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Validating Pasture Heaves as an Equine Model of Neutrophilic Asthma: a Systems Biology Approach

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Asthma is a chronic respiratory disease characterized by reversible airway obstruction, persistent airway hyperresponsiveness, chronic airway inflammation, and chronic airway remodeling. Most adult asthmatics have neutrophilic airway inflammation that correlates to increasing disease severity, and fail to respond to corticosteroid therapies that mitigate other asthma endotypes. Accordingly, there is a need to investigate the molecular mechanisms responsible for neutrophilic asthma. Pasture heaves, a respiratory disease affecting horses housed on pasture in conditions of high heat and humidity, shares the aforementioned characteristics of human asthma, including neutrophilic inflammation. The cause is undetermined, but genetic propensities for reactivity to seasonally inhaled, pasture-associated, aeroallergens are presumed. Complexities of diseases like asthma and pasture heaves, that include temporal interactions between environmental and genetic factors, lend themselves to exploration using -*omics* technologies.

An emergent paradigm in disease pathogenesis views disease as the result of imbalances in a biological system of thousands of proteins that maintain eukaryotic

homeostasis. Consistent with this paradigm, this dissertation describes systematic efforts to identify groups of proteins in the bronchoalveolar lavage fluids of horses with pasture heaves that are altered in a manner that influences neutrophilic airway inflammation, and are similarly changed in human asthma. This is the first use of -*omics* technologies to investigate pasture heaves. This was accomplished first by improving functional annotation of the equine genome by providing functional annotation for an equine oligoarray, thereby facilitating future functional modeling of equine gene products. Next, through comparative modeling of protein functions in normal bronchoalveolar lavage fluid (BALF) proteomes from horse, human, and mouse, we demonstrated conservation of protein functions in lung fluids across these species. Finally, comparative modeling of pasture heaves-affected and non-diseased BALF proteomes demonstrated that proteins in diseased BALF favor airway neutrophilic inflammation by increasing neutrophil migration, chemotaxis, adhesion, detachment, transmigration, and degranulation, while reducing activation, cell spreading, infiltration, phagocytosis, respiratory burst, apoptosis, and clearance. Collectively, these molecular events contribute to airway neutrophilic inflammation in pasture heaves, and are conserved in human asthma. This method further validates pasture heaves as a robust model for human neutrophilic asthma, and highlights proteins of potential clinical and therapeutic relevance.

DEDICATION

To Mom and Dad, for believing in your little miss "selfie-do." I love you, always.

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CHAPTER I

INTRODUCTION

Asthma is a genetically heterogenous, chronic lung disease that affects adults and children of all ages, and can present in a variety of clinical forms. It is caused by a combination of complex and incompletely understood environmental and genetic interactions [1]. Accounts of asthmatic symptoms have been in medical literature from the time of Hippocrates [2], and by the late 19th century, physicians accepted asthma as a distinct disease with a specific set of causes, clinical changes, and treatment [3]. Over time, understanding and monitoring of asthma has improved. In the 1980s, the CDC began conducting National Health Interview Surveys monitoring the prevalence of asthma, which has been increasing since the start of surveillance [4]. Currently, the CDC estimates that 8% of American adults and 9.3% of American children have asthma, 18.7 million and 6.8 million people, respectively [5].

The four characteristics that define asthma are: chronic inflammation, persistent airway hyperresponsiveness (AHR), reversible airway obstruction, and airway remodeling. Environmental and other factors (such as inhaled aeroallergens, irritants, and viruses) provoke airway inflammation in asthmatics [6]. There is an underlying chronic inflammation present in asthmatic patients that occurs even in the absence of continued allergen exposure [7]. Many inflammatory cells play a role in the inflammation of asthma, including mast cells, neutrophils, eosinophils, T-lymphocytes, macrophages,

neutrophils, and epithelial cells [8]. Traditionally, airway inflammation in asthma was thought to be predominantly eosinophilic, but more recent literature indicates that greater than 50% of adult asthmatics have a neutrophilic inflammation, correlating to increasing severity of disease [9-11].

Airway hyperresponsiveness (AHR) describes a condition of increased airway narrowing in response to a bronchoconstrictive challenge [12].Originally thought to be due solely to inflammation, two components to airway hyperresponsiveness are now recognized: persistent and variable AHR. Variable AHR, named for the fact that this type of AHR is variably present, is believed to relate to the inflammatory events of the airway, which vary depending on stimulus. By contrast, persistent AHR refers to AHR that remains even after removal of the inciting agent/allergen/antigen for protracted periods. Persistent AHR is associated with structural changes in the airway [13].

The asthma diagnosis hinges upon documentation that airway narrowing during an asthmatic episode is reversible, either spontaneously or through treatment. Airflow obstruction is caused by a variety of changes in the airway, including bronchoconstriction, edema, mucus plug formation, and airway remodeling. Although the airway obstruction is reversible, the histopathological lesions of asthma, collectively termed "airway remodeling," are not fully reversible [14, 15]. Remodeling changes contribute to a thickening of airway epithelium and airway smooth muscle with subsequent reduction of airway diameter. These changes result in fixed airflow obstruction with asthma progression. Remodeling also results in compromised epithelial barrier function, mucus gland hypersecretion, goblet cell hyperplasia and metaplasia, and

airway fibrosis [16-21]. Significantly, airway remodeling is known to correlate to the clinical severity of asthma [22-24].

Many animal species have been used as models of asthma, which are crucial to the continual development of new therapeutic mechanisms and furthering understanding of disease. The complex nature of asthma makes it difficult to find an animal model that possesses all key facets of asthma, including reversible airway obstruction, persistent airway hyperresponsiveness, chronic inflammation, and airway remodeling; while remaining cost-effective, easy to use, and with findings that translate directly to humans. The rat, guinea pig, and mouse are the classic, laboratory animal models. They are relatively inexpensive and convenient to keep, but in these models, disease is not naturally occurring and must be induced. Rodents have significant limitations as asthma models because, with continued antigen stimulation, they develop tolerance to antigen sensitization protocols used to induce disease, and airway hyperresponsiveness does not persist when antigen sensitization protocols are discontinued [25]. Larger asthma models have been induced in the dog, sheep, swine, cattle, and monkey, whose greater size facilitates lung function testing. However, like rodents, these models are not naturally occurring. Only the horse and cat develop a spontaneous asthma-like syndrome that demonstrates a high level of similarity to the human condition [15]. The most interesting difference between the two is that cats have a predominantly eosinophilic inflammation [26-28], while horses possess a neutrophilic inflammation [29-31].

Recurrent airway obstruction (RAO), also known as ‘equine heaves’ or ‘chronic obstructive pulmonary disease (COPD),' is an asthma-like disease of horses, and is one of the most commonly diagnosed conditions affecting the equine lung [32]. RAO is a

spontaneous, naturally-occurring progressive allergic respiratory disorder characterized by periods of acute airway obstruction, followed by periods of remission [33]. Two forms of the disease have been described: traditional RAO, or "barn-associated RAO," which occurs more commonly in temperate climates [34]; and "pasture heaves," or "summer pasture-associated recurrent airway obstruction" (SPARAO), which affects horses on pasture during the summer in hot and humid climates such as the southeastern United States and Great Britain [35, 36]. The incidence of RAO is rare in warm and dry climates [34]. In barn-associated RAO, disease typically occurs during the winter months, in stabled horses exposed to organic molds found in hay [37], while in pasture heaves disease is associated with horses housed primarily on pasture during spring and summer months. The direct cause of pasture heaves is unknown, but inhalation of pollens or molds are thought to be the main contributors [38]. Clinical exacerbation has also been correlated with increases in temperature, humidity, fungal spore counts, and grass pollens [39].

During disease exacerbation, horses suffer respiratory distress episodes similar to severe human asthmatics, displaying wheezing, coughing, and labored breathing as an airway response to inhaled aeroallergens [40]. During periods of disease remission, RAO-affected horses appear clinically normal, with airway function and cytology similar to nonaffected horses [41]. Significantly, their airways remain hyperresponsive to stimuli, and remodeling is persistent [42]; findings that are consistent in humans with neutrophilic asthma [43].

Like human asthma, RAO and pasture heaves are spontaneous diseases that occur in an outbred population, in response to an environmental aeroallergen challenge. RAO

and pasture heaves possesses all four of the key facets of human asthma: chronic airway inflammation, persistent airway hyperresponsiveness, reversible airway obstruction, and airway remodeling [31, 39, 44-46]. Additionally, the airway inflammation associated with RAO and pasture heaves is predominantly neutrophilic, similar to severely affected human asthmatics. Combined, these facets of RAO and pasture heaves make the diseases an excellent model for human asthma, particularly the neutrophilic phenotype. Based upon these clinical similarities, we hypothesize that horses with pasture heaves share protein networks that are relevant to asthmatic AHR and neutrophilic inflammation. Accordingly, the objective of this research is to employ genome-scale expression data to identify molecular signatures of asthmatic airway hyperresponsiveness and neutrophilic airway inflammation in pasture heaves horses.

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CHAPTER II

LITERATURE REVIEW

Asthma overview

History and prevalence of asthma

Asthma is a chronic respiratory disorder characterized by variable and recurring episodes of impaired breathing, cough, and wheezing. Exacerbations reflect airflow obstruction, bronchial hyperresponsiveness, and airway inflammation [1]. Incidences of asthma have been noted throughout most of recorded human history. In fact, the word "asthma" in Greek, means "to exhale with open mouth, to pant," and the first time the word appeared was in *The Iliad*, a Homer Greek epic poem. In 460-360 BC, Hippocrates was the first to use it as a medical term, or sign, and the first description of asthma as a disease was in the first century AD, by Aretaeus of Cappadocia [2]. It was not until the late 19th century when physicians accepted asthma as its own distinct disease [3]. As the world became modernized, asthma prevalence began to increase, partially due to increasing acceptance of the disease, improving medical care, and increases in environmental aeroallergens that came along with human advancements. Effective monitoring of asthma really began in the 1980s, through the CDC's National Health Interview Surveys. Today, asthma prevalence continues to increase, now affecting more than 300 million people worldwide, including an estimated 8% of American adults and 9.3% of American children [4]. In addition to being a prevalent disease, asthma is costly.

In 2007, the CDC estimated the cost of asthma $56 billion dollars per year in medical costs and lost revenue[5].

Causes of asthma

Asthma is a multifactorial disease, resulting from a complex, incompletely understood interaction between genes and environmental exposure [6, 7]. Traditionally, asthma was considered an atopic disease, reflecting an inherited predisposition to IgE production in which exposure to allergens causes IgE mediated clinical signs of disease [8]. To date, atopy remains 'the strongest identifiable predisposing factor for developing asthma' with viral respiratory infections being 'one of the most important causes of asthma exacerbation' which 'may also contribute to the development of asthma' [1]. Allergens that have been associated with this form of asthma include fungus, mold, animal dander, cockroach, house dust mite, and pollen [9-12]. However, the propensity to produce IgE in response to allergen challenge (atopy) affects approximately half of the adult population, and most atopic patients fail to develop asthma [8].

Within the last two decades, researchers have implicated many other factors in the development of asthma, including environmental factors such as air pollution, including ozone, particulate matter, and nitrogen dioxide, weather, and irritants [11, 13-16], and lifestyle decisions that include stress, diet, exercise patterns, obesity, tobacco smoke, and infection [17-20]. Greater than 100 genes have been identified that are likely to contribute to the different manifestations of asthma [21]. A limited number of genes have been associated with severe asthma, and before the advancements of genome-wide association studies (GWAS), only a few genes were implicated in development of severe asthma; most significantly, polymorphisms within interleukin 4-receptor alpha (IL4RA) [22],

which binds IL13 and IL4, increasing IgE levels [23, 24]. In two adult steroid-refractory asthma cohorts, amino acid substitutions in the intracellular signaling portion of IL4RA, within the minor alleles at E375A and Q551R, were associated with severe exacerbations and reduced lung function [25]. Another study concluded that the IL4-589T allele is a risk factor for near fatal, life-threatening asthma attacks [26].

Since 2007, there have been extensive studies to identify susceptible candidate genes based on genome-wide association studies (GWAS), but for severe asthma, results remain unclear [27]. In 2007, Chupp et al. conducted a GWAS of asthmatics stratified by severity of disease, and concluded that YKL-40 and its encoding gene CHI3L1 may be new biomarkers for severity of asthma [28]. YKL-40 is a chitinase-like protein that is involved in inflammation and remodeling [29]. Ober et al. performed a GWAS in 2008, confirming that CHI3L1 was a susceptibility gene for asthma, airway hyperresponsiveness, and reduced lung function, and that elevated YKL-40 levels were biomarkers for asthma and decreased lung function [29]. In 2010, two GWAS that included patients with severe, refractory asthma revealed Th2-like genes of importance on chromosome 5q, including RAD50, IL13, and TSLP [30, 31]. The protein encoded by RAD50 is involved in DNA double-strand break repair and has no known function directly related to asthma [30], TSLP has been shown to induce the production of Th2-attracting chemokines, such as TARC and CCL17, and prime Th2 T cell development [32], and the involvement of IL13 in many aspects of allergic asthma, including inflammation and remodeling been investigated for decades [24, 33]. Association with HLA-DQB1, a human leukocyte antigen that has been shown to be involved in antigenic presentation [34], was also noted in the 2010 GWAS conducted by Li et al. [30].

