid, [2H₃]phosphatidylethanolamines (PtdEs) 34:1, [2H₅₆]PtdEs 28:0, [2H₁₁]phosphatidylincholines (PtdCs) 34:1, [2H₅₆]PtdCs 28:0, [2H₁₁]phosphatidylserines (PA) 34:1, [2H₅₆]phosphatidylglycerols (PGs) 32:0, and bromocriptine as internal standards. Direct infusion lipidomics were used with high-resolution (0.2 to 3 ppm mass error) data acquisition, with an orbitrap mass spectrometer (Thermo Q Exactive, ThermoFisher Scientific). In negative ion electron spray ionization (ESI) mode, the anions of cPAs, ethanolamine plasmalogens, PtdEs, fatty acids, lysophosphoethanolamines, PGs, phosphatidylinositol, and phosphatidylserines were monitored. In positive ion ESI, the cations of choline plasmalogens and PtdCs, sphingomyelins, and oxidized glycerophosphocholines were quantitated, and lipid identities were validated by mass spectrometry. The cations and anions of bromocriptine were used to monitor for potential mass axis drift, ensuring the accuracy of the high-resolution mass measurements. Between injections, the transfer line was washed with successive 500 µL washes of 100% methanol and 100% hexane/ethyl acetate (3:2) to eliminate any potential memory effect from adhering lipids.

**Total protein content, SP-D, and haptoglobin**

BALF and serum SP-D concentrations were analyzed using a commercially available human SP-D ELISA kit (Biovendor). BALF samples were diluted 184X with 0.9%, saline as previously described. Serum samples were diluted 11X with the supplied buffer, according to the manufacturer’s instructions. Serum haptoglobin concentrations were measured using a commercially available equine haptoglobin kit and diluted 1:10,000 according to the manufacturer’s instructions (Mybiosource). All samples were run in triplicate, and only results with a coefficient of variation of less than 5% were accepted. The sum of serum SP-D and haptoglobin was calculated because previous studies showed it may be used to improve differentiation between horses with MEA-N and controls.

**Statistical analysis**

A paired t test was used to determine whether significant differences (P < .05) existed between each asthma group and their respective age-matched control group. Relative lipid levels are presented as R values (mean ± SEM), which represents the ratio of endogenous lipid peak intensity to the peak intensity of an appropriate internal standard. Data for SP-D were log-transformed for statistical analysis.

**Results**

**Clinical evaluation data**

Samples from 46 horses with potential EA were collected. Thirty samples met the inclusion criteria for EA whereas 16 samples were excluded because of incomplete data or the presence of another concurrent lower respiratory condition (eg, exercise-induced pulmonary hemorrhage, infection). Among the samples included in the study, 18 horses had MEA (8 horses had neutrophilic inflammation and 10 horses had eosinophilic inflammation) and 12 horses had SEA (10 of which had a neutrophilic airway inflammation whereas 2 had a normal BALF cytology but were considered pauci-cellular SEA; Table 1).

The group of horses with MEA-N included 3 Thoroughbreds, 1 Morgan, 1 Standardbred, 1 Warmblood, 1 Paint Horse, and 1 mixed breed; and consisted of 4 mares, 1 colt, and 3 geldings ranging from 2 to 20 years of age (mean ± SD, 9 ± 6 years). The age-matched group of healthy horses included 4 American Quarter Horses, 2 Thoroughbreds, 1 Haflinger, and 1 mixed breed; and consisted of 6 mares and 2 geldings ranging from 3 to 20 years of age (mean ± SD, 9 ± 6 years).

The group of horses with MEA-E included 5 American Quarter Horses, 2 Thoroughbreds, and 3 mixed-breed horses; and consisted of 1 colt and 9 mares ranging from 1 to 25 years of age (mean ± SD, 13 ± 8 years). The age-matched group of healthy horses included 4 American Quarter Horses, 4 mixed breeds, 1 Standardbred, and 1 Paint Horse; and consisted of 8 mares, 1 colt, and 1 gelding ranging from 1 to 25 years of age (mean ± SD, 13 ± 8 years).

