ORIGINAL RESEARCH

Modeling the pasture-associated severe equine asthma bronchoalveolar lavage fluid proteome identifies molecular events mediating neutrophilic airway inflammation

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Background: Pasture-associated severe equine asthma is a warm season, environmentally-induced respiratory disease characterized by reversible airway obstruction, persistent and non-specific airway hyper-responsiveness, and chronic neutrophilic airway inflammation. During seasonal exacerbation, signs vary from mild to life-threatening episodes of wheezing, coughing, and chronic debilitating labored breathing.

Purpose: In human asthma, neutrophilic airway inflammation is associated with more severe and steroid-refractory asthma phenotypes, highlighting a need to decipher the mechanistic basis of this disease characteristic. We hypothesize that the collective biological activities of proteins in bronchoalveolar lavage fluid (BALF) of horses with pasture-associated severe asthma predict changes in neutrophil functions that contribute to airway neutrophilic inflammation.

Methods: Using shotgun proteomics, we identified 1,003 unique proteins in cell-free BALF from six horses experiencing asthma exacerbation and six control herdmates. Contributions of each protein to ten neutrophil functions were modeled using manual biocuration to determine each protein's net effect on the respective neutrophil functions.

Results: A total of 417 proteins were unique to asthmatic horses, 472 proteins were unique to control horses ($p<0.05$), and 114 proteins were common in both groups. Proteins whose biological activities are responsible for increasing neutrophil migration, chemotaxis, cell spreading, transmigration, and infiltration, which would collectively bring neutrophils to airways, were over-represented in the BALF of asthmatic relative to control horses. By contrast, proteins whose biological activities support neutrophil activation, adhesion, phagocytosis, respiratory burst, and apoptosis, which would collectively shorten neutrophil lifespan, were under-represented in BALF of asthmatic relative to control horses. Interaction networks generated using Ingenuity® Pathways Analysis further support the results of our biocuration.

Conclusion: Congruent with our hypothesis, the collective biological functions represented in differentially expressed proteins of BALF from horses with pasture-associated severe asthma support neutrophilic airway inflammation. This illustrates the utility of systems modeling to organize functional genomics data in a manner that characterizes complex molecular events associated with clinically relevant disease.

Keywords: pasture-associated severe equine asthma, *Equus caballus*, horse, proteomics, neutrophil functional genomics

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Introduction

Human asthma is a heterogenous disease with varied clinical phenotypes.[1] Though Th2-dominated asthma characterized by eosinophilic inflammation[2,3] has been well recognized for decades, Th17-mediated neutrophilic airway inflammation has been increasingly recognized in a sizeable group of asthmatic human patients.[4–6] However, neutrophilic asthma and eosinophilic asthma are not mutually exclusive. Associations have been established between airway neutrophilic inflammation and increased severity of signs in severe asthma,[7–9] corticosteroid-resistant asthma,[10–12] asthma exacerbations,[13] nocturnal asthma,[14] "asthma in smokers",[15] occupational asthma,[16] and "sudden onset" fatal asthma.[17] These associations between neutrophils and asthma severity in humans highlight a need to understand how neutrophils contribute to asthma pathophysiology in order to identify new and effective therapeutics.[18,19]

Two naturally occurring, environmentally-induced asthma syndromes affect outbred populations of animals: severe equine asthma and feline asthma.[20,21] Two forms of severe equine asthma are recognized: a barn-associated form, commonly referred to as "heaves", is exacerbated by mold and dust when horses are maintained in stalls in continental climates,[22] while a pasture-associated form (aka recurrent airway obstruction, summer pasture associated recurrent airway obstruction) affects horses grazing pastures during conditions of high heat and humidity and was originally described in the southeastern United States.[23] Airway inflammation in both barn-associated, and pasture-associated equine asthma, may be devoid of eosinophils, and is consistently and predominantly neutrophilic.[22,24–28] Other features shared with human asthma include genetic linkage,[25] reversible bronchoconstriction triggered by environmental aeroallergen challenge, airway remodeling, airway hyper-responsiveness, chronic airway inflammation, and responsiveness to β2-adrenoceptor agonists and corticosteroids. These clinical parallels have led to the recognition of equine asthma as a unique spontaneous model of neutrophilic asthma.[22,24–27,29–32]

Neutrophilic inflammation has been demonstrated to correlate with poor outcome in other chronic human lung diseases in addition to asthma, including bronchiolitis obliterans in lung transplant recipients,[33] and COPD,[34] where the correlation is independent of viral or bacterial infection. Neutrophil elastase also correlates with declining lung function in cystic fibrosis.[35] Neutrophilic inflammation has been linked to poor prognosis in other organs, for example, intracranial hemorrhage,[36] alcoholic hepatitis,[37] myocardial infarction,[38–42] pulmonary embolism,[43] peripheral vascular disease following angioplasty, tumor recurrence in hepatocellular carcinoma,[44,45] colorectal tumors,[46] and lung cancer.[47] Accordingly, identifying proteins that contribute to neutrophilic airway inflammation in horses with severe pasture asthma contributes to the identification of molecular signatures that herald advancing disease, and identifies targets for investigating directed therapies to moderate neutrophilic inflammation.

In systems modeling, homeostasis within a network of eukaryotic proteins (proteostasis) protects the body against disease and allows for healthy cellular development and aging.[48] Disease then reflects challenges to the integrity of this network of interacting proteins (proteome) that direct the generation, maintenance, and removal of proteins to achieve normal function. A growing area of airway research centers on the relationship between airway stress diseases, including COPD, emphysema, asthma, and disorders of proteostasis.[49] Congruent with the concept of disordered proteostasis, we hypothesize that changes in the protein composition of fluid in the airways of horses with pasture-associated severe asthma are collectively predicted to alter functional attributes of neutrophils in ways that contribute to neutrophilic airway inflammation.

Building upon our prior evidence that protein functions in normal bronchoalveolar lavage fluid (BALF) are conserved across horse, human, and mouse,[50] identifying how changes in the BALF proteome contribute to neutrophilic airway inflammation will enable recognition of shared regulatory events in human asthma and severe equine pasture asthma. This in turn informs our understanding of the similar roles of neutrophils in human asthma and severe equine pasture asthma. Here, we employed systems modeling of the BALF proteome of horses experiencing exacerbation of severe pasture asthma, and that of asymptomatic controls exposed to identical aeroallergen challenge and environmental conditions, to evaluate differences in the regulation of ten neutrophil functions: migration, activation, chemotaxis, adhesion, cell spreading, transmigration, infiltration, phagocytosis, respiratory burst, and apoptosis.