Hawkins et al. (2012) identified the IL6R coding SNP rs2228145 (Asp358Ala) as a potential genetic modifier of lung function in asthma, and as a novel genetic marker of asthma severity [35]. IL6R has been correlated with increased levels of inflammation through cytokine signalling dysregulation resulting in the activation of janus kinase 2 (JAK2) and STAT3 [35, 36]. GWAS have also identified the 17q12-21 locus encoding ORMDL3 which regulates the regulating metalloproteases, chemokines, OAS, and ATF6 [37], and GSDMB which regulates apoptosis, as predominantly associated with childhood-onset asthma [38]. Significantly, a GWAS performed by Moffatt et al. (2010) showed increased ORMDL3 expression only in childhood-onset asthma, and not in adult-onset asthma, or severe asthma, and that elevation of serum IgE levels only had a minor role in development of asthma [31]. Continued efforts to elucidate the genetic underpinnings of asthma are extensive and the diversity of these findings is consistent with the emerging concept of differences in asthma pathophysiology directing grouping by endotypes.

Signs, symptoms, and diagnosis of asthma

Impaired breathing during asthma exacerbations is largely reflective of asthma's three cardinal components: reversible airway obstruction (bronchoconstriction), airway inflammation, and airway hyperresponsiveness (AHR) to aeroallergens [39, 40]. Symptoms include shortness of breath and chest tightness, as well as clinically evident wheezing. Airway inflammation and mucus accumulation contributes to coughing. Asthma severity ranges from mild, with occasional disease exacerbation, to more severe disease that adversely affects the patient's quality of life.

Asthma diagnosis hinges on documentation of 1) airway obstruction that is reversible, typically in response to ß2-adrenoceptor agonists; 2) AHR to non-specific spasmogens (eg methacholine or hypertonic saline); 3) chronic airway inflammation; and 4) a medical history consistent with shortness of breath, chest tightness, cough, recurrent wheezing, nocturnal worsening of disease, and symptom triggers [41-43]. Advancing disease is associated with histologic changes in small and large airways that are collectively termed airway remodeling and are diagnosed using endobronchial biopsy [44]. Airway remodeling in asthma includes subepithelial fibrosis, increased airway smooth muscle mass, goblet and mucus gland hyperplasia, angiogenesis, loss of cartilage integrity, inflammation, and epithelial alterations. As asthma severity increases, the severity of airway obstruction, AHR, inflammation, and remodeling also progress [45].

Salient features of asthma

Reversible airway obstruction

The clinical manifestations of asthma including shortness of breath, wheezing, and coughing, are directly caused by airway obstruction [46]. Airflow obstruction results from a variety of changes that collectively contribute to airway narrowing, including bronchoconstriction, airway edema, mucus hypersecretion, and airway remodeling [47]. Airway remodeling refers to a set of histologic changes that include airway obstruction, goblet cell hyperplasia and metaplasia, mucus hypersecretion, thickening of airway epithelium, airway fibrosis, and smooth muscle proliferation [44, 48-52]. The precise contribution of these factors varies between asthmatic patients, resulting in diverse clinical expression, disease severity, and varying therapeutic responses [46].

The asthma diagnosis hinges upon documentation of airway obstruction that is reversible. Two cell types are predominantly responsible for the changes that lead to airway obstruction: airway epithelial cells and airway smooth muscle cells [53]. When an asthmatic patient inhales, environmental stimuli that include aeroallergens, viruses, and pollutants perturb airway epithelial cells, stimulating neurologic pathways that result in acetylcholine mediated constriction of airway smooth muscle [53, 54]. Concurrently, these stimuli also act on airway epithelial cells that produce inflammatory mediators and contribute to goblet cell metaplasia, mucus secretion, and airway smooth muscle contraction [54-56]. Goblet cells within the epithelium secrete mucus and epithelial cells produce inflammatory mediators, including cytokines and interleukins [54-56]. The resultant overproduction of mucus ultimately leads to the formation of mucus plugs, which account for significant mechanical obstruction of the airways [46]. In addition, inflammatory mediators increase capillary membrane permeability that promotes interstitial edema [46] and direct the infiltration of inflammatory cells [48], further contributing to airway narrowing. Inflammatory cascades also cause surrounding airway smooth muscle to become more contractile [46]. However, airway inflammation alone is not enough to explain neither the severity nor the progression of asthma [57, 58]. In fact, repeated bronchoconstriction without additional inflammation has been shown to be sufficient to induce airway remodeling, opening the door to investigations of the role of airway smooth muscle as a major mediator of asthmatic bronchoconstriction as well as inflammation [59, 60].

Airway smooth muscle (ASM) is a pivotal cell type in the overall pathogenesis of asthma [61]. ASM is the major cell type responsible for bronchoconstriction and in

addition, mediates airway inflammation, airway hyperresponsiveness, and chronic lung function impairment [61, 62]. These latter effects are discussed in more detail in the following sections. Airway smooth muscle is increased in both large and small airways in asthmatic patients [63-65], and correlates to asthma severity [63, 66]. Asthmatic ASM also has a greater velocity of contraction [67, 68], but even in the absence of changes in contractile properties, increased muscle mass is a major factor in asthmatic airway obstruction [69]. In addition to acetylcholine-mediated smooth muscle contraction, the diversity of inflammatory mediators released in asthma have both direct and indirect effects on augmenting airway smooth muscle contraction [46].

Airway hyperresponsiveness

Airway hyperresponsiveness (AHR) describes a condition of exaggerated airway narrowing in response to a bronchoconstrictive challenge, and it is a universal and defining feature of asthma [70, 71]. There is wide variability of intensity of AHR between patients, and even within the same patient, but intensity often correlates to severity of disease [72]. AHR is complex in its mechanisms; originally thought to be due solely to inflammation. Two semi-independent components of airway hyperresponsiveness are now recognized: persistent and variable AHR [62, 73].

Variable AHR is acute, transient, and reversible, and is believed to relate to the inflammatory events of the airway, which vary depending on stimulus [72, 74]. The variable component of AHR is associated with current allergen exposure, asthma activity, and disease severity [75]. Although the link to airway inflammation is strong, the precise mechanisms between airway inflammation and AHR are not entirely clear. By contrast, persistent AHR refers to AHR that remains even after removal of the inciting

agent/allergen/antigen for protracted periods. Persistent AHR is related to chronicity of disease and to the structural changes in the airway that define airway remodeling, including subepithelial thickening, smooth muscle hypertrophy, and extracellular matrix deposition [72, 74]. Some patients with mild or episodic asthma may only exhibit variable AHR and not persistent AHR [75].

Measurement of AHR is influenced by the methods used to detect it, which are divided into direct or indirect stimulation. Direct stimulation of the airway, predominantly used to study persistent AHR, is usually performed using methacholine or histamine, which act directly on airway smooth muscle (ASM), causing a contractile response that narrows the airways [72, 76]. A variety of indirect activators are used to detect variable AHR, including aerosolized hypertonic saline and mannitol, and adenosine monophosphate, which acts on mast cells to release mediators [74]. Histamine also elicits indirect stimulation, as it activates sensory fibers in the airway, leading to a reflex response that amplifies bronchospasm [74].

During much of the previous three decades, the dominant opinion was that asthma was a predominantly inflammatory disease. This opinion was formed based on the conclusions of studies that noted a relationship between AHR, inflammation, and remodeling [77]. However, that theory has been challenged by studies reporting a lack of a relationship between lung function, inflammation, and morphology [78, 79]. One such study, by Kariyawasam et al. (2007), evaluated the effects of allergic inflammation on AHR 24 hours and 7 days after an antigen challenge. After 24 hours there was AHR present in response to a methacholine as well as an increase in eosinophilia, but at 7 days, when the eosinophils were back at baseline levels, procollagen cells, a marker for airway

remodeling, were present [80]. This suggested that the structural changes of the airway are greater contributors than inflammation to AHR, and that eosinophils are not causative of AHR [80]. New computational models have shown that AHR is the result of complex interactions among ASM, remodeling, inflammation, load, and breathing patterns, but many functional features have been ascribed to ASM [69, 81, 82].

Airway inflammation

In asthmatics, airway inflammation can be caused by exposure to environmental and other factors (such as inhaled aeroallergens, irritants, and viruses) [83], but there is an underlying chronic inflammation present that occurs even in the absence of continued allergen exposure and obvious asthmatic symptoms [84, 85]. Many inflammatory cells play a role in the inflammation of asthma, including mast cells, neutrophils, eosinophils, T-lymphocytes, macrophages, neutrophils, and epithelial cells [86]. The eosinophil was long considered the effecter cell of airway inflammation, but more recent literature indicates that greater than 50% of adult asthmatics have a predominantly neutrophilic inflammation, correlating to increasing severity of disease [87-89].

Eosinophils are still believed to play an important role in the airway inflammation present in allergic asthma patients, through the release of inflammatory mediators such as leukotrienes, cytokines, and radical oxygen species [90, 91]. There is debate within the literature to the extent of involvement of the eosinophil in the causation of airway hyperresponsiveness, but studies have shown that when asthma patients with a persistent eosinophilia are treated with anti IL-5 mAb, which reduces eosinophil numbers, their quality of life improves, with reduced exacerbations and a reduction in the amount and frequency of their need for systemic corticosteroids [92]. However, some studies

demonstrate that even after this treatment, there is still no reduction in airway hyperresponsiveness, suggesting that eosinophils may not be a prerequisite for airway hyperresponsiveness [58]. Steroid resistance has been described as lack of improvement of lung function and demonstration of airway hyperresponsiveness despite treatment with corticosteroids [93, 94]. Although eosinophilic asthma is generally responsive to corticosteroids, there is evidence that some patients continue to have persistent eosinophilia, thickened subepithelial basement membranes, and higher incidences of asthma-related morbidity despite treatment [95-97]. Contrary to asthmatics with eosinophilic airway inflammation, most cases of steroid resistant asthma are patients with neutrophilic airway inflammation [98, 99].

Most cases of severe asthma have a predominant airway neutrophilia, with numbers that correlate to an increased disease severity [87-89]. Interleukins (IL) -8 and -17, both of which are upregulated in severe asthmatics, have been shown to play an important role in the accumulation of neutrophils at sites of inflammation [100-103]. Neutrophils have been recognized as the major inflammatory cell present in acute severe asthma that requires mechanical ventilation [101]. Further, in fatal asthma attacks, bronchiolar neutrophilic inflammation predominates and eosinophilic inflammation is less prevalent. This finding has led to the hypothesis that fatal attacks of asthma may be triggered by stimuli that elicit neutrophilic inflammatory responses [104].

It is now known that airway smooth muscle is a pivotal cell type mediating airway inflammation. Through the upregulation of cell adhesion molecules including intracellular cell adhesion molecule-1, vascular cell adhesion molecule-1, and CD44, ASM interacts with inflammatory cells including T-cells, mast cells, eosinophils, and

neutrophils [105]. ASM produces numerous cytokines such as IL-1ß, IL-5, IL-6, IL-8, and IL-17. IL-6 is produced when ASM is stimulated by other cytokines, including IL-1ß or TNF-α, and IL-6 can induce ASM hyperplasia [106]. ASM also has other autocrine effects, one example being the presentation of bacterial superantigen toCD4+ T cells to enhance production of IL-13, a proinflammatory cytokine, which can in turn evoke changes in ASM constriction [107]. ASM also produces chemokines that recruit and retain inflammatory cells, including eotaxin, MCP-1, MCP-2, MCP-3, RANTES, CXCL8, CXCL10, fractalkine, and thymus-regulated and activation-regulated chemokine. [108-114]. In addition to typical inflammatory factors, growth factors such as VEGF, BDNF, and TGF-ß can be secreted by ASM cells [115, 116]. Airway smooth muscle responsiveness to and ability to excrete an array of inflammatory mediators is an important part of the inflammatory response in asthma, and the modulation of inflammatory responses by ASM may be a potential therapeutic target.

Histopathology of asthma: airway remodeling

Histopathologic changes in asthmatic lung collectively referred to as airway remodeling were first described in 1922 in fatal cases of asthma, and since then have been documented in all degrees of asthma severity [117]. Airway remodeling refers to the structural histopathological changes that occur in asthmatic airways over time [118]. Unlike airway obstruction, remodeling changes are incompletely reversible [119, 120], and can result in fixed (non-reversible) airflow obstruction as asthma progresses [121]. The most significant remodeling changes contribute to increased airway smooth muscle and thickening of the airway epithelium, but other changes include compromised epithelial barrier function, mucus gland hypersecretion, goblet cell hyperplasia and

metaplasia, and airway fibrosis [44, 48-52]. Significantly, airway remodeling is known to correlate to the clinical severity of asthma [122-124].

Airway smooth muscle thickening

Airway smooth muscle is present in both the large and small airways of both healthy patients and asthmatics [125]. Increased airway smooth muscle (ASM) mass is the most prominent feature of airway remodeling [63], and is thought to be the result of a combination of hyperplasia, hypertrophy, migration of airway smooth muscle cells towards the epithelium, and a decreased rate of apoptosis [66, 126, 127], although ASM hyperplasia seems to be more dominant and significant than hypertrophy [125]. James et al. (2012) proposed that different forms of remodeling of ASM may account for differences in the severity of asthma, with increased hyperplasia correlating to disease severity [117].