The group of horses with SEA included 4 American Quarter Horses, 1 Morgan, 1 Paint Horse, 1 Warmblood, 1 Tennessee Walking Horse, 1 Appaloosa, 1 Arab, and 1 mixed-breed horse; and consisted of 7 mares and 5 geldings ranging from 7 to 26 years of age (mean ± SD, 20 ± 6 years). The age-matched group of healthy horses included 8 American Quarter Horses, 3 mixed breeds, and 1 Paint Horse; and consisted of 11 mares and 1 gelding ranging from 7 to 28 years of age (mean ± SD, 19 ± 6 years).
Table 1—Bronchoalveolar lavage fluid (BALF): cytology differentials, phospholipid content, and serum surfactant protein D (SP-D) concentrations for the different groups of asthmatic horses and their age-matched controls. Represented are horses with (1) mild equine asthma with neutrophilic airway inflammation (MEA-N), (2) severe equine asthma (SEA), and (3) mild equine asthma with eosinophilic airway inflammation (MEA-E).

<table>
<thead>
<tr>
<th>BALF parameters</th>
<th>Control (n = 8)</th>
<th>MEA-N (n = 8)</th>
<th>Control (n = 12)</th>
<th>SEA (n = 12)</th>
<th>Control (n = 10)</th>
<th>MEA-E (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytology</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophil (%)</td>
<td>1 ± 1</td>
<td>10 ± 4a</td>
<td>1 ± 1</td>
<td>47 ± 28a</td>
<td>0 ± 1</td>
<td>1 ± 2</td>
</tr>
<tr>
<td>Eosinophil (%)</td>
<td>0 ± 0</td>
<td>1 ± 1</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>5 ± 3a</td>
</tr>
<tr>
<td>Macrophage (%)</td>
<td>55 ± 13</td>
<td>75 ± 15a</td>
<td>49 ± 14</td>
<td>26 ± 15a</td>
<td>65 ± 20</td>
<td>61 ± 16</td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>45 ± 13</td>
<td>13 ± 15a</td>
<td>51 ± 13</td>
<td>26 ± 16a</td>
<td>35 ± 20</td>
<td>33 ± 15</td>
</tr>
<tr>
<td>Phospholipid (µg/mL BALF)</td>
<td>102 ± 42</td>
<td>84 ± 52</td>
<td>85 ± 50</td>
<td>40 ± 46a</td>
<td>117 ± 53</td>
<td>87 ± 57</td>
</tr>
<tr>
<td>SP-D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Log SP-D (ng/mL)</td>
<td>3.440 ± 0.199</td>
<td>3.503 ± 0.297</td>
<td>3.349 ± 0.211</td>
<td>3.368 ± 0.515</td>
<td>3.378 ± 0.215</td>
<td>3.438 ± 0.221</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD.

aData are significantly different from age-matched controls at P < .05.

BALF differential cell counts for each group of asthmatic horses and their age-matched controls are shown in Table 1. Median age and age range were comparable between the horses with asthma and their age-matched control groups as defined by the study design. The percentage of neutrophils on BALF cytology in the MEA-N and SEA groups, and the percentage of eosinophils on BALF cytology in the MEA-E groups was significantly greater in comparison to their respective age-matched control group (P = .000, P = .000, and P = .001; Table 1). Most MEA-N horses (8/10) had a neutrophil percentage less than 15%. Most horses with SEA (9/12) had more than 20% neutrophils.

Lipid analysis

Total phospholipid content of surfactant (CSPs)

This measurement was expressed as phospholipid content per milliliter BALF recovered for each horse, as described previously.10,26 Data for each group of asthmatic horses and their age-matched controls are presented in Table 1. Although CSP phospholipid content was significantly less in horses with SEA compared to their age-matched controls (P = .017), no significant differences were present between MEA-N or MEA-E horses compared to their age-matched controls (P = .361 and P = .178).