Methods and materials

Animals and BALF sample collection

Archived BALF in this study was collected from six horses with pasture-associated severe equine asthma, and six clinically normal control horses of mixed breeds using a bronchoalveolar lavage catheter (240cm, MILA International, Inc., Florence, KY, USA) under standing sedation. The BAL technique has been previously described.[28] Samples were immediately placed on ice, transported to the laboratory, centrifuged (600x g for 10 minutes) to derive cell-free BALF, and aliquots of supernate were frozen at $-80°C$ for subsequent proteomic analysis.[51,52] All horses were co-housed on pasture, fed identical diets, had no medications within 7 days of sampling, and were sampled in the summer months in Louisiana. Experimental procedures were approved by the Animal Care and Use Committee of Louisiana State University, complying with all federal guidelines overseeing the use of research animals in the United States. All procedures utilized were considered veterinary care best practices.

Horses in this study were a subset of those employed by Costa et al, for which maximal change in pleural pressure (ΔPpl_{max}), BALF cytologic findings, and clinical scores of respiratory effort (CSRE) have been previously described.[28] Asthmatic horses ranged from 10–20 years of age (mean ± SD, 16±4.3 years, two females and four castrated males). Control horses ranged from 7–20 years of age (mean 13.5±6.5 years, two females, three castrated males, and one stallion). Diagnosis of pasture-associated severe equine asthma was based on a history of seasonal remission of clinical disease during cool seasons, followed by episodic and reversible obstructive respiratory disease while grazing pasture during hot humid conditions. Overt respiratory distress was characterized by a CSRE ≥4.5, ΔPpl_{max} ≥24cm H_2O, audible expiratory wheezes in the lung fields, and neutrophilic airway inflammation (≥12%, mean =66%) in BALF. Control horses lacked historical episodic pasture-associated respiratory disease during the summer, had normal bronchovesicular sounds during lung auscultation with a re-breathing bag, CRSE <3, ΔPpl_{max} ≤9cm H_2O, and ≤3%–26% (mean =10%) neutrophils in BALF. Negative bacteriologic culture of BALF was a criterion for inclusion of asthmatic and control horses in this investigation.

Protein isolation, tryptic digestion, and liquid chromatography mass spectrometry/mass spectrometry (LC MS/MS)

Pooled samples containing 100 µg of cell-free BALF protein were created; one from the six control horses and another from the six asthmatic horses. For each pooled sample, 75 µg of protein was analyzed in triplicate using one dimensional LC nanospray ionization as previously described,[54] except that we did not perform differential detergent fractionation. Precursor mass scans were performed using repetitive MS scans immediately followed by three MS/MS scans of the three most intense MS peaks.

Protein identification

Searches were performed using TurboSEQUEST™ (Bioworks Browser 3.3, ThermoElectron). Mass spectra and tandem mass spectra were searched against an in silico trypsin-digested database of equine non-redundant RefSeq proteins downloaded from the National Center for Biotechnology Institute (ver 47) as well as against a reversed decoy database. SEQUEST search results were filtered using a decoy search-based probabilistic method, in which only peptides with a probability <0.05 were considered to be correct. Proteins identified with peptides passing the filter criteria were evaluated for differential expression using an Xcorr resampling technique. Probability of differential expression was calculated for each protein and those proteins with a p-value <0.05 were considered differentially expressed.[55]

Go-based modeling of the effects of BALF proteins on neutrophil function

Systems modeling was performed to test the hypothesis that proteins in the airways of horses with severe pasture asthma are predicted to modify neutrophil functions in a manner that contributes to neutrophilic airway inflammation. Proteins identified in BALF from affected and control horses were biocurated as described in the following pagragraph for their contributions to ten neutrophil functions: migration, activation, chemotaxis, adhesion, cell spreading, transmigration, infiltration, phagocytosis, respiratory burst, and apoptosis. The definitions of these terms are detailed in Table1.

Proteins were individually biocurated according to the principles of the Gene Ontology (GO) consortium,[56] using

Table I Definitions of neutrophil terms employed for biocuration

Activation	The change in morphology and behavior of a neutrophil resulting from exposure to a cytokine, chemokine, cellular ligand, or soluble factor.[120]
Adhesion	The process in which neutrophils attach to vascular endothelium as part of an event in recruitment into acutely inflammatory lesions, or to target tissues/cells following transmigration events.[121,122]
Apoptosis	Regulated cell death.[120]
Cell spreading	The morphogenetic process that results in flattening of a cell as a consequence of its adhesion to a substrate.[120]
Chemotaxis	The directed movement of a neutrophil cell, the most common polymorphonuclear leukocyte found in the blood, in response to an external stimulus, usually an infection or wounding.[120]
Infiltration	The diffusion or accumulation of neutrophils in tissues or cells in response to a wide variety of substances released at the sites of inflammatory reactions.[123]
Migration	The movement of a neutrophil within or between different tissues and organs of the body.[120]
Phagocytosis	An endocytosis process that results in the engulfment of external particulate material.[120]
Respiratory burst	A phase of elevated metabolic activity, during which oxygen consumption increases; this leads to the production, by an NADH-dependent system, of H_2O_2, superoxide anions, and hydroxyl radicals.[120]
Transmigration	The migration of a neutrophil from the blood vessels into the surrounding tissue.[120] Neutrophils pass through endothelium, interstitial tissues, and epithelium before arriving at the target location. No differentiation of stage was made for the purposes of our biocuration.

manual literature searches of each protein in PubMed to identify their effects on the ten neutrophil function terms. Search terms included each protein name paired with the word "neutrophil". An example query to PubMed would be: "annexin A1" AND "neutrophil". If more than 1,000 search results were found for any given protein, the search was refined by adding each of the ten neutrophil functions in turn ("annexin A1" AND "neutrophil", apoptosis). Each relevant paper was read to identify the protein's effect on the neutrophil term. The effect of each protein on each of the ten neutrophil functions was scored as pro (+1), anti (−1), or no net effect (0), as described in GoModeler.[57] In order to model these biological data, the potential of each protein to modify the ten neutrophil functions was approximated by multiplying its expression value by effect

(ie, −1, 0, +1), and the net effect of BALF proteins within each functional category was tabulated; similar to the method used for modeling effects on GO terms via GOModeler.[57] These net effects are represented graphically for asthmatic and control horses in Figure 1. A list of proteins and their relative effects are provided in Supplemental File 1, and a reference list is provided for each in Supplemental File 2.