Increased hyperplasia of airway smooth muscle cells in asthmatics can be explained in part by the stimulus of mitogens, chemical compounds that initiate cell division and mitosis. There are many mitogens, including TGF-ß, IL-1, IL-6, leukotrienes, histamine, serotonin, and others [128]. These mediators function as ASM mitogens, increasing ASM mass by binding to receptor tyrosine kinases, G protein-coupled receptors, and cytokine receptors [129].

Airway epithelial thickening

Increased airway wall thickening has consistently been associated with increased asthma severity [130-132]. Airway wall changes result from a combination of epithelial cell alterations, subepithelial thickening, submucosal gland hyperplasia, and increased

airway smooth muscle mass [48, 65, 133, 134]. Epithelial cell alterations include shedding of the epithelium, goblet cell hyperplasia and metaplasia, and upregulation of cytokines and growth factors [135]. Thickening of the subepithelial layer is characterized by increased deposition of extracellular matrix proteins such as collagens, proteoglycans, and glycoproteins [136, 137], which are produced by fibroblasts and myofibroblasts. These changes occur in the lamina reticularis, and the thickened reticular layer is largely composed of collagen I, III, V, and fibronectin [134].

Epithelial barrier function

The airway epithelium is the interface between the inhaled environment and internal tissues, and its normal function is to form a physical barrier against inhaled particles such as allergens or pathogens [118, 138, 139]. This physical barrier has two primary components, the mucociliary clearance apparatus [140], and epithelial tight junctions [141].

The epithelium of the airways is covered by surface liquid that is composed of a periciliary layer around microvilli, and an overlying mucus layer where inhaled particles are trapped [142]. The mucins that comprise the mucus layer are produced throughout the bronchial tree, by goblet cells, and mucus and serous cells of the submucosal glands [143]. Mucociliary clearance involves the movement of mucus-trapped inhaled particles from the airways towards the oropharynx, by the coordinated beating of the cilia [144]. In the normal lung, ciliated columnar, goblet cells, and Clara cells form a highly regulated and impermeable barrier, made possible through the formation of tight junctions [142]. Tight junctions enable communications between adjacent cells and regulate intercellular transport [145, 146]. Further maintenance of the epithelial integrity is through adherens

junctions, desmosomes, and hemidesmosomes [147]. Disruption of this barrier, the first line of defense in the lung, enables penetration of the airway wall by pathogens and particles [148, 149].

In patients with asthma, there is evidence that the barrier function of the airway epithelium is impaired [146], with loss of columnar cells and enhanced expression of epidermal growth factor receptor in areas of damage [150, 151]. Disruption of tight junctions and loss of junctional proteins has also been recorded in asthmatic patients [152]. Asthmatic patients have increased mucus secretion from goblet cells that are metaplastic and hyperplastic [153], as well as overall increased amounts of mucins in sputum compared to levels in normal induced sputum [154], both which contribute to mucociliary clearance dysfunction, and result in airway obstruction [147].

Repair responses occur after airway epithelial damage, but are often incomplete or abnormal. Incomplete repair can lead to a scenario not unlike a chronic wound [139], where secondary growth factors are secreted, driving structural changes linked to airway remodeling. This includes abnormal subepithelial collagen deposition, which may contribute to an alternative protective barrier in the absence of a functional epithelium [147].

Goblet cell hyperplasia/metaplasia, mucus gland hypersecretion

Goblet cells produce and secrete mucin glycoproteins (MUC) into the airway lumen, which in turn trap foreign particles leading to removal via the mucociliary escalator. Thirteen mucin proteins have been identified in human airways [129], and the predominant mucin is MUC5AC, which is increased in asthmatic patients relative to healthy patients [155]. Mucin production must be tightly regulated, as overproduction can

impair proper mucociliary clearance, and eventually contribute to airway obstruction [149].

Goblet cell hyperplasia has been consistently demonstrated in mild, moderate, and severe cases of asthma, correlating with increasing disease severity [119, 156] and is widely acknowledged as a key component of airway remodeling in asthma [50, 119, 123, 124]. One study demonstrated that in mild asthma the hyperplastic goblet cells increase mucin storage while in moderate asthma the mucin is overstored and secreted, concluding that altered secretion of mucin may be related to asthma severity [119]. IL-9 and IL-33, two cytokines that have been implicated in many aspects of asthma pathogenesis, may play a role in triggering goblet cell hyperplasia and the induction of mucus hypersecretion [157, 158]. IL-9 has also been implicated in promoting allergic inflammation, mast cell growth and survival, airway remodeling, and airway hyperresponsiveness in asthmatic patients [159-161]. Levels of IL-33, an alarmin cytokine, increase with asthma severity [162], and the gene encoding IL-33 has been identified as a major susceptibility loci for asthma in GWAS [31, 163-165]. In addition to its role in goblet cell hyperplasia, IL-33 has been implicated in airway remodeling, Th2-type cell differentiation, promoting allergic inflammation and asthma pathogenesis [162, 166].

There are some inconsistencies throughout the literature with regards to the use of metaplasia and hyperplasia, but hyperplasia refers to proliferation of the same cell type, while metaplasia refers to cellular differentiation to an alternate cell type. Often, metaplasia is referred to as hyperplasia. The overall consequences of these abnormalities

are increased mucus production, airway narrowing due to mucus plugs, and increased airway wall thickness [63].

Airway fibrosis

Subepithelial collagen deposition is a primary histopathologic alteration observed in asthmatic lungs [122], and airway fibrosis has been reported in all severities of asthma [51, 167]. There are conflicting views within the literature in regards to the correlation between the presence of collagen deposition and asthma severity. Many studies have associated subepithelial fibrosis with measurements of asthma severity, frequency, duration of symptoms, and the degree of airway hyperresponsiveness [51, 52, 122, 123, 168-171]. However, other studies have not shown a correlation [124, 172]. Additionally, some severe asthmatics do not have airway fibrosis [122], and some nonasthmatics do [173]. However, measurements of limited airway distensibility correlate well with the magnitude of subepithelial fibrosis [174].

Airway fibrosis plays a role in the pathophysiology of incompletely reversible airway obstruction that occurs in some asthmatics [175]. Although this process was initially described as basement membrane thickening, it is now apparent that the true basement membrane is not grossly altered [134]; instead, the lamina reticularis, which is just above the basement membrane, is thickened by interstitial collagens [117]. In normal lungs, this collagen layer is approximately 5mm thick, but in some asthmatic lungs, it has been reported to increase to 7-23mm [176]. There are inconsistent results with respect to enhanced collagen deposition in the submucosa, which is between the subepithelium and smooth muscle [169, 172]. Abnormalities have also been noted in elastin, proteoglycans, and cartilage [177].

Treatment of asthma

The most important and efficacious component of asthma therapy is antigen avoidance. While certain individuals, particularly those with seasonal allergies and asthma may be helped by allergen immunotherapy, overall there is no conclusive benefit for this therapy in individuals with year round asthma [178]. In addition, asthma exacerbations are well known to follow viral respiratory infections, leading to value in measures that mitigate viral spread and infection, including respiratory viral vaccination [179, 180].

In humans, asthma is treated primarily with inhaled short and long acting ß2-adrenoceptor agonists, which inhibit airway narrowing and closure; and inhaled and oral preparation of corticosteroids which target the inflammatory components of disease. ß2-adrenoceptor agonists are effective bronchodilators due to their ability to antagonize airway smooth muscle contraction [181]. ß2-receptor activation is mediated by increased cAMP levels. ß2-adrenoceptor agonists bind to ß2-adrenergic receptors to activate a G-protein coupled receptor pathway via adenylyl cyclase type 9 (ADCY9). The ADCY9 gene contains a common coding variant, ILe^{772}Met, which alters adenylyl cyclase activation in response to ß2-adrenoceptor agonists, regulating airway smooth muscle relaxation [182].

Corticosteroids suppress inflammation in asthma through the activation of many anti-inflammatory genes, and repression of proinflammatory genes. Corticosteroids diffuse across cell membranes, binding to glucocorticoid receptors in the cytoplasm [183, 184]. Once bound, the glucocorticoid receptor changes conformation, is released, and crosses into the nucleus [185], where it homodimerizes, binding to glucocorticoid

response elements in the promoter region of steroid-responsive genes [186]. This binding initiates gene transcription through interaction with transcriptional coactivator molecules such as cyclic AMP response element binding protein-binding protein (CREB). CREB has histone acetyltransferase activity, which causes the acetylation of core histones, initiating chromatin remodeling, and recruitment of RNA polymerase II, resulting in gene activation [187, 188]. Genes that are activated by corticosteroids include anti-inflammatory proteins such as glucocorticoid-induced leucine zipper, mitogen-activated protein kinase phosphatase 1 (MPK-1), lipocortin-1, secretory leukocyte inhibitory protein, IL-10, IL-12, and IL-1 receptor antagonist [186, 189, 190]. Corticosteroids also inhibit many inflammatory molecules, including inflammatory cytokines such as IL-2, IL-3, IL-4, IL-5, IL-6, IL-13, IL-15, TNF-α, GM-CSF, and TSLP; chemokines such as CCL1, CCL5, CCL11, and CXCL8; inducible nitric oxide synthase; inducible cylcooxygenase; inducible phospholipase A2; endothelin-1; neurokinin; intracellular cell adhesion molecule-1; and vascular cell adhesion molecule-1 [190, 191]. This occurs in two main processes: 1) through glucocorticoid receptors interacting with repressor molecules to reduce histone acetylation , chromatin remodeling, and RNA polymerase II actions of proinflammatory transcription factors [192], and 2) through recruitment of histone deacetylase 2 which deacetylizes the glucocorticoid receptor, allowing suppression of activated inflammatory genes within the nucleus [186].

After an asthma diagnosis is made, treatment is initiated in steps correlating to severity of disease and relief of asthma signs and symptoms. These steps are recommended by the Global Initiative for Asthma:1) an as-needed inhaled short-acting beta-2 adrenoceptor agonist (SABA), 2) regular low dose inhaled corticosteroid and an

as-needed SABA, 3) adults- maintenance dose of a combination low dose inhaled corticosteroid/long acting beta-2 adrenoceptor agonist (LABA) and an as-needed SABA or combination low dose inhaled corticosteroid/formoterol as maintenance and rescue; children - moderate dose inhaled corticosteroid plus as needed SABA, 4) adults - combination low dose inhaled corticosteroid/formoterol as maintenance and rescue, or combination medium dose inhaled corticosteroid/LABA and an as-needed SABA; children- referral to specialist, 5) referral to specialist [193]. When signs and symptoms have been ameliorated for 2-3 months, titration to the lowest maintenance treatment is recommended [193].

While an exhaustive review of asthma therapeutics is not relevant to the focus of this dissertation, second-line asthma therapies include anticholinergic bronchodilators such as atropine and glycopyrrolate, which can be administered parenterally and nebulized, and ipratroprium which is nebulized. The methylxanthine bronchodilator, theophylline, is now infrequently administered to asthmatics due to the heightened risk of cardiac arrhythmias at increased doses [194].

Adjunctive therapies for control of refractory asthma include the leukotriene receptor antagonists, montelukast and zafirlukast [195, 196], and the 5-lipoxygenase inhibitor, zileutonsuch, that inhibits leukotriene synthesis [197]. Leukotriene inhibitors are potent anti-inflammatory agents that also limit airway smooth muscle constriction by interfering with the actions of LTA_4, LTC_4, LTD_4, and LTE4 [198-203]. Mast cell stabilizers of the cromone class (cromolyn) are limited to cromolyn in the United States, which is administered by nebulization. This agent agents block calcium channels essential for mast cell degranulation, stabilizing the cell and thereby preventing the

release of histamine [204, 205]. Omalizumab is a humanized anti-IgE monoclonal antibody used in patients with elevated IgE levels and allergies to a known allergen [206]. Omalizumab works to reduce asthma exacerbations, but has a relatively small effect on airflow [207]. These adjunctive therapeutic agents allow for a reduction in amount of corticosteroids and ß2-adrenoceptor agonists given to patients, but they are highly specific, and only affect a small subset of patients.

Therapeutic controversies in asthma

Inhaled ß2-adrenoceptor agonists are among the oldest and most commonly prescribed treatments for asthma, and they can be divided into three classes: short-acting ß2-adrenoceptor agonists (SABA), long-acting ß2-adrenoceptor agonists (LABA), and ultra-long ß2-adrenoceptor agonists [208]. SABAs, such as albuterol, isoproterenol, terbutaline, and fenoterol, provide rapid symptom relief with bronchodilation, but their short duration of action makes them inadequate for extended relief. LABAs, such as formoterol and salmeterol, are designed to alleviate symptoms for longer durations, but ultra-long ß2-adrenoceptor agonists, including indacaterol and vilanterol, aim to maintain efficacy while improving dosing convenience with a longer duration than LABAs [209].