Surfactant lipidomic analysis

The composition of surfactant (CSPs) from horses with SEA was significantly different compared to that of age-matched control horses (Figure 1). Relative levels of the following sphingolipids were elevated significantly in surfactant from SEA (P < .01): ceramide (Cer) d18:1/16:0 (N-[hexadecanoyl]-sphing-4-enine), Cer d18:1/24:0 (N-[tetrasanoyl]-sphing-4-enine), and hydroxy ceramide (OH-Cer) d18:1/24:0 (N-[2-hydroxy-tetrasanoyl]-sphing-4-enine). Furthermore, surfactant from horses with SEA was characterized by a significant decrease in specific PG species (P < .01): PG 30:0, PG 32:1, PG 34:3, and PG 36:2. Last, relative levels of surfactant cPA 16:0 and DG 36:2 were significantly greater in surfactant from SEA horses compared to age-matched controls.

Fewer compositional changes were noted in surfactant from horses with MEA compared to their age-matched control horses (Figure 1). No significant compositional changes were seen within the class of sphingolipids in MEA-N or MEA-E horses compared to their age-matched controls. In the PG class, relative levels of PG 34:3 were decreased significantly in MEA-N but not MEA-E compared to age-matched control horses. Similar to horses with SEA, relative levels of cPA were significantly greater in MEA-N horses compared to age-matched controls. These changes were not present in horses with MEA-E.

Plasma lipidomic analysis

In plasma from horses with neutrophilic airway inflammation (both SEA and MEA-N), relative levels of cPA and phyto-Cer (40:0) were significantly greater compared to age-matched control horses (Table 2).

In plasma from horses with MEA-E, significantly greater relative levels of Cer d18:1/24:0, OH-Cer d18:1/24:0, and phyto-Cer 40:0 were present when compared to age-matched control horses (Table 2). Levels of cPA and DG in MEA-E horses and their controls were too low and too variable to be measured.

Protein analysis

In horses with SEA, serum SP-D concentrations were significantly greater compared to controls (Table 2, P = .019), and 10 of 12 horses had a serum SP-D concentration greater than 43 ng/mL. Both horses with low serum SP-D had a BALF neutrophil percentage greater than 50%. An SP-D concentration greater than 43 ng/mL was reported previously as a threshold to differentiate mildly or moderately asthmatic horses from controls.4 No significant difference was seen in BALF SP-D in SEA horses compared to control horses (P = .994). In horses with MEA-N or MEA-E, neither serum nor BALF SP-D concentrations were significantly different compared to age-matched control horses (Tables 1 and 2; MEA-N: P = .338 and P = .878, respectively; MEA-E: P = .726
and $P = .646$, respectively). Only 3 of 8 MEA-N and 3 of 10 MEA-E horses had serum SP-D concentrations greater than 43 ng/mL.

Serum haptoglobin concentrations were significantly greater in horses with SEA compared to their age-matched controls ($P = .045$), whereas no significant difference was observed in MEA-N or MEA-E horses versus control horses ($P = .302$ and $P = .244$) (data not presented).

The sum of SP-D and haptoglobin was significantly greater in the SEA group compared to age-matched controls ($P = .033$), but not in the MEA-N or MEA-E groups versus controls ($P = .148$ and $P = .406$).

Table 2—Relative levels of plasma lipids and serum surfactant protein D (SP-D) concentrations for the different groups of asthmatic horses and their age-matched controls. Represented are horses with (1) mild equine asthma with neutrophilic airway inflammation (MEA-N), (2) severe equine asthma (SEA), and (3) mild equine asthma with eosinophilic airway inflammation (MEA-E).