Ingenuity® pathway analysis of the effects of BALF proteins on neutrophil functions

To identify central molecules in small sub-networks and support our manual biocuration findings using literature searching in PubMed, proteins were also analyzed for their contributions to neutrophil function using Ingenuity® Pathway Analysis (IPA, Qiagen NV, Venlo, the Netherlands). Neutrophil functions that were curated in IPA included migration, activation, chemotaxis, adhesion, infiltration, phagocytosis, and respiratory burst, and are defined in Table1. Because IPA does not accept equine gene products, human, mouse, and rat orthologs were first identified for each equine protein using Ensembl BioMart© (Ensembl 93 set)[58] and then employed for analysis. Most proteins had human orthologs, but mouse, and then rat were used to find orthologs when orthologous human proteins could not be identified. IPA Core Analysis (Ingenuity® Version 01–13, July 2018) was chosen for analysis as all pathways, ontologies, and interactions are supported by manually curated literature, providing a robust and standardized platform for interpreting biologic function from lists of differentially expressed gene products.[59]

Results
Neutrophil relevant BAL proteome
We identified 1,003 proteins from horse BALF: 417 proteins unique to asthmatic BALF, 472 proteins unique to control BALF, and 114 proteins common to both groups. A total of 108 proteins were identified as significantly differentially expressed between asthmatic and control groups ($p<0.05$). Using manual biocuration, 93 of the 1,003 proteins identified in BALF were determined to have evidence of biological activities that regulate one or more of ten neutrophil functions. These proteins and their respective effects on neutrophil functions are listed in Supplemental File 1. Manual literature searching initially identified a total of 21,533 papers, 395 of which contained

Effects of BALF proteins on neutrophil function
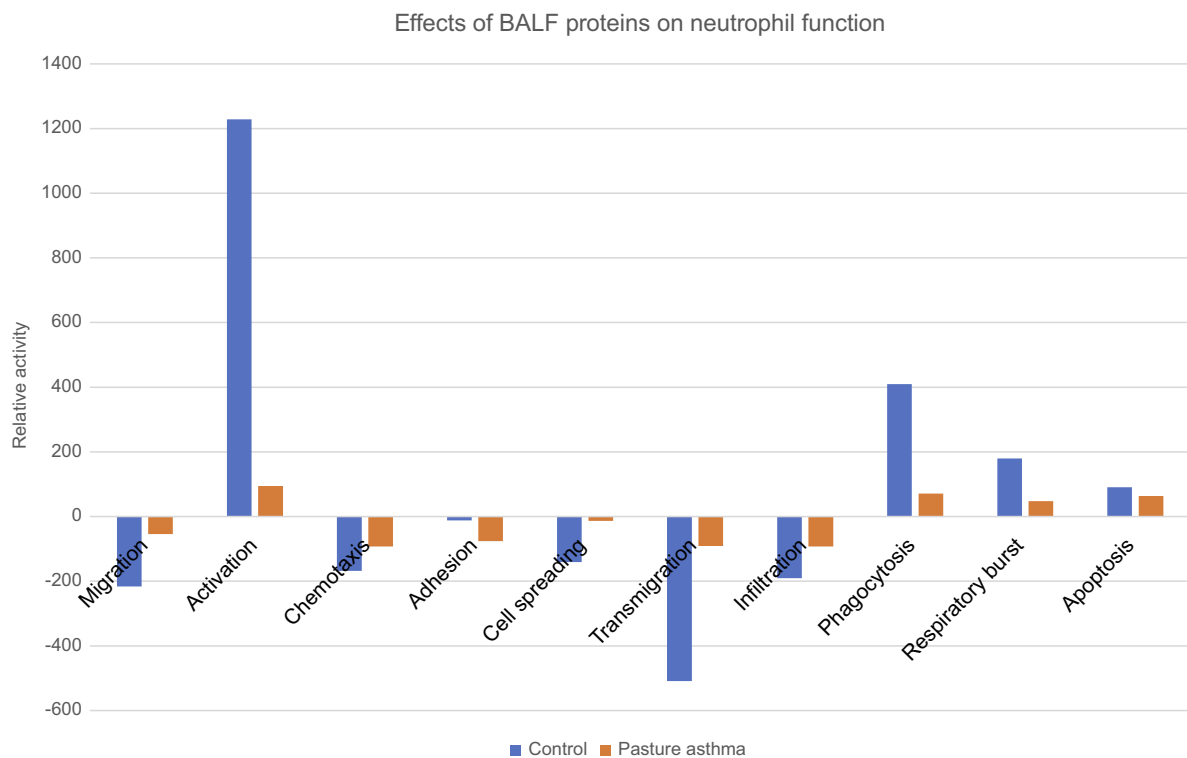
Figure 1 Cumulative effect of bronchoalveolar lavage fluid (BALF) proteins on neutrophil functions in asthmatic vs control horses. A total of 1,003 proteins were reviewed to identify their effects on ten neutrophil functions. Peer-reviewed manuscripts were read and actions involving ten neutrophil functions were scored for each protein as pro (+1), anti (−1), no effect (0), or no data. The contribution of each protein to each neutrophil function was calculated as the product of magnitude of expression multiplied by its effect (ie, −1, 0, +1), then these values were tabulated for category of neutrophil function. Bars represent the predicted net effect of BALF proteins on the respective neutrophil function.

relevant information on a specific protein and neutrophil function.

