Mild equine asthma is frequently encountered in performance and pleasure horses,1–3 and the disease has been related to respirable organic dust exposure.1,4 Current treatments rely principally on a combination of pharmacological and environmental management. Drugs used to control airway inflammation are mainly glucocorticoids; however, treatment may not be effective,5 and their use in competing horses is complicated by potential adverse effects and drug residues. Therefore, control and prevention of equine asthma without pharmacologic intervention are key.6

The main source of dust exposure to horses is hay, the most commonly used forage in horses.4 Feeding horses low-dust forages, such as hay pellets or haylage, can decrease dust exposure in the horse’s breathing zone by 60% to 70% when compared with hay and by 90% when compared with poor-quality hay, respectively.7,8 But the resolution of neutrophilic inflammation may require months of low-dust conditions.9,10 Airway inflammation in human asthmatics is thought to reflect a failure to resolve inflammation, thereby preventing a return to homeostasis after an inflammatory trigger.11

The fatty acid composition of an individual’s diet influences the formation of inflammatory mediators related to many chronic diseases, such as...
asthma in humans. Omega-3 polyunsaturated fatty acid (Ω-3) and omega-6 polyunsaturated fatty acid (Ω-6) intake determines cell membrane composition and differences in the dietary intake of these fatty acids can modify downstream production of pro- and anti-inflammatory mediators. Proresolving lipid mediators (PRLMs) derived from Ω-3 are central to the resolution of inflammation, at least in part owing to increased apoptosis and clearance of inflammatory neutrophils by efferocytosis. In humans, lipoxin A₄ (LxA₄) and resolvin E₁ (RvE₁) increase neutrophil apoptosis in vitro. In murine models of airway inflammation, resolvin D₁ (RvD₁) decreases neutrophilic inflammation and enhances efferocytosis. To our knowledge, the role of PRLMs in the resolution of airway inflammation in asthmatic horses has not been reported.

Haylage is more abundant in Ω-3, compared with hay; therefore, it may provide additional benefit to equine airway health beyond that of reducing dust exposure. The purpose of this study was to compare airway inflammation and plasma PRLM concentration in horses transitioned from a high-dust, low Ω-3 diet (hay) to a low-dust, high Ω-3 diet (haylage) or low-dust, low Ω-3 diet (hay pellets) and to determine the effect of PRLMs on apoptosis and efferocytosis of equine neutrophils in vitro.

We hypothesized that horses transitioning from a high-dust, low Ω-3 diet to a low-dust, high Ω-3 diet would exhibit a faster resolution of bronchoalveolar lavage fluid (BALF) neutrophilia than horses transitioned to a low-dust, low Ω-3 diet (hay pellets), and that horses fed with the diet high in Ω-3 will have higher plasma concentrations of PRLM. Furthermore, we hypothesized that PRLMs (LxA₄, RvD₁, and RvE₁) would increase the apoptosis of equine neutrophils and their efferocytosis by alveolar macrophages in vitro.

Materials and Methods

Study design

A prospective trial was conducted with 20 healthy horses from the university teaching herd. Fifteen mares and 5 geldings with a mean ± SD age of 16 ± 6 years and a mean weight of 485 ± 41 kg were kept in dry lots and had free access to round bales of hay from covered feeders for at least 6 weeks prior to the start of the study. The horses were housed in 2 separate dry lots at the same facility but did not share a fence line. The study was performed during the winter (February and March 2019), so the dry lots remained free of grass. Horses were not randomized to groups but rather allocated by housing requirements to be fed grass-alfalfa mix haylage (n = 10) or hay pellets (10) for 6 weeks. The horses fed haylage were introduced to the new forage gradually over 7 days. Horses in the hay pellet group were fed timothy-alfalfa mix pellets (DuMor; Purina Animal Nutrition). Horses were fed forage based on an estimated intake of approximately 2% of body weight/d. They had free access to clean water. Baseline assessment conducted during the period of round bale hay feeding consisted of physical examination, bronchoalveolar lavage (BAL), and blood collection on each horse. Horses were deemed healthy based on a normal physical examination findings and history of normal respiratory health in at least the past 2 years. Sample collection was repeated after 3 and 6 weeks on the new forage (haylage or hay pellets). The Purdue University Animal Care and Use Committee approved all procedures (protocol No. 1111000181).