<table>
<thead>
<tr>
<th>Plasma or serum parameters</th>
<th>Control (n = 8)</th>
<th>MEA-N (n = 8)</th>
<th>Control (n = 12)</th>
<th>SEA (n = 12)</th>
<th>Control (n = 10)</th>
<th>MEA-E (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cPA 16:0</td>
<td>0.890 ± 0.100</td>
<td>5.660 ± 0.980</td>
<td>0.800 ± 0.093</td>
<td>1.440 ± 0.150</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>DG 36:2</td>
<td>0.510 ± 0.050</td>
<td>0.480 ± 0.085</td>
<td>0.480 ± 0.770</td>
<td>0.960 ± 0.100</td>
<td>0.500 ± 0.057</td>
<td>0.440 ± 0.048</td>
</tr>
<tr>
<td>Cer d18:1/24:0</td>
<td>0.340 ± 0.043</td>
<td>0.340 ± 0.035</td>
<td>0.380 ± 0.043</td>
<td>0.390 ± 0.028</td>
<td>0.340 ± 0.090</td>
<td>0.500 ± 0.087</td>
</tr>
<tr>
<td>OH-Cer d18:1/24:0</td>
<td>0.340 ± 0.043</td>
<td>0.330 ± 0.036</td>
<td>0.330 ± 0.035</td>
<td>0.400 ± 0.028</td>
<td>0.270 ± 0.064</td>
<td>0.530 ± 0.093</td>
</tr>
<tr>
<td>Phyt-Cer 40:0</td>
<td>0.320 ± 0.047</td>
<td>0.560 ± 0.079</td>
<td>0.340 ± 0.062</td>
<td>0.300 ± 0.054</td>
<td>0.320 ± 0.092</td>
<td>0.560 ± 0.090</td>
</tr>
<tr>
<td>Hex-Cer 24:0</td>
<td>0.320 ± 0.027</td>
<td>0.350 ± 0.036</td>
<td>0.300 ± 0.030</td>
<td>0.270 ± 0.018</td>
<td>0.260 ± 0.045</td>
<td>0.290 ± 0.022</td>
</tr>
<tr>
<td>DHC 16:0</td>
<td>0.190 ± 0.015</td>
<td>0.190 ± 0.019</td>
<td>0.120 ± 0.023</td>
<td>0.200 ± 0.016</td>
<td>0.180 ± 0.029</td>
<td>0.250 ± 0.022</td>
</tr>
<tr>
<td>SP-D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Log SP-D (ng/mL)</td>
<td>1.403 ± 0.228</td>
<td>1.594 ± 0.400</td>
<td>1.468 ± 0.406</td>
<td>1.961 ± 0.392</td>
<td>1.609 ± 0.421</td>
<td>1.553 ± 0.377</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD.

Cer = Ceramide; cPA = Cyclic phosphatidic acid; DG = Diacylglycerol; DHC = Dihydroceramides; Hex-Cer = Hexosyl ceramide; NA = not applicable (insufficient number of results); OH-Cer = Hydroxy ceramide; Phyt-Cer = Phyto ceramide.

*Data are significantly different from age-matched controls at $P < .01$. Each group of asthmatic horses had their specific age-matched control group. The same hatch pattern is used for all control groups. Cer = Ceramide.

Please refer to the explanation for shorthand notation at the beginning of this article.
Discussion

Surfactant from horses with SEA was significantly altered in lipid content and composition. In comparison, only a few compositional surfactant changes were observed in horses with MEA-N. Interestingly, horses from all asthma groups had significant changes in their plasma lipidomic profile when compared to age-matched control horses. Data from a larger population of asthmatic horses are needed to determine whether these changes could be used for diagnostic, prognostic, or therapeutic purposes. The following discussion addresses some of the potential causes and consequences of these lipid alterations.

A deficit in surfactant phospholipid content was present in SEA but not MEA-N affected horses. This confirms our previous findings of a surfactant deficit in horses with SEA. A surfactant deficit can be caused by 3 main mechanisms: an immaturity of type 2 alveolar cells (ie, premature animals), an injury to type 2 alveolar cells, or excessive surfactant degradation. Degeneration of type 2 alveolar cells and accumulation of lamellar bodies have been reported in SEA. Neutrophilic airway inflammation and subsequent surfactant degradation via enzymes and oxidative stress are also conceivable. Disease duration, inflammatory response, and tissue remodeling are more pronounced in SEA compared to MEA and may explain the surfactant deficit in SEA horses.