Modeling effects of BALF proteins on neutrophil function

By modeling effects of the 93 proteins that were determined, by biocuration, to modify neutrophil functions, increases in neutrophil migration, chemotaxis, cell spreading, transmigration, and infiltration were predicted in asthmatic relative to control horses (Figure1). We found that proteins in control BALF exert a net negative regulation of migration, chemotaxis, cell spreading, transmigration, and infiltration while proteins in BALF of asthmatic horses facilitate a partial release of the negative regulation of these neutrophil functions, as compared to BALF proteins from control horses. This results in a protein milieu in BALF of asthmatic horses that is less inhibitory to migration, chemotaxis, transmigration, and infiltration than the proteins in BALF of control horses. By contrast, a net decrease in protein activities responsible for neutrophil activation, adhesion, phagocytosis, respiratory burst, and apoptosis was identified in the BALF of asthmatic relative

to control horses. For these neutrophil functions, there is a net positive regulation of activation, phagocytosis, respiratory burst, and apoptosis by proteins in control BALF, while proteins in asthmatic BALF affect an incomplete decrease in the net positive modulation that characterizes homeostasis. Finally, neutrophil adhesion was decreased by the proteins in both asthmatic and control BALF, though this net effect was comparatively greater in asthmatic than control BALF. A summary of proteins contributing to each of the neutrophil functions segregated by increase, decrease, or no net effect is included within Supplemental File 1. References included in the manual biocuration are available in Supplemental File 2.
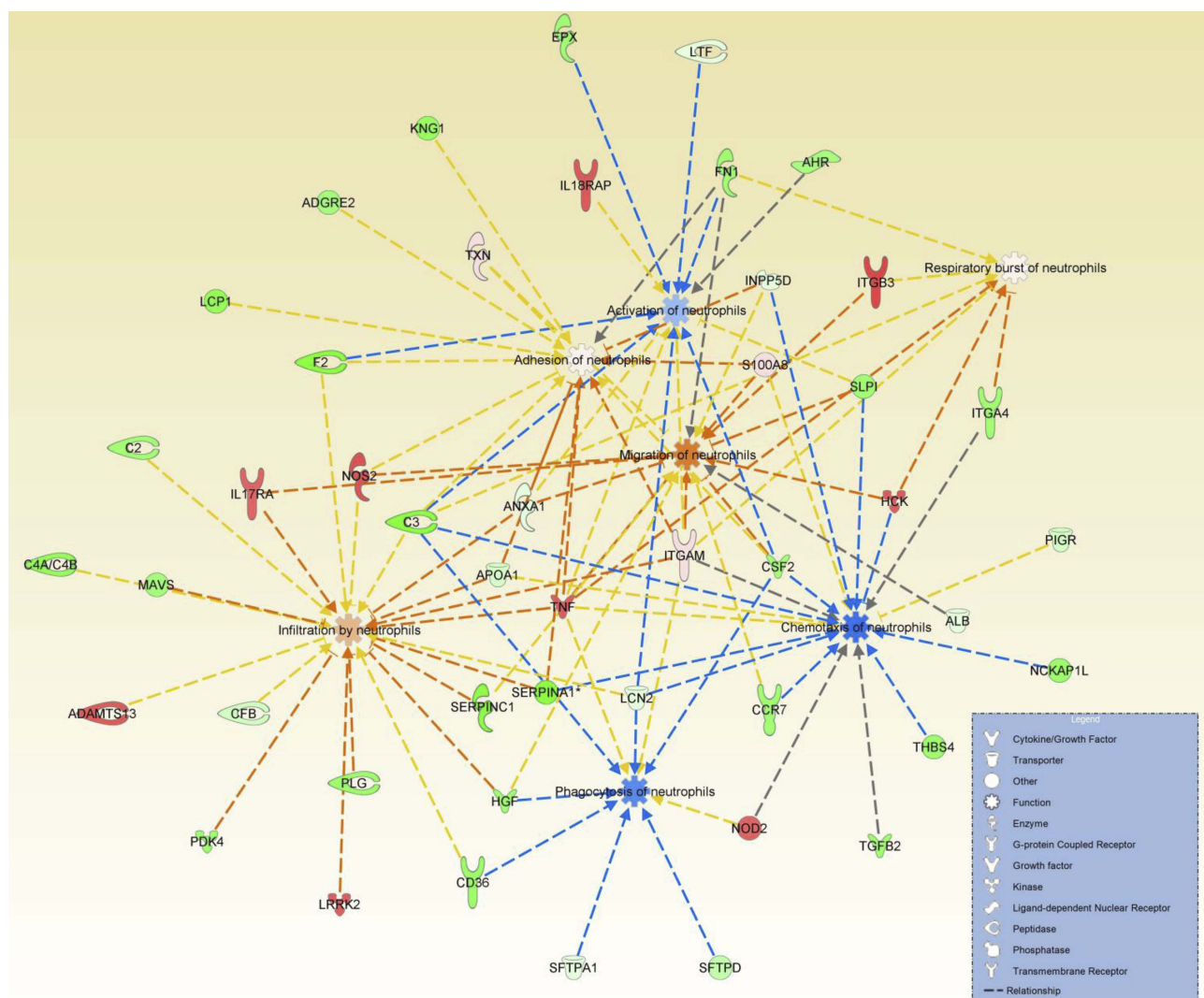
Neutrophil interactive network

The 1,003 proteins identified in BALF were also modeled for their effects on neutrophil functions using IPA. Of the 1,003 proteins, 129 did not have human, mouse, or rat orthologs, resulting in 874 proteins for IPA analysis. Forty-six proteins were identified to have an influence on neutrophil migration, activation, chemotaxis, adhesion, infiltration, phagocytosis, and respiratory burst. An

interaction network of proteins and their impact on neutrophil functions is presented in Figure 2. IPA modeling predicted a net increase in neutrophil migration, adhesion, infiltration, and respiratory burst, and a net decrease in neutrophil activation, chemotaxis, and phagocytosis. Differences in proteins annotated to each neutrophil function in IPA relative to manual curation are detailed in Table 2.

Discussion

Though at least 50% of human asthmatics demonstrate neutrophilic inflammation in BALF, which correlates to increased clinical severity and resistance to corticosteroid therapy,[1,4,60] regulatory mechanisms responsible for neutrophilic asthma are poorly characterized. Complicating this effort is the difficulty in attaining purified populations of airway neutrophils from human asthmatics, and the fact that bronchoscopy stimulates non-specific airway neutrophilia,[61] resulting in a predominance of investigations that employ peripheral blood neutrophils. However, there is evidence that peripheral blood neutrophils do not behave in the same way as airway neutrophils.[62–64] Deciphering regulatory mechanisms associated with neutrophilic airway inflammation and the major protein effectors responsible for these changes has the potential to identify molecular events that herald advancing airway

Figure 2 Ingenuity® Pathway Analysis (IPA) predicted decreases in three and increases in four neutrophil functions. An interactive network was derived using IPA by screening 1,003 proteins in bronchoalveolar lavage fluid (BALF) of asthmatic vs control horses for their impact on neutrophil activity. Green shades indicate relative downregulation of proteins in asthmatic BALF (and relative upregulation in control BALF), while red shades indicate relative upregulation of proteins in asthmatic BALF (and relative downregulation of proteins in control BALF). Blue indicates predicted decreases in neutrophil functions, while orange shades indicate predicted increases. More intense (darker) colors indicate greater increases or decreases. Together, this network indicated net downregulation of neutrophil activation, chemotaxis, and phagocytosis, and upregulation of neutrophil migration, adhesion, infiltration, and respiratory burst. Broken lines indicate a relationship between the proteins and the neutrophil functions.