Clinical score

Horses were trailered to the laboratory located 2 miles from the farm and allowed at least 30 minutes to acclimate before the examination. On physical examination, a clinical score (range, 0 to 21) based on cough, nasal discharge, respiratory efforts, and auscultation was determined as previously described.

Blood collection and processing

Blood was collected by jugular venipuncture with a vacutainer into evacuated tubes containing EDTA. Within 1 hour of blood collection, samples were centrifuged at 1,500 X g at room temperature for 10 minutes. Plasma was aspirated carefully and placed into plastic tubes with no additives. Plasma was stored at −80 °C until lipid quantification was performed.

BAL

Horses were sedated by administration of butorphanol (0.02 mg/kg, IV) and xylazine hydrochloride (0.2 to 0.5 mg/kg, IV). A sterile BAL tube (Bivona Medical Technologies; 300-cm length; 10-mm outer diameter) was passed through the nose and wedged into a distal bronchus. Two hundred fifty milliliters of sterile saline (0.9% NaCl) solution was instilled and recovered into 1 aliquot by use of 60-mL syringes. The BALF was immediately placed on ice and processed within 1 hour of collection. Total cell count was measured with a hemocytometer. Cytospin slides were prepared and processed with modified Wright stain. Differential cell counts were determined by enumerating 600 cells/horse by a single individual unaware of forage assignment (CJO). Horses were returned to the farm once recovered from sedation.

Particulate exposure measurements

Exposures to particulate matter (PM) smaller or equal to 1 μm (PM₁), PM smaller than 2.5 μm (PM₂.₅), and PM smaller than 10 μm (PM₁₀) in the horse’s breathing zone were measured with a real-time particulate monitor (OPC-N₂2; Alphasense) for 20 minutes on 2 occasions: when the horses ate dry hay from round bales and while the horses ate the assigned low-dust forage (hay pellets or haylage). The monitor was secured to the horse’s neck using a breakaway halter, and the inlet of the sampling tube was secured to the noseband of the halter to sample dust at the breathing zone of the horse. The horse was free to move around, eat, and drink as usual (Supplementary Appendix S1).
Plasma lipids quantification

Lipid mediators were analyzed using targeted liquid chromatography–tandem mass spectrometry (LC-TMS) from plasma samples at the Metabolite Profiling Facility, Discovery Park, Purdue University. Five hundred microliters of plasma was transferred to a 5-mL vial and spiked with standards including 500 pg of LxA4-d5 (Cayman Chemical), 500 pg of RvD1-d5 (Cayman Chemical), 2,500 pg of prostaglandin E₂ (PGE₂; PGE₂-d4; Cayman Chemical), and 250 pg of RvE1-d4 (Cayman Chemical). Methanol (2 mL) was added to the spiked samples to extract lipids from the plasma. Samples were vortexed for 1 minute and centrifuged at 20,913 X g for 10 minutes to precipitate the proteins. The supernatant was collected and transferred to a new vial to be evaporated and stored at –80 °C until analysis. The dried lipid extracts were reconstituted with 50 μL of methanol and water at a 1:1 volume ratio and submitted for targeted quantification by LC-TMS. Twenty microliters of the reconstituted sample was delivered to a column (ACQUITY UPLC BEH C18; Waters Corp; 1.7 μm; 2.1 X 100 mm) through a multisampler (G7167B: Agilent Technologies) into a triple quadrupole mass spectrometer (QQQ6470A MassHunter B.06.00; Agilent Technologies) equipped with electrospray ionization jet stream ion source (Agilent Technologies). The binary pump flow rate was set at 0.3 mL/min by use of water and 0.1% formic acid as mobile phase A and acetonitrile and 0.1% formic acid as mobile phase B. The liquid chromatography column was pre-equilibrated for 1 minute with 20% B, and a linear gradient to 100% B was set in 28 minutes and then returned to 20% B in 2 minutes and re-equilibrated for 3 minutes. Concentrations in ng/mL of plasma were obtained by calculating the ratio of the areas of the endogenous and the deuterated internal standard, then multiplied by the concentration of the internal standard. For molecules without deuterated internal standards, calibration curves were done with 5 serial dilutions of the stock solution starting at 100 µg/mL as the highest concentration with a dynamic range and linear ion intensity response (R² = 0.99) of the calibration curves were observed for over 4 orders of magnitude. Limit of detection (LOD) and limit of quantification (LOQ) of the analyzed compounds were as follow: palmitic, palmitoleic, stearic, oleic, linoleic acids had an LOD of 1 ng/mL and LOQ of 5 ng/mL; linolenic acid had an LOD of 0.1 ng/mL and LOQ of 1 ng/mL; arachidonic acid, eicosapentaenoic acid (EPA) and DHA had an LOD of 0.01 ng/mL and LOQ of 0.1 ng/mL; PGE₂ and RvD1 had an LOD of 0.1 and 0.05 ng/mL and LOQ of 1 and 0.3 ng/mL, respectively; an LOD of 0.5 ng/mL was obtain for RvE1 and 0.25 ng/mL for protectin D1, maresin 1, LxA4, no LOQ was calculated for these last compounds. Data processing was performed by use of dedicated software (Agilent Technologies).