For decades, debates have occurred regarding the safety of ß2-adrenoceptor agonists in asthma therapy. In the 1960s and 1970s, asthma mortality epidemics were associated with two SABAs, fenoterol and high-dose isoproterenol, but these epidemics subsided after the SABAs were withdrawn from the market [210, 211]. In 1993, Taylor et al. discovered that chronic use of inhaled fenoterol increased asthma exacerbations, airway hyperresponsiveness, and decreased lung function in asthmatic patients, indicating that chronic administration of short acting ß2-adrenoceptor agonists treatment was

deleterious to long term asthma control [212]. Also in the early 1990s, the United States Food and Drug Administration (FDA) requested a surveillance trial to evaluate the safety of LABAs in 60,000 subjects; this was called the Salmeterol Asthma Multicenter Research Trial (SMART) [213]. The SMART study was terminated early when analysis revealed an increase in life-threatening asthma exacerbations and death in subjects taking LABA compared to placebo [213]. This resulted in a black box warning for all inhaled LABAs regarding the risk of severe and life-threatening asthma exacerbations [214]. Changes to labels included the contraindication of LABAs without the concomitant use of inhaled corticosteroids and recommendations that LABAs be used only when necessary to achieve and maintain control [214, 215]. There is an ongoing FDA-mandated international LABA safety study, with expected results in 2017 [214]. This study is designed to assess the safety of a regimen of LABAs plus inhaled corticosteroids as compared to corticosteroids alone [214, 216]. Studies have revealed that with LABA therapy in conjunction with inhaled corticosteroids does not result in increased risk of death, but in a beneficial effect on asthma control and risk of exacerbation, suggesting that adverse reactions occur in a small population [217-219].

Other side effects have been documented with frequent or repetitive use of ß2-adrenoceptor agonists. Frequent use of ß2-adrenoceptor agonists increases airway hyperresponsiveness to allergen [220, 221], causes tolerance to the protective effect on bronchoconstriction [220] and decreases asthma control [222]. More recent evidence indicates that repetitive use of either short-acting or long-acting ß2-adrenoceptor agonists leads to reduced bronchoprotection and sensitization of excitation-contraction signalling pathways in airway smooth muscle cells [223].

When inhaled corticosteroids were introduced to asthma management, large improvements were observed in symptom control, lung function, number of exacerbations, and mortality [224]. Most asthmatic patients are well controlled with low doses of inhaled corticosteroids [225], but 5-10% of severe asthmatics are refractory to corticosteroids. Investigations determined that treatment failure corresponded to the presence of neutrophilic airway inflammation in these patients, with or without eosinophils, increased tissue injury and remodeling, and are part of the noneosinophilic asthma endotype [226]. Although this is a small population of people, the increased disease severity associated with corticosteroid refractory asthma accounts for approximately half of the total cost of healthcare, and the majority of asthma-related deaths [227]. Adverse effects associated with corticosteroid use, include immune suppression, cataracts [228], growth deceleration [229], skin thinning and bruising [230], osteoporosis[231], and dysphonia [190], although most patients do not experience them, as they tend to be a result of improper use, inhaler technique, or high doses [193, 232].

Since the introduction of ß2-adrenoceptor agonists and corticosteroids as asthma therapeutics, few novel therapeutic agents have effectively been translated to clinical use from animal investigations [233]. Many drugs have done well in animal trials, only to fail in human testing stages. For example, animal models provided evidence that interleukin-13 (IL-13) was necessary to induce all features of allergic asthma, regardless of the other Th2 cytokines. IL-13 was shown to be upregulated in asthmatics[234], and was supported in animal models of mice [235], cynomolgus monkeys [236], and sheep [237]. Significant research and funding was invested in biotherapeutics that target IL-13 and its pathways, but clinical trials have been disappointing [238, 239]. Difficulties in the

clinical translation of drugs from the traditional mouse model to the human condition are not unique to asthma. The FDA has acknowledged the limitations of current animal models across many therapeutic areas, including asthma, and that there is a need for new models and approaches for further drug development [240].

Asthma endotypes

Traditionally, asthma classification schemes have been based on subjective parameters and objective assessments that reflected whether disease signs were intermittent, mild persistent, moderate persistent, or severe persistent [241]. These cases were all thought to be similar, only differing in their degree of severity, and were accordingly treated with the same medications, while employing different doses, frequencies, or routes of administration [242]. For decades, clinicians have recognized different asthma phenotypes, classifying them based on observable characteristics including clinical signs, physiologic and morphologic changes, biochemical characteristics, and response to treatment [241]. These facets remained without formal characterization. In 2006, it was proposed that asthma be recognized not as a single disease, but as a complex and heterogeneous syndrome composed of a disease variants, each with their own pathophysiologies [21]. This has yielded an effort towards classifying asthma by distinct functional or pathophysiological mechanism, or 'endotypes', rather than by overtly observable characteristics. With this method of classification based upon pathophysiology, it is possible for an endotype to be present in more than one phenotype, consistent with the natural spectrum that characterizes disease. Accordingly, the umbrella term "asthma" may be eventually replaced by terms that more

accurately describe the actual pathophysiology of diseases that manifest as with signs of asthma [243].

Currently, the asthma syndrome is divided into several distinct asthma endotypes, including, but not limited to: allergic asthma, aspirin-sensitive asthma, severe late-onset hypereosinophilic asthma, allergic bronchopulmonary mycosis, viral-exacerbated asthma, premenstrual asthma, API-positive preschool wheezer, airflow obstruction caused by obesity, severe steroid dependent asthma, cross-country skiers' asthma, elite athlete asthma, infection-induced asthma, steroid-insensitive eosinophilic asthma, and noneosinophilic (neutrophilic) asthma [241, 244, 245]. As research continues, and more endotypes are elucidated and further classified, this effort advances asthma classification towards personalized medicine [246].

Neutrophilic airway inflammation in asthma

Allergic asthma is the classic form of persistent asthma, and has been investigated in great detail. Typically it has a childhood onset and is accompanied by allergic features, including sensitization to allergens and allergic rhinitis[241]. The inhalation of allergens by a predisposed individual triggers TH2 immune deviation, driving eosinophilic inflammation and tissue damage, and resulting in airway hyperresponsiveness and release of inflammatory mediators. This causes the clinical signs, including acute bronchoconstriction [21, 241], which are largely sensitive to corticosteroid use. Research over the past two decades has focused on these allergic pathways and inflammatory profile.

The development of allergic asthma consists of three phases: induction, early-phase asthmatic reaction (EAR), and late-phase asthmatic reaction (LAR) [247]. The

induction phase occurs after the patient is first exposed to an inciting allergen. The allergen is then taken up by antigen presenting cells, and is presented to allergen-specific T and B cells in the lymph nodes [248]. Activation of T helper cells by the antigen presenting cells leads to the production of cytokines that regulate B cells in their production of immunoglobulin (Ig) E [249]. Once IgE is synthesized, the antibodies circulate in the blood before binding to high affinity receptors present on both mast cells and peripheral basophils [250]. When a patient is reexposed, the allergens cross-link to the bound IgE, which triggers the production and release of mediators such as histamine, prostaglandins, leukotrienes, and cytokines [250], beginning the EAR. The early phase is an immediate response that lasts approximately 30-60 minutes, and is characterized by constriction of airway smooth muscle cells, vascular leakage, mucus production, enhanced airway hyperresponsiveness, and recruitment of inflammatory cells [251]. The late phase occurs 4-6 hours later, and is characterized by a second period of bronchoconstriction, excessive airway inflammation, increased bronchiovascular permeability, enhanced airway hyperresponsiveness, mucus secretion, and through chronicity, airway remodeling [167, 252, 253]. Approximately half of allergic asthma patients develop a late-phase response after allergen challenge [254].

Traditionally, it was thought that all asthmatics had allergic asthma, and associated eosinophilic inflammation. However, more recent literature estimates that greater than 50% of asthmatics have neutrophilic inflammation, correlating to more severe disease [87-89]. The airways in severe asthma have been described as having characteristics of a chronic wound, with evidence of on-going epithelial injury and repair [255]. A cause of increased recruitment of neutrophils is through IL-8, a neutrophil

chemoattractant, which is expressed in airway epithelial cells [256]. IL-8 is synthesized and secreted is via epidermal growth factor receptor (EGFR) activation [257, 258], a key factor of epithelial repair that is correlated to asthma severity [259]. The increased recruitment and subsequent increased total number of activated neutrophils [260], correlate with increased airway damage [261]. Further, with neutrophilic asthma, there is a shift from the Th2 phenotype recognized in traditional allergic asthma to Th1-like patterns with increased expression of tumor necrosis factor alpha (TNFα) [262] and interferon-gamma (INFγ) [263]. TNFα also increases IL-8 synthesis [257], and itself can increase chemotaxis of neutrophils [262, 264].

For an asthmatic to be considered part of the noneosinophilic, or neutrophilic asthma endotype, they must have the following essential pathogenic mechanisms: activation of the innate immune response, abnormal activation of histone deacetylase 2, abnormal recruitment of neutrophils, increased survival of neutrophils [265] and demonstrate reduced corticosteroid responsiveness [99]. The gold standard for diagnosing neutrophilic asthma is through induced sputum measurements, with numbers of neutrophils correlating to disease severity [266].

Mainstay therapies that are efficacious for traditional allergic asthma are not as effective in patients with neutrophilic disease. As disease severity increases, and numbers of neutrophils increase, corticosteroid refractoriness also increases [99]. Additionally, treatment with corticosteroids has been shown to promote neutrophil survival, further increasing the magnitude and duration of inflammation [267]. These treatment challenges that accompany severe neutrophilic asthma highlight the need for suitable animal models

to investigate the mechanistic basis of asthma that is accompanied by neutrophilic airway inflammation.

Animal models of asthma

Complex multifaceted diseases such as asthma necessitate investigations at the whole-organism level. Animal models of human disease address this need by allowing researchers to examine differences in the complex *in vivo* interactions that characterize diseased and non-diseased whole organ systems [233]. Animals have been used extensively to study mechanisms of asthma, the activity of asthma related genes and pathways, and to estimate safety of asthma therapeutics [268, 269]. These investigations have produced much of the current knowledge regarding the mechanisms that are responsible for asthma. However, the complexity of asthma, and the varying endotypes, make finding an animal model that mimics all characteristics of disease difficult [270]. The four key characteristics of asthma, airway inflammation, reversible airway obstruction, airway hyperresponsiveness, and airway remodeling, should be a minimum standard in an animal model of asthma, along with exhibition of all clinical signs shown by humans, including wheezing and coughing.

Many different animal models have been used to investigate asthma, including mice, rats, guinea pigs, ferrets, rabbits, cats, dogs, pigs, sheep, nonhuman primates, cattle, and horses, and each have their strengths and weaknesses. The most frequently used models are rodent models [271]. The only two species to have spontaneous, natural occurring asthma like syndrome are cats and horses [120], although dogs do have a spontaneous, exercised induced form of disease. Each of these species has sequenced

reference genomes of varied refinement that facilitate genome-based investigations [272-281].

Murine model

Mice are the most popular animal for modeling allergic responses in the airways [62, 271]. Many protocols are employed in murine models of asthma, but the most common, traditional approach is using antigen sensitization via ovalbumin (OVA), usually given intraperitoneally with an adjuvant, followed by an inhalation challenge [233, 282]. This approach results in an acute asthma-like,Th2-like, inflammatory response. Cytokines including interleukins 4, 5, 13, and 9, are released, promoting eosinophilic inflammation. The importance of dendritic cells, Th2 cells and eosinophils to the allergic response have been mapped out primarily using this mouse model [283].

More recently, researchers have attempted to develop "long term" mouse models through repeated sensitization to OVA, or to multiple human allergens, such as house dust mites [284], cockroach antigens [285], *Aspergillus fumigatus* [286], and ragweed [287]. In these protocols, mice are continually exposed to antigen for up to 12 weeks. Chronic models have been met with limited success, with development of tolerance to antigen, resulting in diminishing airway inflammation and airway hyperresponsiveness [288, 289]. This model fails to stimulate chronic inflammation and epithelial alterations, both important characteristics of human asthma that are not present in rodents. These models do yield a degree of airway remodeling, but it is largely due to fibrosis, and not airway smooth muscle hypertrophy, which is the predominant histologic change that correlates to worsening asthma severity in humans [290, 291].

There are some advantages to the mouse model; mainly cost and convenience. Mice are inexpensive to acquire, have a short and successful reproductive cycle, are easy and inexpensive to house, and are easy to handle. There is also a wide availability of genetically characterized inbred strains, at low cost, which allows for focused mechanistic research, and examination of particular pathways and genes [292, 293], and transgenic mice are useful to characterize key events and effector molecules that regulate airway inflammation [293].

However, it is important to note the significant disparity that exists between antigen sensitized mice and human asthma. Not only must the disease phenotype be induced in the mouse [233], but their response is strictly allergy-based with a predominance of eosinophilic inflammation, and does not provide information of relevance to severe, neutrophilic inflammation. Sensitization via ovalbumin injection is completely different from the naturally occurring human disease in which sensitization occurs via the respiratory tract, and OVA does not trigger asthma in humans [294]. Even after sensitization, an OVA-mouse does not truly develop asthma, but demonstrates certain cellular and pathophysiological features that have some similarities to parts of the asthma condition, namely a massive influx of inflammatory cells, particularly eosinophils, airway hyperresponsiveness, and an IgE response [283]. Further, strains of mice can exhibit vast differences in the extent to which they develop immune responses to the same sensitizer [295], with levels of antigen-specific IgE varying between strains [296], as well as differences in the degree of eosinophilic inflammation, airway wall thickening, collagen deposition, and airway hyperresponsiveness [295, 297]. Like other laboratory animals, mice are housed in clean and controlled laboratory environments

where they are not exposed to the same environmental allergens that contribute to disease in humans [283].