Table 2 Comparison of proteins curated by Ingenuity® Pathway Analysis (IPA) vs manual biocuration. Bolded proteins were common between the two methodologies

Neutrophil category	Proteins curated in IPA	Proteins manually curated
Migration	• ALB – albumin • **ANXA1 – annexin A1** • **CCR7 – C-C chemokine receptor type 7** • **CSF2 – colony stimulating factor 2** • **FN1 – fibronectin I** • **HCK – tyrosine-protein kinase** • HGF – hepatocyte growth factor • **IL17RA – interleukin 17A receptor** • INPP5D – inositol polyphosphate-5-phosphatase D • **ITGAM – integrin alpha M** • ITGB3 – integrin beta 3 • **NOS2 – nitric oxide synthase 2** • **S100A8 – S100 calcium binding protein A8** • SERPINC1 – serpin family C, member I • SLPI – secretory leukocyte peptidase inhibitor • **TNF – tumor necrosis factor** • TXN – thioredoxin	• alpha-I-acid glycoprotein 2 • alpha-I-acid glycoprotein-like • alpha-I-antiproteinase 2-like isoform X1 • **annexin A1** • apolipoprotein A-I-like • apolipoprotein A-IV • **C-C chemokine receptor type 7** • complement factor B • dnaJ homolog subfamily C member 27 • EGF-like module-containing mucin-like hormone receptor-like 2-like • **fibronectin isoform X1** • gelsolin • **granulocyte-macrophage colony-stimulating factor precursor** • hemopexin • integrin alpha-4 • **integrin alpha-M isoform X1** • **interleukin-17 receptor A isoform X1** • **nitric oxide synthase, inducible** • phosphatidylinositol 4-phosphate 3-kinase C2 domain-containing subunit alpha isoform X1 • polypeptide N-acetylgalactosaminyltransferase I • **protein S100-A8** • prothrombin • pulmonary surfactant-associated protein D • reactive oxygen species modulator I-like • thioredoxin • toll-like receptor 8 precursor • **tumor necrosis factor** • **tyrosine-protein kinase HCK**

(*Continued*)

Table 2 (Continued).

Neutrophil category	Proteins curated in IPA	Proteins manually curated
Activation	• AHR – aryl hydrocarbon receptor • **ANXA1 – annexin A1** • **C3 – complement C3** • **CSF2 – colony stimulating factor 2** • EPX – eosinophil peroxidase • F2 – coagulation factor II, thrombin • FN1 – fibronectin 1 • **IL18RAP – interleukin-18 receptor accessory protein** • ITGAM – integrin alpha M • LCN2 – lipocalin 2 • **LTF – lactotransferrin** • SLPI – secretory leukocyte peptidase inhibitor • **TNF – tumor necrosis factor**	• alpha-1-acid glycoprotein 2 • alpha-1-acid glycoprotein-like • alpha-1-antiproteinase 2-like isoform X1 • anionic trypsin • **annexin A1** • annexin A5 isoform X2 • apolipoprotein A-I-like • apolipoprotein A-IV • beta-2-glycoprotein 1 • C-C chemokine receptor type 7 • complement C2 • **complement C3** • complement component C1q receptor • complement factor B • cytochrome c • dnaJ homolog subfamily C member 27 • dystrophin • **granulocyte-macrophage colony-stimulating factor precursor** • haptoglobin • hexokinase-2 • **interleukin-18 receptor accessory protein isoform X1** • **lactotransferrin precursor** • leptin receptor-like • mitogen-activated protein kinase kinase kinase 2 • myoglobin • nck-associated protein 1-like • phosphatidylinositol 4-phosphate 3-kinase C2 domain-containing subunit alpha isoform X1 • plasma protease C1 inhibitor • polymeric immunoglobulin receptor • prothrombin • ral guanine nucleotide dissociation stimulator • reactive oxygen species modulator 1-like • serotransferrin precursor • thioredoxin • toll-like receptor 1 precursor • toll-like receptor 8 precursor • tropomyosin alpha-1 chain-like • **tumor necrosis factor**

(Continued)

Table 2 (Continued).

Neutrophil category	Proteins curated in IPA	Proteins manually curated
Chemotaxis	• APOA1 – apolipoprotein A1 • **C3 – complement C3** • CCR7 – C-C chemokine receptor type 7 • **CSF2 – colony stimulating factor 2** • **HCK – tyrosine-protein kinase** • INPP5D – inositol polyphosphate-5-phosphatase D • **ITGA4 – integrin alpha 4** • ITGAM – integrin alpha M • LCN2 – lipocalin 2 • NCKAP1L – Nck-associated protein 1 like • NOD2 – nucleotide-binding oligomerization domain-containing protein 2 • **PIGR – polymeric immunoglobulin receptor** • **S100A8 - S100 calcium binding protein A8** • SERPINA1 – serpin family A, member 1 • SLPI – secretory leukocyte peptidase inhibitor • TGFB2 – transforming growth factor-beta 2 • THBS4 – thrombospondin-4 • **TNF – tumor necrosis factor**	• alpha-1-acid glycoprotein 2 • alpha-1-acid glycoprotein-like • alpha-1-antiproteinase 2-like isoform X1 • amyloid beta A4 precursor protein-binding family A member 2 • ankyrin repeat domain-containing protein 11 • annexin A1 • annexin A2 • annexin A5 isoform X2 • complement C2 • **complement C3** • complement component C1q receptor • fibronectin isoform X1 • gamma-aminobutyric acid type B receptor subunit 2 • gelsolin • **granulocyte-macrophage colony-stimulating factor precursor** • hemopexin • hepatocyte growth factor isoformX1 • integrin alpha-3 isoform 1 • **integrin alpha-4** • leptin receptor-like • microtubule-associated protein 2 isoform X8 • nitric oxide synthase, inducible • phosphatidylinositol 4-phosphate 3-kinase C2 domain-containing subunit alpha isoform X1 • plasma protease C1 inhibitor • **polymeric immunoglobulin receptor** • **protein S100-A8** • prothrombin • pulmonary surfactant-associated protein D • thioredoxin • **tumor necrosis factor** • **tyrosine-protein kinase HCK** • tyrosine-protein kinase JAK3 • utrophin-like • vitamin D-binding protein

(Continued)

Table 2 (Continued).