Compositional surfactant changes were most pronounced in SEA horses, but were also present in MEA-N horses. Phospholipid molecular species in the PG class and cPA/DG differed between the SEA and MEA-N groups compared to their age-matched control groups. Surfactant in SEA was also characterized by alterations within the class of sphingolipids, notably by increases in several Cer molecular species. Each of these compositional changes are contextualized in the following paragraphs.

In the class of surfactant sphingolipids, significant increases were observed in 2 ceramides (Cer d18:1/16:0 and Cer d18:1/24:0), 1 dihydroceramide (Cer 18:0/16:0), and 1 OH-Cer (OH-Cer d18:1/24:0), but only in SEA. Sphingolipids represent a relatively small percentage in the spectrum of lipid classes contained in lung surfactant. Nevertheless, they contribute to essential functions such as membrane integrity, intracellular signaling, and mediation of cellular processes. Imbalances in sphingolipids have been implicated in the pathophysiology of lung diseases in people, including human asthma. Ceramides are a crucial component in the metabolism of sphingolipids. Ceramides with 16 and 24 carbon fatty acid chains, which were the most affected in our study, are known to be the most abundant ceramides in the lungs. Increased ceramide levels have been described in rodent models of asthma. Similar to findings in our study, ceramides (Cer 18:1/16:0, Cer 18:1/24:0) and dihydroceramide (Cer 18:0/16:0) were elevated in the lung and BALF of mice challenged with house dust mites. A recent study showed increased ceramide levels in human patients with severe asthma and a possible correlation with disease severity. Increased pulmonary ceramide levels could be the result of augmented de novo synthesis, amplified sphingomyelinase activity, or enhanced recycling. Studies in human medicine and rodent asthma models suggest that increased de novo synthesis is an important contributor. Reasons for elevated surfactant ceramide levels in horses with SEA remain to be determined.

In the class of surfactant PGs, multiple molecular species were decreased in SEA (PG 30:0, PG 32:1, PG 34:3, and PG 36:2) whereas only one (PG 34:3) was decreased in MEA-N. PG is the second most abundant phospholipid class in surfactant after phosphatidylcholine. Its concentration in the lung is greater compared to other tissues and it can serve as a marker for surfactant maturity in neonatal infants. A reduction of surfactant PG was reported previously in horses affected clinically by SEA and in some human patients with asthma. PG plays a role in biophysical properties of surfactant, such as adsorption at the air–liquid interface and film stabilization during the respiratory cycle. In addition, it participates in innate immune functions of surfactant. The predominant molecular species of PG in surfactant is PG 16:0/18:1. Alterations in the PG profile of asthmatic horses could be related to the action of secretory phospholipases, damage by oxidative stress, or degradation through other inflammatory processes. Further studies are needed to assess these possibilities. A decrease in select PG molecular species may affect surfactant properties, such as PG’s role in decreasing surface tension (which would exacerbate bronchoconstriction) or its role in immune modulation (which may increase susceptibility to infection).

Relative levels of cPA were significantly greater in the surfactant and plasma from horses with neutrophilic airway inflammation (SEA and MEA-N). Interestingly, the increase in plasma cPA levels was approximately 5 times greater in MEA-N compared to SEA. We previously reported increased cPA and DG levels in surfactant from asthmatic horses, but did not perform plasma analysis at the time. Reports in horses indicate that these markers are not specific to asthma because they may be elevated in some infections or ongoing inflammation. Phospholipid synthesis and hydrolysis yields cPA, lysophosphatidic acid (LPA), and DG, which intervene in lipid signaling and modulation. In human patients, polyunsaturated LPA and autotaxin levels are elevated in BALF, but not in plasma, of asthmatic subjects. The autotaxin and LPA pathways play a role in immune regulation and asthma. cPA is a natural analog of LPA and targets some of the same receptors; its effects are similar or opposite to those exerted by LPA. The role of cPA in EA remains to be determined.