In vitro assessment of neutrophilic apoptosis

Neutrophils were collected from blood samples obtained by jugular venipuncture and isolated with a discontinuous density gradient (Percoll; Sigma-Aldrich Corp) and underwent centrifugation within 2 hours of collection. One aliquot of neutrophils was incubated for 30 minutes in culture media only (RPMI-1640; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Hyclone; Thermo Fisher Scientific) as a negative control, or culture media spiked with a PRLM (i.e., LXA4 at 100nM, RvE1 at 10nM, or RvD1 at 10nM), or phorbol myristate acetate (Thermo Fisher Scientific) at 100nM as a positive control to cause neutrophil apoptosis at 37 °C in 24-well cell culture plates. Concentrations of PRLMs were selected based on similar studies in other species. After incubation, the neutrophils were washed and resuspended in PBS solution. Apoptosis was quantified by flow cytometry (CytoFlex; Beckman Coulter) with a fluorescein isothiocyanate annexin V apoptosis detection kit (BD Biosciences) and propidium iodide was used to exclude dead cells following the manufacturer’s instructions. Gating was done manually by use of unstained controls and a dedicated software (PlateAnalyzer; Purdue University Cytometry Laboratories). Cells positive for both green and violet stains were considered macrophages that had phagocytosed neutrophils.

In vitro assessment of efferocytosis

Alveolar macrophages were isolated from BALF by cell culture in complete medium (RPMI-1640; Thermo Fisher Scientific) for 4 hours at 37 °C in a humid chamber with 5% CO₂, as previously described. Neutrophils isolated the day before (as already described) were incubated in complete medium (RPMI-1640; Thermo Fisher Scientific) for 16 hours at 37 °C in a humid chamber with 5% CO₂ in 24-well cell culture plates to allow the neutrophils to age and become naturally apoptotic as previously described. After the incubation period, neutrophils were stained green (Celltracker; Thermo Fisher Scientific) and alveolar macrophages stained stained violet (Tag-it Violet; Biologend) following manufacturer’s instructions. Stained neutrophils were coincubated with stained alveolar macrophages from the same animal in 24-well cell culture plates, at an approximate ratio of 3:1. Cells were counted by use of a hemocytometer to achieve the appropriate ratio. Cells were coincubated and treated with PRLM (LXA4 [100nM], RvE1 [10nM], or RvD1 [10nM]) for 30 minutes at 37 °C with 5% CO₂. Following incubation, cells were washed with PBS solution by use of a 1,000-µL pipette twice to eliminate all the neutrophils that were not phagocytized. Cells were detached from the plate with a cell scraper (Thermo Fisher Scientific). Samples were analyzed by use flow cytometry (CytoFlex; Beckman Coulter), and data analyses were performed with dedicated software (Purdue University Cytometry Laboratories).