Neutrophil category	Proteins curated in IPA	Proteins manually curated
Adhesion	• ADGRE2 – adhesion G protein-coupled receptor E2 • **ANXA1 – annexin A1** • **APOA1 – apolipoprotein A1** • **C3 – complement C3** • **CSF2 – colony stimulating factor 2** • F2 – coagulation factor II, thrombin • **FN1 – fibronectin 1** • INPP5D – inositol polyphosphate-5-phosphatase D • **ITGAM – integrin alpha M** • **KNG1 – kininogen 1** • **LCP1 – plastin 2** • **NOS2 – nitric oxide synthase 2** • **S100A8 – S100 calcium binding protein A8** • SERPINA1 – serpin family A, member 1 • **TNF – tumor necrosis factor** • **TXN – thioredoxin**	• A disintegrin and metalloproteinase with thrombospondin motifs 13 • alkaline phosphatase, tissue-nonspecific isozyme isoform X1 • alpha-l-antiproteinase 2-like isoform X1 • **annexin A1** • **apolipoprotein A-I-like** • apolipoprotein A-IV • C-C chemokine receptor type 7 • CD2-associated protein • **complement C3** • dnaJ homolog subfamily C member 27 • EGF-like module-containing mucin-like hormone receptor-like 2-like • fibrinogen gamma chain • **fibronectin isoform X1** • gelsolin • **granulocyte-macrophage colony-stimulating factor precursor** • hemopexin • hepatocyte growth factor isoformX1 • integrin alpha-4 • **integrin alpha-M isoform X1** • **kininogen-1 isoform X2** • **nitric oxide synthase, inducible** • plasma protease C1 inhibitor • **plastin-2** • **protein S100-A8** • **thioredoxin** • **tumor necrosis factor** • tyrosine-protein kinase JAK2 isoform X1
Cell spreading	• N/A	• apolipoprotein A-I-like • apolipoprotein A-IV • serum albumin precursor • tumor necrosis factor • tyrosine-protein kinase HCK

(Continued)

Table 2 (Continued).

Neutrophil category	Proteins curated in IPA	Proteins manually curated
Transmigration	• N/A	• annexin A1 • annexin A2 • antithrombin-III isoform X1 • catenin alpha-3 isoform 1 • C-C chemokine receptor type 7 • hepatocyte growth factor isoformX1 • integrin beta-3 precursor • plasma protease C1 inhibitor • polymeric immunoglobulin receptor • tumor necrosis factor
Infiltration	• ADAMTS13 – ADAM metallopeptidase with thrombospondin type 1 motif 13 • **ANXA1 – annexin A1** • APOA1 – apolipoprotein A1 • C2 – complement C2 • C3 – complement C3 • **C4A/C4B – complement C4A/CFB** • CD36 – platelet glycoprotein 4 • CFB – complement factor B • F2 – coagulation factor II, thrombin • **HGF – hepatocyte growth factor** • **IL17RA – interleukin 17A receptor** • ITGAM – integrin alpha M • LCN2 – lipocalin 2 • **LRRK2 – leucine-rich repeat kinase 2** • MAVS – mitochondrial antiviral-signaling protein • **NOS2 – nitric oxide synthase 2** • PDK4 – pyruvate dehydrogenase kinase 4 • **PLG – plasminogen** • SERPINA1 – serpin family A, member 1 • SERPINC1 – serpin family C, member 1 • **TNF – tumor necrosis factor**	• alpha-1-acid glycoprotein 2 • alpha-1-antiproteinase 2-like isoform X1 • **annexin A1** • annexin A2 • antithrombin-III isoform X1 • argininosuccinate synthase • aryl hydrocarbon receptor • BK potassium ion channel • cadherin-1 • **complement C4-A** • dystrophin • Fc receptor alpha-chain • gelsolin • granulocyte-macrophage colony-stimulating factor precursor • group IIF secretory phospholipase A2 • haptoglobin • **hepatocyte growth factor isoformX1** • **interleukin-17 receptor A isoform X1** • **leucine-rich repeat kinase 1** • **nitric oxide synthase, inducible** • pancreatic alpha-amylase • peroxiredoxin-1 • plasma protease C1 inhibitor • **plasminogen isoform X1** • pulmonary surfactant-associated protein D • thioredoxin • **tumor necrosis factor** • vitamin D-binding protein

(Continued)

Table 2 (Continued).

Neutrophil category	Proteins curated in IPA	Proteins manually curated
Phagocytosis	• **C3 – complement C3** • CD36 – platelet glycoprotein 4 • **CSF2 – colony stimulating factor 2** • HGF – hepatocyte growth factor • ITGAM – integrin alpha M • LCN2 – lipocalin 2 • **NOD2 – nucleotide-binding oligomerization domain-containing protein 2** • **SFTPA1 – surfactant protein A1** • **SFTPD – surfactant protein D** • **TNF – tumor necrosis factor**	• alpha-1-acid glycoprotein 2 • alpha-1-acid glycoprotein-like • alpha-fetoprotein precursor • ankyrin repeat domain-containing protein 11 • annexin A1 • ceramide kinase • ceruloplasmin isoform X2 • complement C2 • **complement C3** • complement component C1q receptor • complement factor B • complement factor I • Fc receptor alpha-chain • ferritin light chain • fibronectin isoform X1 • gelsolin • **granulocyte-macrophage colony-stimulating factor precursor** • hemopexin • leptin receptor-like • **nucleotide-binding oligomerization domain-containing protein 2** • plasma protease C1 inhibitor • **pulmonary surfactant-associated protein A precursor** • **pulmonary surfactant-associated protein D** • toll-like receptor 1 precursor • **tumor necrosis factor**

(*Continued*)

Table 2 (Continued).