Mice also have well-documented disparities in the branching pattern, airway smooth muscle mass, and type and location of inflammatory cells relative to human asthma [298]. Histologic abnormalities are also largely isolated to airways in humans, but mice also show lung parencyhmal and pleural involvement [299]. Humans and mice also have different transcriptional responses to acute inflammatory insults [300], which limit direct translation of results. For example, mice lack the gene that codes for interleukin-8 [301], a neutrophil chemotactic factor which has been implicated in severe asthma [88]. One of the most important differences between humans and mice is that antigen responsiveness in mice is not persistent [302]. That is, when the allergen exposure ceases, the inflammatory process, and airway hyperreactivity abate, and require an additional sensitization period to re-establish airway inflammation and hyperresponsive to antigen challenge. In humans, these facets of asthma persist despite extensive periods of antigen withdrawal [80].

While it is now recognized that the original concept of asthma as a Th2-mediated disorder is overly simplistic, and that the mouse model fails to appropriately model the asthma syndrome, mouse models continue to be the primary model used to evaluate new therapies [283]. It is now generally recognized that mice are suitable for modeling traits associated with asthma, rather than modelling the entire asthma syndrome, or even a specific endotype [269]. Alternative murine models are being used and developed, including models demonstrating limited biphasic inflammatory responses and neutrophilic inflammation. Models with two different asthmatic phases have used

sensitization protocols with cockroach antigen [303] and chronic intratracheal ovalbumin [304, 305]. In 2012, Vaickus et al. demonstrated an early airway response occurring 1.5 hours post-challenge with cockroach antigen, and a second phase occurring 24 hours later. The first phase was characterized by inflammatory mediators such as CXC chemokines, mucin production, and recruitment of neutrophils to the lung, and the second was characterized by accumulation of new mucin in pulmonary epithelial cells; eosinophils remained constant throughout the study [303]. Nabe et al. (2005) established a late-phase model with airway obstruction that peaked at 30 minutes, and returned at 2-8 hours. Their study required four intratracheal exposures to ovalbumin [305]. The 1st through 3rd challenges resulted in airway inflammation characterized by infiltration of eosinophils and CD4+ cells, while the 4th challenge induced neutrophilic inflammation into the airway [305]. These two investigations are representative of the current information regarding late-phase allergic responses in mice, where additional investigations are needed to address these somewhat contradictory findings regarding the timing and inflammatory profiles of the late phase reaction in this species.

Rat model

Rats are also popular models of allergic airways disease. In the past, rats were used more frequently to model asthma, but in recent years, mouse genetics and associated technologies have improved exponentially, increasing the popularity of the mouse as a model [271]. Just as in mice, the asthma-like disease exhibited by rats can only be induced, and they are easily sensitized to antigens such as ovalbumin, house dust mites, and *Ascaris* [306, 307]. Challenge results mostly in a Th2-like response, with

predominant airway eosinophilia and IgE [308], but there can be involvement of neutrophils, mast cells, and lymphocytes [309-311].

Neutrophilic inflammation can occur in rat models of asthma, but there are variations between strains. In Brown Norway rats subcutaneously OVA-sensitized, challenge with aerosolized OVA results in neutrophilia present 3-14 hours after challenge, followed by a peak in neutrophilia and initial evidence of eosinophil infiltration at 24 hours post-challenge [309, 312]. Another common mechanism to induce neutrophilic pulmonary inflammation in Sprague-Dawley rats is via intranasal LPS sensitization and challenge [313, 314].

The popular rat model has some distinct advantages. Key facets of asthma, including airway hyperresponsiveness, airway inflammation, and airway obstruction can be easily reproduced in rats [311, 315]. Other similarities between rats and humans include immediate and late asthmatic responses after a nonspecific challenge with methacholine, acetylcholine or serotonin, IgE production, and inflammatory cell accumulation [311, 316]. Recent studies on OVA sensitized rats with long term challenge with either inhaled *Aspergillus fumigatus* or ovalbumin, demonstrated airway remodeling changes including goblet cell metaplasia, airway smooth muscle hypertrophy and hyperplasia, collagen deposition, and epithelial cell damage, along with increased airway responsiveness and inflammation [315, 317, 318]. Compared strictly to mice, rats have a size advantage, making measurement of physiological outcomes such as airway hyperresponsiveness and acute responses to allergens easier to obtain. Finally, like other small laboratory animals, rats are inexpensive and easy to handle and care for.

Disadvantages of the rat as an animal model are similar to those in the murine model. Most significantly, disease is not spontaneous and must be induced with antigens and adjuvants. There are many strains available, and response following sensitization and challenge varies between strains [309, 310]. Finally, when compared to the mouse, species-specific immunological reagents are not as abundant [319].

Guinea pig model

Although mice are the most common animal used to model asthma, beginning more than 100 years ago, guinea pigs were the original animal model of asthmatic disease [268]. Like mice and rats, guinea pigs must also undergo antigen sensitization protocols to develop asthma-like disease. Guinea pigs are readily sensitized to OVA, and accordingly most guinea pig models involve serial intraperitoneal injections of OVA [320, 321]. Other antigens have been used to induce experimental asthma, including intramuscular injections of bovine serum albumin [322], intradermal injections of trimellitic anhydride (TMA) [323], and cockroach antigens [324]. There are also chronic sensitization protocols involving a longer, month-long OVA sensitization period that result in some airway remodeling changes [320].

Histologically and clinically, the guinea pig model has similarities to human asthma [325, 326]. Guinea pigs exhibit bronchoconstriction, cough, airway hyperresponsiveness, inflammation, and remodeling with exposure to antigen [327]. Airway remodeling changes from chronic OVA challenge include increased smooth muscle mass, epithelial thickness, bronchiolar collagen, and goblet cell hyperplasia [320]. Guinea pigs develop both early and late asthmatic responses, where both eosinophilic and neutrophilic inflammation can be observed [326, 328]. Guinea pigs are a preferred model

for evaluating anti-asthmatic drugs in allergic bronchial asthma, due to similarities in their airway anatomy and response to inflammatory mediators [329]. Guinea pigs are small, docile, inexpensive and easily handled, but large enough for researchers to isolate portions of airways for testing.

Guinea pigs have limitations as a model of asthma. Significantly, the asthmatic condition in guinea pigs must be induced by antigen sensitization [329]. There is also a scarceness of genetically modified animals, inbred strains, and guinea pig-specific reagents [271].

Rabbit model

Rabbits have been used as a model of asthma since the 1970s, although overall, they are infrequently used. Like prior models, in rabbits the asthma-like disease can only be induced following a sensitization protocol, and is not a spontaneous disease. Pinckard et al. (1977) was the first to sensitize rabbits. Their protocol included weekly intraperitoneal injections of neonatal rabbits with bovine serum albumin in conjunction with *Corynebacterium parvum* adjuvant [330]. This results in production of antigen-specific IgE [331], and exhibition of airway signs following aerosol challenge. Since the 1970s, this protocol has been modified to include a number of various antigens for sensitization, predominantly ovalbumin [332] but also including ragweed [333], house dust mite [334], horseradish peroxidase [335], *Alternaria tenuis* [336], or even a combination of antigens [337].

The rabbit model has considerable advantages over traditional rodent models, with its larger size allowing for easier lung function testing, nonlethal monitoring of physiological changes, and sample collections, including bronchoalveolar lavages [338].

Rabbits are easy to handle and readily available [339]. Furthermore, the ability to sensitize the rabbits at birth, and follow them through to adulthood allows researchers to investigate processes that contribute to development of allergic disease [331]. With chronicity, airway remodeling also occurs, with thickening of the epithelial layer, mucosa, and submucosa of the lung [340]. Like the human allergic asthma endotype, rabbits exhibit airway hyperresponsiveness, and demonstrate both acute and late phase reversible airway obstruction, as well as eosinophilic inflammation [336, 338, 341]. However, if rabbits are not immunized as neonates, the late phase airway responses that are present in a majority of human asthmatics do not occur [331, 339]. Additionally, rabbits lack submucosal glands and their goblet cells are less abundant than in humans [342]. Rabbits are also more costly than the traditional rodent species used to study asthma, and require more space for housing [331]. Tools and reagents to use in rabbits, as well as knock out or transgenic rabbits, are also less available [331, 339].

Canine model

Dogs have been extensively used to investigate asthma. Although dogs readily develop spontaneous allergic disorders, they typically manifest themselves as dermatitis, not asthma [343]. Rarely, and unlike most animal models, spontaneous asthma-like disease does occur in dogs. The syndrome occurs in specific environments: Alaskan sled dogs in subzero temperatures [344], and dry air environments [345], and is exercise-induced. These would likely be superb models for the cross-country skiers' asthma and elite athlete asthma endotypes, respectively, but are not as useful for studying severe asthma. Most canine studies employ an induced model of disease; performed using aerosol sensitization to *Ascaris suum* larvae [346, 347] or neonatal sensitization to

ragweed [348]. Although the exercise-induced disease of dogs is useful as a model of certain exercise-induced human asthma endotypes, the canine *Ascaris suum* model is not a naturally occurring disease process like that of asthma in humans. It is interesting that *Ascaris suum*, the roundworm of pigs, and *Ascaris lumbricoides*, the round worm of people may indeed be a single or very closely related species, and that *Ascaris suum* can attain patent infections in both dogs and humans, making this model of unique relevance in asthma investigations [349, 350].

There are many benefits to using a canine asthma model. Following sensitization, many key features of human asthma, including reversible airflow obstruction, inflammation, mucus hypersecretion, airway hyperresponsiveness, and cough are demonstrated [348, 351]. Similar to severe asthmatics, pulmonary inflammation consists mainly of neutrophils [352-354], though it can also be eosinophilic [353, 355]. As in human asthma, the canine syndrome affects both central and peripheral airways, with constriction of both central and distal airways [356]. Additionally, their relatively large size and easy handling is ideal for repeat bronchoscopies and procedures that involve mapping the airways or long-term exposure to allergens [357].

The canine model is not a perfect model for human disease and has distinct disadvantages. With exception of the exercise-induced form of canine asthma, the disease must be induced from chronic antigen sensitization. When sensitized, dogs predominantly exhibit an acute phase response, as the presence of a late-phase response is not routinely found after antigen challenge [348, 357]. The acute phase is characterized by airway constriction [358], including peripheral airway constriction [359], mucus hypersecretion [360], tachypnea [361], and a sensitized cough reflex [362]. The late phase can be

reliably produced using a wedged bronchoscope method to deliver antigen directly to the airways [363]. Further, airway remodeling has not been extensively investigated with the canine asthma model. However, in canine models of COPD with long-term exposure to sulfur dioxide gas, hypertrophy and hyperplasia of airway mucus glands, thickening of the airway epithelium, mucus hypersecretion, airway smooth muscle thickening, and airway wall thickening have been observed [364, 365]. Genetically manipulated canine lines are rare. While this is considered a disadvantage in research, it is relevant that asthma is a multifactorial disease that reflects complex genetic and environmental interactions. Accordingly, spontaneous asthma-like diseases in outbred animal populations, such the spontaneous disease of sled dogs, are powerful and valid models in which the complex interaction of environment and genetics responsible for asthma can be investigated. Particular disadvantages of the dog include limited immunologic reagents, and as a larger animal species, increased animal, housing, and maintenance costs [271].

Ovine model

Sheep have been used to study asthma both through inducible allergic responses to house dust mite [366] and through natural sensitization to *Ascaris suum* [367]. Sensitization protocols involving house dust mite generally involve a subcutaneous injection protocol [366]. In sheep, inhalation of antigen elicits early and late-phase bronchoconstriction and airway hyperresponsiveness [368, 369], accompanied by eosinophils, neutrophils, and lymphocytes [367, 370].

Additionally, sheep have structural similarities to human lungs, allowing the use of methodologies and equipment used for pulmonary function testing in humans. The branching pattern and size allow repeat bronchoalveolar lavages or biopsies, and accurate

measurement of pulmonary function [367]. Alveoli, airway size, cartilage distribution, sensory nerves, capillaries, collagen, bronchial glands, and goblet cells are similar to humans [367]. Lung function is also similar, including airflow, resistance, breathing rates, tidal volume, and compliance [371]. Sheep also develop wheezing and coughing in response to antigen sensitization and challenge. Chronic HDM sensitization protocols result in airway remodeling changes including increased collagen, airway smooth muscle proliferation, goblet cell hyperplasia, and epithelial cell hyperplasia [369, 370].

A 2008 study with chronic *A. suum* challenge demonstrated irreversible bronchoconstriction that was nonresponsive to albuterol, a finding that is more consistent with human COPD than asthma [367]. Other limitations to using the induced asthma-like disease in sheep include a lack of inbred and genetically manipulated sheep, and very limited immunologic reagents [371]. Additionally, like other large animals, animal costs, housing, and maintenance become progressively higher with increasing animal size.