Statistical analysis

An a priori sample size calculation indicated that a sample size of 20 horses (10 horses/for-age group) would provide 80% power to detect a clinically relevant difference of 5% in BALF neutro-
phil proportions between groups at a significance level of $\alpha = 0.05$. Data analyses and graphs were performed with various computer software packages (ProcGLIMMIX version 9.4; SAS Institute; GraphPad Prism version 8.4.2; GraphPad Software Inc; MetaboAnalyst version 3.0; Wishart Research Group). An exploratory analysis of correlations between the various lipid mediators, age, BALF neutrophil proportion, and BALF total nucleated cell count at baseline was performed by calculating Spearman rank correlations with unadjusted $P$ values. Generalized linear models were constructed to examine the effect of forage assignment on BALF cytology over time and to compare in vitro neutrophil apoptosis and efferocytosis between forage groups and in vitro treatments. All models were controlled for age. Tukey-adjusted values of $P < 0.05$ were considered significant.

Results

Horses

One horse from the haylage group was removed from the study because of the development of pneumonia during study day 42 (week 6). Both feeding protocols were well tolerated by the horses, and no adverse effects were observed during the study. The mean age (95% CI) of the horses from the pellet and haylage groups was 17.4 (14.4 to 20.4) years and 14.9 (11.9 to 17.9) years, respectively. Clinical score (mean [95% CI]) was not different between groups at baseline (pellet = 3.9 [2.8 to 5.5]; haylage = 5.2 [3.8 to 7.1]; $P = 0.541$) or week 6 (pellet = 1.9 [1.0 to 3.5]; haylage = 4.6 [3.1 to 7.0]; $P = 0.083$; Supplementary Appendix S2) but was significantly ($P = 0.025$) lower in the horses fed pellets at week 6 than at baseline.

Dust exposure

Breathing-zone measurements of PM1, PM2.5, and PM10 were available for 8 horses when eating from hay in round bales. Four of these horses were subsequently assigned to the hay pellet group, and 4 were assigned to the haylage group. Breathing-zone measurements of exposure were obtained for 10 horses while eating pellets and 9 horses eating haylage. Dust exposures to PM1, PM2.5, and PM10 in the horses’ breathing zone were significantly higher when horses were eating round bales of hay (baseline measurement; $P < 0.005$) than when consuming hay pellets or haylage (week 6 measurement; Figure 1) but were not different between horses eating pellets and haylage ($P > 0.055$; Supplementary Appendix S3).

Airway cytology

Horses eating hay from round bales exhibited a mild degree of neutrophilic airway inflammation (BALF neutrophils = 12%). Horses fed haylage experienced a marked and gradual decrease in BALF neutrophil proportions between baseline and week 6 ($P = 0.002$), while horses fed pellets experienced a mild but nonsignificant decrease ($P = 0.283$; Figure 2; Supplementary Appendix S4). At week 6, horses eating haylage had significantly lower BALF neutrophil proportions than those eating pellets ($P = 0.014$). BALF cytology data for macrophages, lymphocytes, mast cells, and eosinophils showed no effect of time or forage (Table 1).

Plasma lipid quantification

The only quantifiable PRLM was RvD1. The other targeted PRLMs, including RvE1, LxA4, ma-

![Figure 1](image-url)
resin 1, and protectin D1, were under the LOD (0.5 ng/mL). No effect of forage or time was observed on RvD1, arachidonic acid, EPA, docosahexaenoic acid (DHA), oleic acid, linoleic acid, palmitoleic acid, palmitic acid, or PGE2 (P > 0.250; Supplementary Appendix S5). Stearic acid concentration in plasma was affected by forage over time (P = 0.046), with a significant decrease at week 6 in horses eating haylage when compared with baseline (P = 0.048; Figure 3). Linolenic acid concentration decreased over time regardless of forage assignment (P = 0.010).

There was a negative correlation between PGE2 and the age of the horses (r[16] = –0.486; P = 0.041). Both DHA and EPA were positively correlated to each other, and DHA was negatively correlated with BALF total nucleated cell count (Supplementary Appendix S6). Plasma concentrations of palmitoleic acid, palmitic acid, oleic acid, linoleic acid, arachidonic acid, and stearic acid were significantly correlated (Supplementary Appendix S7).