Neutrophil category	Proteins curated in IPA	Proteins manually curated
Respiratory burst	• **C3 – complement C3** • FN1 – fibronectin 1 • **HCK – tyrosine-protein kinase** • ITGA4 – integrin alpha 4 • **ITGAM – integrin alpha M** • ITGB3 – integrin beta 3 • **TNF – tumor necrosis factor**	• alpha-1-acid glycoprotein 2 • alpha-1-acid glycoprotein-like • apolipoprotein A-II • apolipoprotein A-IV • C-C chemokine receptor type 7 • complement C2 • **complement C3** • complement component C1q receptor • complement factor B • dnaJ homolog subfamily C member 27 • EF-hand calcium-binding domain-containing protein 3 • granulocyte-macrophage colony-stimulating factor precursor • group IIF secretory phospholipase A2 • haptoglobin • **integrin alpha-M isoform** • lactotransferrin precursor • leptin receptor-like • mitogen-activated protein kinase kinase kinase 2 • phosphatidylinositol 4-phosphate 3-kinase C2 domain-containing subunit alpha isoform X1 • protein S100-A8 • pulmonary surfactant-associated protein A precursor • pulmonary surfactant-associated protein D • thioredoxin • toll-like receptor 1 precursor • **tumor necrosis factor** • **tyrosine-protein kinase HCK**

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Table 2 (Continued).

Neutrophil category	Proteins curated in IPA	Proteins manually curated
Apoptosis	• N/A	• alpha-1-acid glycoprotein-like • alpha-1-antiproteinase 2-like isoform X1 • amyloid beta A4 precursor protein-binding family A member 2 • annexin A1 • annexin A5 isoform X2 • antithrombin-III isoform X1 • argininosuccinate synthase • bcl-2-related protein A1 • cytochrome c • dnaJ homolog subfamily C member 27 • granulocyte-macrophage colony-stimulating factor precursor • lactotransferrin precursor • leptin receptor-like • nitric oxide synthase, inducible • phosphatidylinositol 4-phosphate 3-kinase C2 domain-containing subunit alpha isoform X1 • protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), beta isoform • thioredoxin • tumor necrosis factor

disease, as well as avenues for pharmacologic intervention. Systems modeling provides a unique opportunity to translate a large amount of data from high throughput technologies, including proteomics and transcriptomics, into a better understanding of the mechanistic basis of complex biological phenomena and disordered proteostasis.

We report the predicted impact of changes in protein expression in BALF on neutrophil functions in horses with pasture-associated severe equine asthma. This study provides insight into the molecular events that contribute to neutrophilic airway inflammation, a characteristic of both equine asthma and human asthma that is not adequately addressed by rodent models. Our results indicate that, relative to the control BALF, proteins in BALF from asthmatic horses are predicted to increase neutrophil migration, chemotaxis, cell spreading, transmigration, and infiltration. These functions favor the movement of neutrophils into the airways, congruent with airway neutrophilic inflammation that is characteristic of pasture-associated severe equine asthma. Increased neutrophil migration,[65] chemotaxis,[66,67] cell spreading,[68] and infiltration[60,69] have similarly been reported in human asthma. Our findings are also consistent with increased neutrophil migration[70] and chemotaxis[71,72] reported in barn-associated equine asthma. Despite expanding knowledge of how individual proteins modify neutrophil movement in asthma, changes in neutrophil transmigration that occur during neutrophilic forms of asthma are not documented and the majority of knowledge is extrapolated from investigations of neutrophil transmigration across other epithelial surfaces.[73] In asthma, increased airway neutrophils are considered a priori evidence of increased neutrophil transmigration.[17,74,75] Increased transmigration of neutrophils into the airway is also a feature of several inflammatory diseases, including bronchitis and COPD.[74]

Apolipoprotein A-1, polymeric immunoglobulin receptor, and haptoglobin were highly expressed proteins in the BALF of control horses. Apolipoprotein A-1 decreases neutrophil migration and cell spreading,[76,77] polymeric immunoglobulin receptor decreases neutrophil chemotaxis and transmigration,[78] and haptoglobin decreases infiltration.[79] Accordingly, decreased expression of these proteins in the BALF of horses with pasture-associated severe asthma relative to expression levels in control BALF had a strong influence on the predicted release of inhibition for these neutrophil functions by proteins in the BALF proteome of diseased horses. Apolipoprotein A-1

modulates lung health and contributes to protective pathways in lung biology, decreasing severity of respiratory diseases including asthma.[80–82] This protein has been considered as a potential novel therapeutic agent for asthmatics.[80,81,83] Congruent with our findings in the BALF of horses with severe asthma, BALF and serum samples from human asthmatics and murine models also demonstrate decreased levels of apolipoprotein A-1.[82,84] Further, in agreement with our findings of increased polymeric immunoglobulin receptor in the BALF of horses with severe asthma, this protein, which transports dimeric IgA across mucosal epithelial cells,[85] is also decreased in the bronchial epithelium of human asthma patients.[86] Finally, haptoglobin, which was decreased in the BALF of horses with severe asthma, is an acute phase glycoprotein involved in tissue repair and airway remodeling in asthmatic patients.[87] While some studies have demonstrated decreased expression in asthmatics relative to controls,[87] others have shown expression levels that decrease after initial allergen challenge, but then increase 24 hours post-challenge, suggesting a replenishing of the protein over time.[88]

Protein expression changes in the BALF proteome of asthmatic horses are predicted, collectively, to decrease neutrophil activation, adhesion, phagocytosis, respiratory burst, and apoptosis. Similar decreases in neutrophil phagocytosis and apoptosis have been identified in asthmatic patients,[89–93] and decreased apoptosis of airway neutrophils has been identified in horses with the barn-associated form of severe equine asthma.[94,95] Decreased apoptosis contributes to airway neutrophilic inflammation that characterizes human and equine asthma by increasing neutrophil persistence in the airway. Similarly, neutrophil activation and phagocytosis program neutrophils for death,[96–98] such that decreasing these activities contributes to neutrophil persistence and neutrophilic airway inflammation. In general, neutrophil adhesion is increased in asthmatic patients, primarily due to an increase in cellular adhesion molecules.[99] Our findings, which indicate that the BALF proteome of asthmatic horses would reduce adhesion in asthmatic relative to control horses, contradict these findings in human asthma, but are congruent with the airway neutrophilic inflammation that characterizes equine pasture asthma. This difference may reflect changes in the neutrophilic phenotype induced by ex vivo manipulations or could reflect a compensatory mechanism in the BALF proteome designed to moderate increases in neutrophil adhesion that lead to tissue damage. Significantly,

differences may reflect the relatively small sample size of horses included in this study.