Non-human primate models

Approximately fifty years ago, researchers began to use rhesus monkeys sensitized to *Ascaris suum,* an intestinal parasite, to study allergen-induced airway responses [372]. *Ascaris* exposure promotes an IgE and a Th2-like immune response [373], and subsequent airway challenge results in immediate and late phase asthmatic reactions, as well as eosinophilia and hyperresponsiveness [374, 375]. Other non-human primate models for allergic asthma have been developed in rhesus and cynomolgus monkeys using human allergens such as house dust mite and birch pollen antigens [376, 377].

There are many advantages to using nonhuman primates as a model of asthma. Monkeys are genetically and physiologically similar to humans, with similar pulmonary anatomy, histology, and size. Human protein and monoclonal antibody therapeutics can be studied because of the close similarity of species [378], and they are therefore the most suitable asthma model for investigating immune regulation and to test new immunomodulatory therapies in asthma [379]. Additionally, the innervation of airways in monkeys is similar to that of the human [380]. Clinical signs such as coughing and dyspnea, as well as all four hallmark features of human allergic asthma are observed in nonhuman primate models, including airway obstruction, eosinophilic airway inflammation, airway hyperresponsiveness, and airway remodeling through goblet cell hyperplasia, epithelial hypertrophy, and thickening of the basement membrane [377, 379]. Airway smooth muscle hypertrophy and hyperplasia was also noted in chronically sensitized monkeys [381]. Both an acute and late-phase bronchoconstrictor response is present in monkey models [374].

Despite those advantages, the nonhuman primate model has limits. Most significantly, nonhuman primate asthma models are induced, and are not completely representative of the spontaneous process of disease in humans. Airway inflammation is predominantly eosinophilic [382], making monkeys a poor model for severe neutrophilic endotypes of asthma. There is also a large expense for care for the animals, food, medical care, and housing. Further, there is a great deal of effort required to maintain the model, as sensitization develops over 18 months and requires frequent handling of animals to continue exposure to antigens [382]. Further, many ethical issues surround the use of captive primates.

Feline model

Cats have a spontaneous form of asthma, called "feline asthma syndrome," which makes the feline model an interesting and valuable model for asthma research [383]. Diagnosis of feline asthma is generally made from a combination of clinical signs, radiographic findings, and response to treatment [384]. Radiographic signs of disease include bronchial wall thickening and increased bronchial or interstitial lung patterns [385]. Most affected cats respond quickly to bronchodilators or steroids such as prednisolone.

The feline model has many advantages compared to other asthma models; most notably that it is a spontaneous disease. Feline asthma also has many immunologic, physiologic and pathologic similarities to human asthma, including reversible airway obstruction, airway hyperresponsiveness, airway remodeling, airway smooth muscle thickening, goblet cell hyperplasia, allergen-specific IgE, and eosinophilic airway inflammation [380, 386]. There are also clinical similarities between feline and human asthma, including recurrent episodes of coughing, wheezing, and dyspnea [385, 387, 388].

However, there are some disadvantages to using cats to study asthma. In general, cats do not handle respiratory distress well, and limited interventions can be tolerated [388]. Genetic and molecular tools are underdeveloped for feline models [380]. The eosinophilic response of cats makes them an excellent, naturally occurring, model of allergic asthma, but as the predominant inflammatory cell type is the eosinophil, they are not a suitable model for the neutrophilic asthma endotype. Although cats experience an

obvious acute response to allergens, characteristics of the late phase response are not well characterized in cats.

Equine model

Horses spontaneously develop a chronic respiratory disease, recurrent airway obstruction (RAO), which is triggered by aeroallergens including dust and hay [389-391]. Two separate disease syndromes have been described. Traditional RAO, which mainly occurs in temperate climates [389], and pasture heaves which generally occurs in hot and humid climates [392, 393]. Occasionally horses can be affected with both forms of disease [393]. Horses must be predisposed genetically, and be exposed to certain environmental events for disease to occur [394]. In RAO-affected horses, disease is reliably triggered by challenge with moldy hay, and is typically associated with increased aeroallergen exposure coupled with inadequate air circulation [395]. Exacerbation is particularly common when horses are housed in stalls and barns with poor air quality, with poor quality, dusty, or moldy hay [396]. Inhalation of pollens and outdoor molds are thought to be the main cause of the summer condition [397], but clinical exacerbation is also associated with increases in temperature and humidity [398].

Both forms of disease are useful as models for severe, neutrophilic asthma. Like human asthmatics, horses have clinical signs that range in severity from exercise intolerance to coughing, wheezing, tachypnea and dyspnea [397]. All four key facets of human asthma are demonstrated in the RAO model, including chronic airway inflammation, airway hyperresponsiveness, reversible airway obstruction, and airway remodeling [396, 399-402]. The same facets are present in the pasture heaves model, and airway hyperresponsiveness has also been experimentally documented [403]. With both

diseases, affected horses have goblet cell metaplasia and hyperplasia, smooth muscle proliferation, airway fibrosis, and mucus hypersecretion [120, 399, 404, 405], all of which are changes present in the asthmatic airway. Airway inflammation is primarily neutrophilic, but some horses can present a moderate eosinophilic airway infiltration [406]. Horses have a long lifespan on the scale of 25-30 years which allows researchers to investigate disease progression over years, even decades [407]. The effect of aging on immunological models is disregarded with rodent models, but both innate and adaptive immune functions change with age [408]. Further, RAO is a disease of middle aged to older (>20 years) horses, and horses that are 7 years of age or older are 6-7 times more likely to develop RAO than younger animals [409]. Ages are similar for pasture heaves-affected horses; Costa et al. (2009) noted a median age of 15 years in the horses involved in their study [410]. This is in keeping with adult forms of asthma in which neutrophilic airway inflammation predominates [87-89]. Finally, like other large models, the size of horses allows for easier sampling, including pulmonary function testing [402, 411], methacholine challenges [403, 411, 412], lung lavages [401], and bronchial and lung biopsies [413].

Although the horse is the most complete model for investigating the human asthma syndrome, especially the neutrophilic disease endotype, using horses as an animal model is not free of drawbacks. Immunological reagents are limited for use in the horse, including availabilities of antibodies [414]. Due to their size, drugs, facilities, equipment, veterinary care, and food has a higher cost. However, an equine tissue bank has been developed for respiratory research, so researchers lacking the facilities and funding to keep a research herd of horses can still study this model [401].

Recurrent airway obstruction and pasture heaves

Horses naturally develop a chronic, asthma like disease called "recurrent airway obstruction," (RAO), also known as "heaves." RAO is a spontaneous allergic respiratory disorder affecting mature horses, with exacerbations of acute airway obstruction followed by periods of remission [415]. Two syndromes have been described. Traditional RAO, or barn-associated RAO, which occurs commonly in temperate climates [389], and pasture heaves, also known as summer pasture associated recurrent airway obstruction (SPARAO), which occurs in hot, humid climates such as the Southeastern United States and the United Kingdom [392, 393]. Horses can infrequently be affected by both forms of disease [393]. Affected horses, during disease exacerbation, exhibit respiratory distress episodes similar to those affecting asthmatics, ranging in severity from exercise intolerance and coughing to tachypnea, dyspnea, and wheezing. Like human asthmatics, RAO is characterized by chronic airway inflammation, airway obstruction, and airway remodeling [393, 397]. Airway obstruction results from accumulation of mucus, inflammation, and bronchoconstriction. Both RAO and pasture heaves have previously been called chronic obstructive pulmonary disease (COPD), but this term has been discarded, as human COPD has minimally reversible airway obstruction and airway hyper-reactivity is not a predominant facet of disease [416].

RAO is the most common chronic respiratory condition affecting mature horses housed in the barn in the northern hemisphere [391], but only occurs rarely in warm and dry climates such as California and Australia [389]. In 1998, an epidemiological study estimated that RAO affected 43% of winter stabled horses [417]. Risk factors of disease include increased aeroallergen exposure coupled with inadequate air circulation, direct

exposure to aeroallergens, or stalls and barns with poor air quality, with poor quality, dusty, or moldy hay [396]. Disease is reliably triggered by challenge with organic molds found in hay, and clinical signs can be alleviated by reducing dust exposure and improving ventilation in the stall [395]. Risk is also associated with increasing age. Horses that are 7 years of age or older are 6-7 times more likely to develop RAO than younger animals [409]. Exposure to urbanized environments with air pollution is also a risk factor for RAO [392].

Opposite of traditional RAO, pasture heaves is reported in mature horses housed on pasture during spring and summer months, with disease remission occurring during the winter [418]. Inhalation of pollens and outdoor molds are thought to be the main cause of the summer condition [397], but clinical exacerbation is also associated with increases in temperature and humidity[398]. Mature horses that are kept on pasture for more than 12 hours a day are more likely to develop pasture heaves [419, 420]. For both diseases, clinical remission occurs when horses are removed from the inciting environmental allergens [389, 397].

Both syndromes are heterogenous diseases that result from a predisposing genetic factor and environmental interactions [394]. The precise immunopathological mechanisms and antigens responsible for causing the clinical syndrome in susceptible animals have not been determined[390, 402]. As with asthma endotypes that group asthma by the respect underlying pathophysiologic mechanisms, this likely reflects a complex multifactorial pathophysiology that varies among subsets of horses with both the barn and pasture associated diseases. Intradermal tests have confirmed hypersensitivity to

numerous antigens, but skin hypersensitivity does not necessarily signify a causal relationship for RAO [395].

RAO and pasture heaves-affected horses present with similar clinical signs, which result from airflow obstruction, which occurs secondary to diffuse bronchoconstriction [391]. The most common signs are flared nostrils, tachypnea, coughing that worsens during exercise, intermittent serous nasal discharge, exercise intolerance, and anxiety. A heave line, reflecting hypertrophy of the external abdominal oblique muscle is evident in the most severely affected horses [416]. In RAO, clinical signs are associated with horses stabled during the winter months, but pasture heaves clinical signs first appear during spring months, becoming more prominent during late summer, and entering remission during the winter [420].

Pathophysiology

Similar to human asthma, pasture heaves and RAO are characterized by four key components: reversible airway obstruction, airway remodeling , airway inflammation, and airway hyperresponsiveness [396, 398-402, 421, 422].

During disease exacerbation, the major cause of airflow limitation is diffuse bronchoconstriction, but mucus plugs, inflammatory cells, and airway remodeling are also components of obstruction [391, 423]. After bronchodilators are administered to horses, there is a rapid improvement of 60-70% in lung function, indicating the reversibility of a majority of obstruction [399, 424]. This is significant to the viability of equine RAO/pasture heaves as a model of asthma, as the diagnosis of human asthma hinges upon the diagnosis of reversible airway obstruction. Airway smooth muscle is the

cell type predominantly responsible for the rapid constriction of airways [425], and is a key cell type in both asthma and equine RAO/pasture heaves [20,27].

Airway remodeling in affected horses is multifaceted, and includes goblet cell metaplasia and hyperplasia, airway smooth muscle proliferation, airway fibrosis, and mucus hypersecretion; all of which are present in asthmatic patients [120, 399, 404, 405, 426]. Furness, et al., (2010) demonstrated metaplasia of goblet cells in noncartilaginous airways of RAO-affected horses [427]. Costa et al. (2000) followed by Polikepahad et al. (2008) also confirmed the presence of goblet cell metaplasia in noncartilaginous airways of pasture heaves-affected horses [406, 426]. Herszberg, et al., (2006) and Mujahid, et al., (2011) used histomorphometry to demonstrate that RAO and pasture heaves, respectively, are characterized by an important increase of airway smooth muscle in small and large airways [400, 405]. Additional research is warranted to clarify the degree to which airway smooth muscle proliferation contributes to airway remodeling and how this process occurs in RAO. Mucus hypersecretion is an important feature of both RAO and pasture heaves, and it is even present during times of disease remission [399, 428]. Mucus accumulation in the airways of both RAO and pasture heaves-affected horses also contributes to exacerbation of clinical signs, as well as neutrophilic inflammation [399, 426, 429]. Costa, et al., (2000), described mucus accumulation as the main change in small airways of pasture heaves-affected horses [426] and Polikepahad, et al., (2008) also suggested that the most prevalent histopathologic change in euthanized pasture heaves-affected horses is mucus accumulation [406]. Pulmonary fibrosis has been described in RAO using light microscopy and electron microscopy [430], but the collagen types involved have not been determined [431].

The inflammatory process in RAO and pasture heaves is characterized by a predominance of neutrophilic infiltration, which can vary in severity [419, 432]. A minor and occasional eosinophilic infiltration may also be present in RAO horses [428]. Airway neutrophils are recruited 3-6 hours after allergen exposure, and are more numerous in RAO-affected horses compared to controls exposed to the same environment [433]. RAO is also characterized by delayed apoptosis of neutrophils, and neutrophil release of elastase, oxygen metabolites, LTB4, and MMP-9 [433-436]. Cytokines such as IL-1B, TNF-a, IL-8, and IL-17 are all increased in RAO affected horses, which are likely partially derived from neutrophils [437-440]. Chronic immune activation is present in neutrophilic human asthma, as in RAO, which persists during remission of disease [441, 442].