One ceramide—namely, phytoceramide 40:0 (phyto-Cer t18:0/22:0 and/or phyto-Cer t20:0/20:0)—was increased significantly in the plasma from all EA groups. Phytoceramides are major sphingolipids in plants and fungi, and as such we cannot exclude dietary influences on the levels we measured.
However, vertebrates do synthesize this subclass of sphingolipids, and these lipids have demonstrated cytoprotective properties. Therefore, the increased levels of phytoceramide we measured may be reflective of a compensatory biochemical mechanism to reduce the inflammation associated with EA. However, the origin and significance of elevated phytoceramide levels in asthmatic horses remains to be determined. In addition, 2 other plasma sphingolipid species (Cer d18:1/24:0 and OH-Cer d18:1/24:0) were significantly greater in the MEA-E group. In human medicine, several studies have looked at sphingolipid levels in blood in relation to inflammatory phenotypes or asthma-risk genotypes. The study by Gy et al. illustrated that SP-D concentrations are significantly greater (> 43 ng/mL) in MEA-N horses versus control horses if airflow inflammation leads to a BALF neutrophil percentage of at least 15%. The majority of SEA horses in our study had a BALF neutrophil percentage of more than 20% and serum SP-D values greater than 43 ng/mL. It is likely that elevated serum SP-D concentrations in EA are tied to the severity of neutrophilic airway inflammation and may be less useful in horses with mild BALF neutrophilia and those with other inflammatory patterns. Causes of increased serum SP-D concentrations in pulmonary diseases include increased alveolocapillary membrane permeability, altered SP-D synthesis or clearance, or changes in its molecular structure. The study did not reveal any significant changes in BALF SP-D concentrations between asthmatic and control horses, making an increase in local SP-D production unlikely.

Our study had several limitations. Horses were age matched, but not necessarily matched regarding other individual factors (ie, sex, body condition score) or environmental factors (ie, housing, nutrition). Although finding a perfect match with regard to individual and environmental factors is desirable, it is very difficult to obtain in practice because it implies sampling a healthy horse from the same client or barn. The sample size in each of the horse groups was small. This was a result of the stringent selection criteria for cases and the time needed to obtain enough samples from participating institutions. Correction for multiple analysis was not performed in our study. This correction would have increased the probability of a type 2 error in our analysis, and thus the likelihood of a false-negative finding. Last, the degree of BALF neutrophilia—particularly in the group of mildly asthmatic horses—was low, because we used the 5% mark as a cutoff between healthy horses versus asthmatic horses. Despite this fact, it was interesting for us to see that even horses with mild neutrophilia had significant changes in the plasma and surfactant lipidomic profile.

In conclusion, surfactant from horses with severe asthma was characterized by significant changes in phospholipid content and composition, whereas only a few compositional alterations were seen in horses with mild asthma. The plasma lipidomic profile was altered significantly in asthmatic horses compared to age-matched controls. The plasma lipidomic profile also varied depending on the BALF inflammatory profile present. These changes warrant further investigation on a larger and more diverse group of asthmatic horses.

Acknowledgments

This study was funded by a grant provided by the Lincoln Memorial University College of Veterinary Medicine. The authors declare that there were no conflicts of interest.

No third-party funding or support was received in connection with this study or the writing or publication of the manuscript.

We thank the veterinarians, students, staff, and horses involved from both Gluck Equine Research Center and Lincoln Memorial University College of Veterinary Medicine for their help throughout the project.

References


AJVR
obtained from asthmatic horses exposed to hay. Am J Vet Res. 2019;80(3):300–305. doi:10.2460/ajvr.80.3.300