While the proteins in BALF of asthmatic horses were predicted to decrease neutrophil activation relative to proteins in the BALF of controls, measures of neutrophil activation are reported to increase in asthma.[100–104] However, neutrophil activation is a complex and multifaceted neutrophil function. For example, respiratory burst is one component of neutrophil activation. We identified BALF proteins that both increase and decrease respiratory burst, predicting a net negative effect on this neutrophil function in asthmatic relative to control horses. While characterization of the respiratory burst of airway neutrophils in asthma has been poorly characterized, our findings are congruent with those of Lacy et al (2003), which identified decreased respiratory burst activity in neutrophils from induced sputum of asthmatics relative to controls.[63] However, this finding must be considered with the knowledge that ß$_2$-agonists and corticosteroid therapy were not withheld from the asthmatic group, whereas our horses with asthma were not medicated. Other authors have demonstrated that respiratory burst of blood-derived neutrophils from asthmatics exceeds that from non-asthmatics.[62,105,106] However, Marcal et al (2004) clarified that significant differences in measures of respiratory burst are influenced by disease severity and are not immediately evident in ex vivo neutrophils, occurring only in more severe asthmatics following ex vivo incubation periods of 25 minutes.[62] Despite evidence that selected measures of neutrophil activation are increased in asthma,[100–104] our modeling indicated that BALF proteome changes in asthmatic horses favor decreased neutrophil activation, which is consistent with neutrophil persistence in the airways and the airway neutrophilic inflammation that characterizes pasture-associated severe equine asthma. However, based upon aforementioned evidence that neutrophils are activated in human asthma, and that the net decrease in neutrophil activation was the most robust of the predicted effects of BALF proteins on neutrophil functions, we reason that proteins in BALF may work collectively to mitigate neutrophil activation that is driven by exogenous factors in neutrophilic asthma.

We identified four proteins, serotransferrin precursor, polymeric immunoglobulin receptor, surfactant protein A, and cytochrome C, which had strong contributions to the decreases in neutrophil activation, phagocytosis, respiratory burst, and apoptosis predicted by our modeling.

Serotransferrin precursor and polymeric immunoglobulin receptor increase neutrophil activation,[107,108] surfactant protein A increases neutrophil phagocytosis and respiratory burst,[109] and cytochrome C increases apoptosis.[110–112] In each case, these proteins demonstrated decreased expression in the BALF of asthmatic relative to control horses. Accordingly, decreases in expression of these four proteins had large effects on decreasing these neutrophil functions in association with asthma in our modeling. Increased expression of thioredoxin in the BALF of diseased horses, which is a negative regulator of neutrophil adhesion,[113] was the largest contributor to the net decrease in neutrophil adhesion predicted by our modeling. While cytochrome C levels have not been specifically examined with regards to asthma, both serotransferrin and pulmonary surfactant-associated protein are decreased in the serum of asthmatic patients relative to controls, which is congruent with our equine findings.[114–116] Pulmonary surfactant proteins have also been demonstrated to inhibit histamine release during allergen challenge and decrease lymphocyte proliferation in asthmatic children, providing a protective role against the pathogenesis of asthma.[117] Thioredoxin, a redox-regulating protein with antioxidant activity, has been observed to be increased in human asthmatics, congruent with findings in asthmatic horses in this investigation.[118,119]

Functional analysis, performed using IPA, revealed 46 proteins with a known relationship with neutrophil functions (Figure 2). These proteins were predicted by IPA to cause an increase in neutrophil migration and infiltration, and a decrease in neutrophil activation and phagocytosis – predictions which directly corroborate our PubMed manual biocuration predictions. In contrast, IPA predicted an increase in neutrophil adhesion and respiratory burst, and a decrease in chemotaxis, while our manual biocuration findings predicted opposite trends for these functions. As previously indicated, chemotaxis is well documented to be increased in human asthma,[66,67] while respiratory burst has been found to be decreased.[63] These findings indicate that increased chemotaxis and decreased respiratory burst identified by the manual curation and associated data modeling in our approach are congruent with findings in human asthma. The IPA-predicted increase in neutrophil adhesion contradicts our manual biocuration findings but is congruent with human asthma literature.[99] Several differences between the databases that support IPA vs our manual effort are likely relevant to these disparities. First, IPA identified only 46 proteins as relevant to modifying

neutrophil functions, while 93 proteins were identified as relevant in our manual curation. Whereas IPA maintains an internal proprietary biocuration effort, our manual biocuration, which was limited to PubMed citations, identified more references that implicated BALF proteins in each neutrophil function, as demonstrated in Table2. Also, IPA relies upon the identification of orthologous human, mouse, or rat proteins from the equine proteins. In our case, 129 proteins that contributed to our curation effort did not, at the time of analysis, have identifiable human, mouse, or rat orthologs, so they were not included in IPA analysis. Of those 129 proteins without an ortholog, our manual biocuration found that eight of them affected neutrophil functions.

Conclusion

Changes in the protein composition of BALF from horses with pasture-associated severe asthma are predicted to increase neutrophil migration, chemotaxis, cell spreading, transmigration, and infiltration, and to decrease neutrophil activation, adhesion, phagocytosis, respiratory burst, and apoptosis. With the exception of adhesion and activation, these predictions mirror changes in neutrophil functions described in both human asthma and barn-associated equine asthma, suggesting that BALF proteins may have a role in moderating the neutrophil activation and adhesion that are reported in human asthma. This investigation confirms our hypothesis that changes in the protein composition of fluid in the airways of horses with pasture-associated severe equine asthma predict functional attributes of neutrophils in the airways of asthmatic horses that contribute to neutrophilic airway inflammation. These data also support the value of pasture-associated severe equine asthma as an animal model of neutrophilic asthma, while demonstrating utility of systems modeling to identify complex molecular events associated with clinical disease in genome scale datasets.

Highlights

Pasture-associated severe equine asthma is characterized by neutrophilic airway inflammation.

Modeling differentially expressed protein functions in BALF from asthmatic horses identifies biologic events that account for neutrophilic airway inflammation.

Systems modeling can organize high throughput data from clinical disease processes in a manner that defines a biologically relevant phenomenon.

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Disclosure

The authors report no conflicts of interest in this work.

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