Inflammatory mediators including histamine, bradykinin, nitric oxide (NO), and endothelin-1 (ET-1) are increased during disease exacerbation in pasture heaves horses [421, 443]. NO, an inflammatory mediator with implications in the development of airway inflammation in human asthma, is derived from inducible NO synthase (iNOS) in airway epithelial cells [444]. Costa et al. (2001) examined the plasma and BALF concentrations of NO and localization of iNOS in pasture heaves horses and found an increase in plasma NO concentration in pasture heaves horses that was not statistically relative to non-diseased control horses [48]. However, iNOS production in bronchial epithelial cells from 3 of 5 lung lobes was significantly elevated in pasture heaves relative to control horses, suggesting that NO may have a role in amplifying the inflammatory response at the surface of airways. Airway hyperresponsiveness is present and is increased when affected horses are exposed to dust, even before changes in lung function

and clinical signs can be observed [445]. One mechanism of airway hyperresponsiveness in pasture heaves horses is mediated by airway sensitivity to endothelin-1, a potent contractor of equine bronchi that is increased in plasma and pulmonary secretions during exacerbations of pasture heaves [410]. Increased ET-1 sensitivity in has also been documented by increased ET-1 receptor expression in bronchial smooth muscle of horses with pasture heaves [22]. In humans, ET-1 is produced by many cells in the body, but the lungs are the primary site of synthesis; the greatest concentration being found in smooth muscle and alveolar septae [446]. The known functions of ET-1 include maintenance of airway tone [415], smooth muscle contraction [415], bronchodilation through the release of prostaglandin E2 from airway epithelium [421], and potent bronchoconstriction through the release of secondary mediators including thromboxanes and platelet activating factor [447]. ET-1 also stimulates DNA synthesis in airway smooth muscle, resulting in proliferation[447], and has proinflammatory effects on airways, including activation of cytokines [448]. ET-1 levels are increased in BALF and plasma of human asthmatics [449] and horses with RAO [415]. Accordingly, ET-1 is an important contributing factor to airway smooth muscle constriction in pasture heaves, and likely to RAO as well.

The persistence of airway hyperresponsiveness in RAO-affected horses has been debated within the literature. Studies have shown that clinically normal, RAO-affected horses kept in low-dust, indoor, controlled environments have persistently hyperresponsive airways when compared to healthy controls housed in the same environments [411, 423, 450]. However, of those studies, one noted higher clinical scores in the RAO-affected group [423]. In direct contradiction of this, other studies

have indicated that airway hyperresponsiveness does not occur when RAO-affected horses are on pasture, in clinical remission [396, 412, 451]. A hyperresponsive reaction occurs in RAO horses in response to non-specific agonists such as histamine [452-454] and methacholine[412]. This persistence of AHR is an important facet of RAO as an animal asthma model, as AHR does not persist in induced murine models of asthma [302].

Pathogenesis of RAO and pasture heaves

The pathogenesis of RAO has been more heavily investigated than that of pasture heaves. It is generally accepted that there is a hypersensitivity component to RAO pathogenesis [389], but compared to other allergic diseases, the underlying immunological mechanisms of RAO are more complex [455], involving both innate and adaptive immunity[456]. The role of IgE in RAO is controversial, with conflicting results within the literature. Some studies showed an increase in serum IgE concentration in RAO horses [457, 458], while other studies showed no significant difference [432, 459], including in bronchoalveolar lavage fluid (BALF) samples [457]. Scharrenberg et al. demonstrated through a genetic study of offspring from two RAO-affected sires that allergen-specific IgE concentrations were higher in offspring of one sire when compared to the other, indicating a genetic effect on allergen-specific IgE levels [460]. Genetic differences and the presence of groups of horses that share similar pathophysiologic mechanisms, akin to the description of endotypes in asthma, may help explain some of the differences within the literature, as many studies contain small sample sizes of genetically outbred horses. Dirscherl et al. demonstrated that relative to control horses, basophils from affected horses were hypersensitized to many antigens, including molds

of the genus *Mucor* [461]. This work substantiates a role for IgE-sensitized inflammatory cells in some horses with RAO, as the gateway to antigen-specific recognition and initiation of the inflammatory cascades, and is consistent with a similar IgE mediated endotype in asthma [reference here for IgE mediated asthma]. Further, *in vitro* stimulation of basophils and pulmonary mast cells is an accepted method of identifying allergens involved in RAO pathogenesis [462-465].

Following exposure to allergens, RAO-affected horses develop airway inflammation characterized by a massive influx of neutrophils [466], and an increase in CD4+ cells in BALF [467, 468]. Helper T cells orchestrate the inflammatory response in RAO, and the intensity of cytokine expression has been correlated to disease chronicity [466]. Cordeau et al. demonstrated that relative to unaffected horses, RAO-affected horses had increased mRNA expression of IL-4 and IL-5, and decreased expression of INF-γ, which is a predominant Th2-type cytokine response, coinciding with the presence of airway obstruction [469]. This is consistent with the Th-2 cytokine profile that has been demonstrated in allergic asthma endotypes [470]. However, characterizing the cytokine profile of RAO into a predominantly Th2 or Th1-mediated response has been difficult, as studies have generated contrasting data. Ainsworth et al. showed the cytokine profile of RAO horses, compared to unaffected horses, had increased expression of INF-γ, but decreased IL-4 and IL-13 in BALF cells [437]. This cytokine profile is consistent with a Th1-type cytokine response, which is more similar to the cytokine profile seen in the neutrophilic endotype of human asthma [263]. Some studies on RAO-affected horses have even demonstrated a mixed response, with increased mRNA expression of IL-4, IL-13, and INF-γ in BALF [438, 471]. The disparity in results could be related to differences

in the clinical stages of the horses and timing of sample collection [468, 471, 472]. In addition, horses with RAO have decreased pulmonary clearance [10211695] and the possibilities of confounding subclinical infection in these populations should be considered. In 2005, Debrue et al. showed that organic dust exposure increases gene expression of IL-17, another T-cell derived cytokine, in the BALF of RAO-affected horses, but not in unaffected horses [440], which may contribute to airway neutrophilia. However, gene expression of IL-8 is also increased several days before IL-17 levels become increased, making it a likely contributor to neutrophilia, at least in earlier stages of RAO [437]. Continued research into the underlying pathogenesis of RAO will likely divulge further information on T cell associated immunological mechanisms. It is clear, however, that T cells play an important role in the pathogenesis of RAO, and one major consequence of their activation is the recruitment of neutrophils into the airways.

Increases in specific disease mediators, including chymase, mucin, and endothelin-1 (ET-1), are correlated with the changes seen during disease. A correlation between high chymase positive mast cell numbers in the bronchial wall and lung fibrosis has been demonstrated [432], suggesting a possible involvement in the airway remodeling associated with RAO. The amount of stored mucin in goblet cells has been shown to increase with severity of airway inflammation in RAO [404]. ET-1, a powerful smooth muscle constrictor and proinflammatory mediator, is increased during RAO exacerbation [415]. This finding has been corroborated in pasture heaves, with increased ET-1 concentrations in plasma, BALF, and pulmonary epithelial lining fluid from pasture heaves affected horses during seasonal exacerbation relative to other control horses, and also to their own ET-1 concentrations during disease remission [406, 410, 421]. Other

inflammatory markers in the airways of RAO horses include myeloperoxidase (MPO), metalloproteinases (MMPs), ascorbic acid and associated markers of oxidative stress. MPO, a neutrophil-derived enzyme, has been found to be increased in RAO-affected horses when compared to control horses, both during disease exacerbation and remission [473]. MMPs including MMP-1, MMP-8, MMP-9, and MMP-13 have been shown to be elevated in BALF and tracheal secretions of horses with RAO during exacerbation, while MMP-2 and MMP-14 have not [474-477]. It is hypothesized that the MMPs that differ between groups may contribute to lung dysfunction in RAO horses, and can be used as diagnostic markers of disease [477]. There are low ascorbic acid concentrations in BALF, which result in the decreased antioxidant capacity of RAO horses compared to control horses [478, 479]. In pasture heaves-affected horses, iNOS, responsible for the production of the inflammatory mediator NO, is increased in airway epithelial cells [443]. Markers of oxidative stress, including oxidized glutathione concentrations, glutathione and ascorbic acid redox ratios, and hydrogen peroxide, have also been identified as increased within the airways and exhaled breath of RAO horses during disease [480-482].

Diagnosis and treatment

In 2011, Kutasi et al. determined that gathering a history and performing a physical examination were not enough to accurately diagnose chronic pulmonary disorders including RAO, yet veterinarians often do not use any tests to confirm diagnosis [483]. The most useful combination of information to accurately diagnose pulmonary allergic diseases in horses includes age of the horse, history, physical examination, respiratory tract endoscopy, and BALF with cytologic analysis [483]. Cytologic analysis of BALF is simple and inexpensive, and can be used to differentiate

between pulmonary infections and inflammation [484]. Horses with RAO/pasture heaves typically have greater than 25% neutrophils present on cytology [426, 485]. Immunological testing has been a topic of debate within the literature, with some studies showing an increased frequency of skin responses in RAO-susceptible horses following intradermal injections of stable dust, mold extracts, and allergens [395, 486], but conflicting results from other studies [390, 487] . The diagnostic value remains questionable, and is significantly less valuable in a clinical setting than the recommended diagnostic methods. While measures of lung function including resistance, compliance and elastance are evaluated in airway research involving RAO and pasture heaves, the equipment necessary to evaluate lung function is not well suited to its use in field practice [391].

Prognosis depends on disease severity at time of diagnosis, and vigilant environmental management. Though RAO and pasture heaves are chronic and progressive, environmental management and medical therapy, including ß2-adrenoceptor agonists and corticosteroids, can be effective in controlling clinical signs [423]. Second line treatments, including anticholinergic bronchodilators such as atropine and glycopyrrolate, and ipratroprium, and the methylxanthine bronchodilator theophylline, have been administered in horses. Because they are both allergic diseases, eliminating the inciting allergens is crucial for effective response to therapy [420]. If disease occurs in a stabled, indoor environment, as is typical with RAO-affected horses, it is best to move the horse to the pasture [451]. If disease occurs in the pasture, as is typical with pasture heaves horses, it is important to move the horse indoors. Problematic for pasture heaves horses is the fact that indoor housing does not fully mitigate their exposure to pasture

aeroallergens. Efforts should be made to reduce the amount of dust in the environment as well, by wetting hay, or switching to a pelleted diet, and by changing the bedding to wood chips or pelleted paper [488, 489]. Exercise should be kept to a minimum during disease exacerbation. Controlling clinical signs, airway inflammation, and airway hyperreactivity are important therapeutic goals [416].

Importance as a model of asthma

RAO and pasture heaves share a common clinical presentation and immunological similarities to severe human asthma. Like asthmatics, horses have all four cardinal facets of asthma, including airway hyperresponsiveness, reversible airway obstruction, airway inflammation, and airway remodeling [396, 399-402]. Further, like human asthmatics, affected horses have airway remodeling that includes goblet cell metaplasia and hyperplasia, smooth muscle proliferation, airway fibrosis, and mucus hypersecretion [120, 399, 404, 405]. Horses are also a genetically outbred population, akin to humans, and treatment and response are similar in humans and asthmatics [401]. However, one facet of asthma is particularly unique to horses relative to animal asthma models. In horses with RAO/pasture heaves, the disease is chronic and progressive, recurring regularly with re-exposure to inciting allergens despite periods in which the horse is removed from the allergen. This facet of disease, reflects persistent sensitivity to the inciting allergens and is a critical feature of asthma that is not found in induced animal asthma models such as rodents that lose antigen responsiveness following withdrawal of the sensitizing antigen [302]. Clinical similarities between asthma and RAO/pasture heaves, association of airway neutrophilic inflammation with more severe forms of asthma [266], together with the absence of airway neutrophilic inflammation,

airway remodeling, and persistent sensitivity to antigen in rodent asthma models [283, 290, 294] highlight the value of RAO/pasture heaves as a research model of neutrophilic asthma. Accordingly, horses with RAO/pasture heaves present a powerful model for investigating the pathophysiologic mechanisms contributing the neutrophilic asthma and for directing future therapeutic discovery.

Systems biology

"Systems biology is about putting together rather than taking apart, integration rather than reduction. It requires that we develop ways of thinking about integration that are as rigorous as our reductionist programmes, but different. ... It means changing our philosophy, in the full sense of the term." - Denis Noble [490]

Systems biology is an attempt to structure knowledge into hierarchical levels, from gene products to whole organisms, and to determine rules that will allow navigation between levels [491], aiming for a system-level understanding of biological systems. Understanding a biological system at a systems-level involves embracing the network of dependent yet interconnected components that comprise a unified, complex organism, as opposed to focusing on the characteristics of isolated parts of a cell or organism [492]. This is a relatively new way to approach molecular biology and genetics, but the approach has been used in other branches of science, including physics, engineering, economics, sociology, psychology, and ecology [492, 493]. Although the definition varies throughout the literature, it is generally accepted that systems biology is interdisciplinary, making use of the principles, knowledge, and tools of biology, computer science, physics, chemistry, engineering, medicine, and genetics, bridging the gaps between disciplines [494].