### In vitro determination of apoptosis and efferocytosis

Apoptosis did not differ between forage groups (P = 0.496) or time points (P = 0.624), nor did it vary with any in vitro PRLM treatment (P > 0.950). The only effect of treatment was observed with an increase in apoptosis with phorbol myristate acetate (positive control), compared with effects of no treatment in both groups (P < 0.001; Figure 4: Supplementary Appendix S8).

Overnight incubation in culture media resulted in apoptosis in roughly 50% of isolated neutrophils (fluorescein isothiocyanate–annexin V apoptosis.
As anticipated, breathing-zone measurements of PM1, PM2.5, and PM10 were not different between low-dust forages, and both pelleted hay and haylage resulted in significantly lower dust exposure when compared with results for the round bale hay. Exposure to PM2.5 and PM10 has been related to visible tracheal mucus score in racehorses. Clinical score did not differ between groups at baseline or week 6, but it was significantly lower at week 6 in horses fed pellets when compared to the same group at baseline. The decrease in clinical score was due to changes in abdominal lift and nostril flare. These scores ranged from 0 (absent) to 1 (mild) in all horses, regardless of forage assignment. With the exception of one, all horses in the pellet group had a score of 0 assigned to both abdominal lift and nasal flare at week 6; however, this finding was unlikely to be clinically relevant and the decrease in clinical score in the group fed pellets was presumably related to lower stress level or part of random variation. Future studies with a larger number of horses will be needed to confirm this result. All horses displayed mild BALF neutrophilia at baseline. These horses were kept on dry lots during the winter and fed round bales of hay for at least 6 weeks prior to the study. Similar results have been described in a population of horses eating round bales of hay during winter, in which 80% of the horses presented with a mild to moderate increase in BALF neutrophil proportions; however, dust exposure was not measured. After 6 weeks on low-dust diets, both groups of horses in the present study showed a decrease in BALF neutrophil proportions, but this decrease was only statistically and clinically significant in horses eating haylage. In previous studies, horses19,10 with severe equine asthma exacerbation placed on a low-dust diet (pasture and pelleted feed) demonstrated an improvement in BAL neutrophilia, but only after 2 to 6 months. Horses with severe equine asthma kept on grass pasture, a diet naturally higher in Ω-3 than dried forages, may exhibit resolution of BALF neutrophilia within 2 months. Similarly, in another study,16 horses with severe asthma eating a low-dust diet (complete pelleted diet) and an Ω-3 supplement for 2 months display significantly greater improvement in clinical signs and BAL neutrophilia when compared with a group that received the low-dust diet with a placebo supplement. Taken together, these results suggest that the presumed higher content in Ω-3 of the haylage fed in the present study contributed to the rapid resolution of BALF neutrophilia, similar to that seen with Ω-3 supplementation and access to pasture.

We were unable to detect most of the targeted PRLMs using LC-TMS. This technique has been used to measure plasma concentrations of PRLM in humans and rodents, but the detection of these molecules remains challenging. These mediators display bioactivity at concentrations in the picomolar and lower nanomolar ranges. The PRLMs such as LXA4, RvD1, RvE1, maresin 1, and protectin D1 may have been present at concentrations and activ-
itly that differed between forage groups but were below our LOD. We were able to measure only plasma concentrations of RvD1 from the horses in this study, and 20 samples were under the LOD (50 pg/mL). The mean plasma concentration at baseline in the horses was 142 ± 169 pg/mL; this concentration is higher than those previously described in humans (24.4 ± 2.5 pg/mL). The RvD1 concentration did not change with the haylage, despite the presumed higher content of Ω-3. The failure to detect a statistical difference between groups is likely due to the high variability of the measurements and relatively small sample size. The only plasma lipid affected by forage was stearic acid, which decreased significantly with the consumption of haylage. Stearic acid is a saturated fatty acid present in forages such as hay and has been reported to increase as forages become more mature. A possibility is that pellets were made from a more mature alfalfa-grass hay mixture compared with haylage. Stearic acid has been associated with inflammatory processes such as osteoarthritis and obesity in human. In vitro studies indicate a proapoptotic effect of stearic acid on macrophages and the capacity to enhance the production of oxygen radicals by neutrophils. Stearic acid was correlated with oleic acid and arachidonic acid, which are lipid mediators associated with inflammatory processes in humans. Thus, compared with pellets, the effect of haylage on stearic acid concentration may reflect decreased dietary intake of this lipid, a potential role of this molecule in airway inflammation, or both.

Prostaglandin E2, a fatty acid that is derived from arachidonic acid, was negatively correlated with age at baseline. The opposite has been reported in humans and mice with an increase in PGE2 production seen with aging. This increase in synthesis has been related to various chronic diseases in elderly humans, such as arthritis, and cancer. On the other hand, PGE2 has been described to prevent allergen-induced bronchoconstriction and to reduce airway hyper-responsiveness and inflammation in bronchial asthma in humans. In horses with severe asthma, diminished production of PGE2 by airway mucosa has been reported, while increased BALF PGE2 concentration has also been found. In the present study, plasma PGE2 concentrations decreased after 6 weeks in the horses fed haylage, but the change did not reach statistical significance. Again, the lack of statistical significance could be related to the small sample size and the high variability of the measurements.

Decreased neutrophilic airway inflammation over the 6-week course of the study as a result of low-dust exposure was expected to be secondary to enhanced neutrophil apoptosis and efferocytosis mediated by PRLMs. Horses fed haylage did indeed exhibit improved resolution of BAL neutrophilia, but we did not find increased neutrophil apoptosis nor increased efferocytosis in this group. Instead, horses on the low Ω-3 pellet diet demonstrated greater alveolar macrophage efferocytosis, potentially indicating that alveolar macrophages maintained an activated state in horses fed pellets due to continued inflammation, while resolution of inflammation in the haylage group resulted in a more quiescent population of alveolar macrophages. Neither apoptosis of neutrophils harvested from the systemic circulation nor efferocytosis of apoptotic neutrophils by alveolar macrophages was affected by PRLM treatments at the concentrations and incubation times used in this study. PRLMs have been reported to affect both apoptosis and efferocytosis in vitro in other animal models and humans. The concentrations of the PRLM and incubation times used in the present study were reportedly effective in previous studies with rodent or human cells, but may not have been sufficient for equine cells. Another potential explanation is that multiple PRLMs are needed to work in concert. Alternatively, other PRLMs such as maresins may be more important in resolving inflammation in the horse. It is also possible that the accelerated resolution of inflammation observed in the horses fed haylage in this study was mainly due to inhibition of transendothelial migration of neutrophils to the lungs by PRLMs. Such an effect would not be apparent with the type of in vitro studies performed in this study.

A limitation of this study was that the groups were made by convenience and not randomized. Other unobserved differences between the groups may have obscured the effect of forage in this study. This latter possibility is unlikely since horses were studied at the same time and were kept in paddocks in close proximity. However, a randomized, crossover study design would have been best to reduce confounding factors. Also, as previously noted, the plasma lipid detection method may not have provided the necessary sensitivity to detect differences in PRLM between the groups, as evidenced by our inability to detect most of the targeted molecules. Larger sample size may have enabled us to detect an effect of forage on those molecules we were able to measure. Finally, Ω-3 and Ω-6 are important components of cell walls. In other species, such as felines and horses, DHA and EPA have been detected in RBCs at higher concentrations than in plasma. Therefore, measuring the concentration of these molecules in RBCs, rather than plasma, could be considered for future studies.

In conclusion, horses transitioning from feeding round bale hay to haylage and pellet fed haylage had a comparable reduction in dust exposure, but only those fed haylage for 6 weeks experienced a significant reduction in BALF neutrophilia. This clinical effect was not accompanied by an in vitro effect of PRLM treatments on neutrophil apoptosis or efferocytosis. The reduction in neutrophilic airway inflammation exhibited by horses fed haylage is greater than expected by the decreased dust exposure alone; however, the mechanism remains unclear.

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Supplementary Materials

Supplementary materials are posted online at the journal website: avmajournals.avma.org