History

The conceptual basis of systems biology dates back to the 17th century, when reductionism and mechanistic thinking were initially developed. The reductionist approach was developed by René Descartes and is based on the idea that complex situations can be reduced into manageable pieces that can then each be investigated and reassembled into a whole [492]. Mechanistic biology also developed during the 17th century, with the idea that natural systems were considered to be merely mechanisms with a more or less complex machinery [494]. These two philosophies led to great discoveries in mathematics and physics, sanctioning the mechanistic thinking and reductionist method as the way to develop a successful scientific approach [492, 494]. Mechanistic biology reached its peak in the early 1900s, when Jacob Loeb wrote that organisms were nothing but complex machines, with mechanisms that must be rigid and invariant [495]. This concept was objected by scientists for two reasons: Aristotelian views, or "the whole is something over and above its parts and not just the sum of them all" [496] and the introduction of "holistic biology" by Jan Smuts, according to which the comprehension of systems as a whole is irreducible [497], meaning "the properties of systems could not be reduced to those of their constituent parts [494]." Although mechanistic reasoning is no longer a prominent biologic methodology, reductionist approaches remain in use [494]. Contemporary biology successfully applied reductionist methods to investigate up to the last molecular bases of biological phenomena [494], but new technologies were needed to define and understand the behavior of systems. The enormous development of fields including molecular biology, genetics, biochemistry, and computer science during the 1900s made possible the scientific and technological

revolution of the 1980s-2000s, with the introduction of the new field of systems biology at the beginning of the 21st century [498, 499]. Table 2.1 highlights some significant events and landmarks leading up to the advent of systems biology.

Prior to 1944, it was believed that proteins were responsible for hereditary characteristics, but Avery et al., through experiments with *D. pneumoniae,* discovered that DNA was linked to heredity [500]. Just a few years later, discovery that DNA had a double-helical structure, allowed scientists to begin to understand the basic mechanisms of DNA replication, protein synthesis, gene expression, and the exchange and recombination of genetic material [501]. The development of recombinant DNA techniques laid the foundation for a new area of scientific research [504], including the identification, mapping, and sequencing of genes. DNA sequencing evolved from the first methods for DNA sequencing, which included chemical sequencing and the more economical Sanger-sequencing [506, 507], to semi-automatic sequencing technologies, to the release of the first automated DNA sequencing machine in 1986 [508]. Sanger sequencing, or shot-gun sequencing, was the predominant form of sequencing for a quarter of a century, until its replacement with next-generation sequencing technologies (NGS) in 2005 [513]. In the late 80s, discussion on creating the Human Genome Project began in earnest, with a meeting from the United States Department of Energy. The launch and implementation of the Human Genome Project promoted new technological improvements, including -*omics* technologies and computational tools, contributing to the rapid development of modern systems biology [494, 514, 515].

Since its beginning in the 1990s, systems biology has seen rapid development. This development is largely due to a combination of three things: the reinforced holistic

approach to biology, the differentiation of life into biological domains, and the advancement of technology, including the internet, to accelerate accessibility of bioinformatics. In 1999, Hartwell et al. indicated that functional modules should be recognized as critical levels of biological organization, and general design principles rule the structure and function of said modules [511]. This use of modular organization and design principles became a new approach to study biological systems, influencing further development of systems biology.

The first genome, *Haemophilus influenzae,* was sequenced in 1995 [510]. Shortly afterwards, the human genome was sequenced (2001) [512], and since then, the number of sequenced genomes has increased exponentially, as technologies improve and costs decrease. For instance, in 2012, 282 permanent drafts of genomes existed in the Genomes OnLine Database (GOLD) [516], and in 2013, an additional 968 more genomes were completed with 4914 permanent drafts. In 2014, there were 2031 additional completed genomes, and 15,653 permanent drafts [517]. Access to these data, along with increases in computational power, technology, and bioinformatics tools, has allowed researchers to describe gene and protein functions and interactions, leading to improvements in clinical diagnostics, understanding of disease risk, therapeutic identification, prenatal testing, discovering disease-related genes, synthetic biology, understanding of viruses and bacteria, and many others [518].

The systems biology approach to asthma

-*Omics* begins with the collection and processing of large volumes of high-throughput data and the subsequent computational and statistical analysis of thousands of proteins, mRNAs, miRNAs, transcripts, etc [519]. With asthma the -*omics* approach has

already been used to identify asthma-associated genes and proteins based on expression values, highlight asthma-related signaling networks, potential biomarkers of disease, evaluate therapeutics, and further describe molecular mechanisms of asthma endotypes [520-524]. Applying unbiased -*omics* methods, coupled with a hypothesis-driven project, is ideal for discovery of new disease pathways and processes. There is no one standard for the application of systems biology, so the strategies used vary depending on the type and amount of data available and the questions to be asked [519].

Genome-wide association studies (GWAS) examine associations between genotype and phenotype, resulting in the unbiased identification of genetic loci. Many GWAS of asthma have been conducted, and the 17q21 locus is frequently associated with disease risk [163, 525, 526]. Transcriptomics, or the systematic, unbiased, quantitative and qualitative characterization of RNA transcripts across the genome, has transformed our understanding of cellular function [527]. Microarrays first made the analysis of the transcriptome possible. Using microarrays it is possible to detect variations in expression of any gene included within the predefined probes on the array chip [528]. The main advantage of microarrays over next generation sequencing methods is the comparatively low running cost [529]. Today most researchers use direct high-throughput next generation sequencing (RNA sequencing) which has become increasingly more cost effective. RNA sequencing (RNA-seq) offers distinct advantages over microarrays, particularly that it covers the whole transcriptome without bias, allowing researchers to identify novel transcripts [530]. Proteomics encompasses the systematic analysis of protein populations through identification, quantification, and analysis [531]. The predominant method used to investigate respiratory diseases is expression proteomics

through either 2DE or LC-MS analysis [532]. In 2DE, proteins are separated in isoelectric focusing strips according to their net charge, and then are separated by molecular mass through polyacrylamide gel electrophoresis (PAGE) [533]. The ideal protein identification method is through MS-peptide mass fingerprinting along with sequencing of selected peptides using tandem mass spectrometry (MS/MS) [532]. LC-MS allows any two samples to be qualitatively and quantitatively compared, providing information about differentially expressed genes, and although 2D PAGE is still commonly used, LC-MS is used for a majority of research work in the respiratory field [532]. Although much can be gained from transcriptomics or proteomics alone, the most valuable information is likely to be from a combination of transcriptomics as a measure of gene regulation, and proteomics as a measure of post-translational information [534]. Although metabolomic studies have also been used to investigate asthma, a majority of the scientific literature is centered around proteomic and transcriptomic discoveries. Systems biology-based research investigations into equine RAO and pasture heaves are just beginning.

Transcriptomics

Most transcriptome profiling to date has been performed with oligonucleotide microarrays [535]. Within asthma, most array-based studies have involved relatively small sample sizes (reviewed in [536]), but have still provided valuable information. Woodruff et al. (2007) used gene expression microarrays to identify and compare gene expression from airway epithelial cells from asthmatic patients, healthy patients, and smokers [537]. Within the same study, they found that IL-13 lead to the upregulation of chloride channel, calcium-activated, family member 1 (CLCA1), periostin, and serine

peptidase inhibitor, clade B (ovalbumin), member 2 (serpin B2) in asthmatics but not smokers. After corticosteroid treatment, all three genes were downregulated, but FK506-binding protein 51 became upregulated; a gene that was found to be inversely correlated to poor response to corticosteroids [537]. In a study on childhood wheezers (Kapitein et al., 2008), gene expression microarrays were used to examine CD4+ T cells from blood samples collected from transient wheezers, persistent wheezers (considered asthmatics), and healthy controls. Both affected groups had increased expression of several genes with documented effects on airway hyperresponsiveness and allergic responses. For example, a) complement component 5 receptor 1 (C5R1), b) a gene that produces interleukin-12 through macrophages and monocytes, driving type-1 adaptive immune responses [538] and c) heat shock protein A1A, which has been shown to correlate to increasing severity of asthma and neutrophil count in both sputum and plasma [539]. In 2013, Fu et al. performed gene expression microarray analysis on asthmatics with and without systemic inflammation, a characteristic that has been associated with neutrophilic airway inflammation in asthma [540]. Those with systemic inflammation showed evidence of increased serum levels of C-reactive protein and IL-6. 449 genes were identified that were significantly altered in sputum between the two groups. These genes were involved with IL-1, TNF-α, NF-Kß, and Kit receptor pathways, and were related to the presence and severity of neutrophilic inflammation [540].

Currently, most researchers are using RNA-sequencing technology over microarrays to investigate asthma. A comparison of RNA-seq profiles of endobronchial specimens from subjects with atopic asthma and healthy controls revealing 46 differentially expressed genes (DEGs) [520]. This same study conducted pathways

analysis of the 46 DEGs, revealing networks associated with cellular movement, cell death, and cellular morphology. Within their DEGs, only a few had previously been associated with asthma, including SLC26A4 (pendrin) , periostin, chloride channel regulator 1 (CLCA1), and serpin peptidase inhibitor, clade B, member 2 (SERPINB2) [520]. A recent study examining RNA-seq profiles of nasal brushings from asthmatics and controls showed that airway nasal gene expression profiles can identify subjects with asthma that is driven by IL33 [541]. In 2011, Baines et al. investigated asthma phenotypes using gene expression profiling of induced sputum with hierarchical clustering of the expression profiles [542]. Within their experiment, they were able to describe three distinct phenotypes: 1) chronic airflow obstruction and poorly controlled asthma, with increased exhaled nitric oxide, sputum eosinophilia, and decreased lung function 2) severe airflow obstruction, higher sputum neutrophils, and decreased lung function 3) higher sputum macrophages and lower eosinophils and neutrophils with normal lung function. In the same study, they described overexpression of IL-1, TNF, and NF-Kß, which correlated to increasing severity of clinical signs and neutrophilic inflammation [542].

Potential mechanisms underlying glucocorticoid treatment in asthma have been examined using RNA-seq. One study included endobronchial biopsy samples from patients before and after prednisolone or placebo treatment. In this study, treatment with prednisolone resulted in 15 differentially expressed genes, with two genes also associated with airway hyperresponsiveness (FAM129A and SYNPO2) [543]. In a separate study, the effect of dexamethasone on airway smooth muscle responses was investigated,

revealing 316 glucocorticoid-responsive DEGs including DUSP1, KLF15, PER1, TSC22D3, C7, CCDC69, and CRISPLD2 [544].

MicroRNAs (miRNAs) are single-stranded RNA species that constitute a class of noncoding RNAs emerging as key regulators of gene expression [545]. Aberrant expression of miRNAs has been associated with pathogenesis of diseases including heart disease, congenital organ defects, neurodegenerative diseases, and are being used to investigate pulmonary diseases including asthma (reviewed in [545]).

In 2012, Jardim et al. analyzed human bronchial epithelial cells from mild asthmatics and healthy controls using a microRNA (miRNA) microarray [522]. Of 66 total differentially expressed miRNAs, three, miR-let7f, miR-487b, and miR-181c were significantly higher in asthmatic patients. miR-203 was significantly underexpressed in asthmatics, and through network analysis, AQP4, a potential gene inhibited by miR-203, was identified. After confirmation of results via RT-PCR, Jardim et al. demonstrated an inverse relationship between miR-203 and AQP4, where asthmatics had decreased miR-203 correlating to increased expression of AQP4 [522]. These results contrast those of an earlier study conducted by Williams et al. (2009), where no differences between miRNA profiles of airway biopsies from mild asthmatics and healthy controls were detected [546]. However, a later study conducted in 2012 by Solberg et al., used miRNA microarrays to analyze bronchial epithelial brushings from steroid naive asthmatics, steroid-using asthmatics, and healthy controls, finding a completely different subset of differentially expressed miRNAs [547]. This study also examined the effects of exposure of IL-13 and subsequent administration of corticosteroids on miRNA expression. IL-13 caused repression of miR-34/449 family members, miRNAs with established roles in

airway epithelial cell differentiation, but corticosteroids did not improve the repression [547]. A 2013 study investigating alterations in bronchoalveolar lavage fluid (BALF) exosomal miRNA profiles in healthy subjects and asthmatics before and after exposure to an environmental challenge showed substantial differences in miRNA profiles between groups, but not following environmental challenge [548].

Proteomics

Most proteomic approaches to human asthma are performed using sputum samples, but some have been performed on lung biopsy samples, BALF, and plasma. Recent examples of each are described here. In 2005, Wu et al. analyzed BAL fluid after segmental allergen challenge using SDS-PAGE, followed by LC-MS/MS, resulting in the identification of 160 differentially expressed proteins (DEPs) [549]. One protein, MMP-9, was validated as a potential biomarker of disease. Cederfur et al. (2012) applied galectin affinity chromatography to analyze BALF, identifying 175 DEPs, including PIGR, SP-B, and SP-A2, and many galectin-binding glycoproteins [550]. O'Neil et al. (2011) investigated the proteome of bronchial biopsies taken from asthmatics [523]. Compared to the proteome of healthy controls, the asthma bronchial proteome demonstrated upregulation of several cytokines including SCGB1A1 and TNFSF13B; the latter is associated with both TH1 and TH2 inflammation [551, 552]. Other known asthma-relevant proteins that were upregulated in the asthmatic proteome relative to controls included SERPINA1, thrombins, fibrinogens, and LMNA [523]. Singh et al. (2012) analyzed plasma samples from asthmatics exposed to allergen challenge to identify potential markers specific to the early or late asthmatic phase, but no specific markers could distinguish the two responses [553]. Using a shotgun proteomics approach,

Gharib et al. (2011) analyzed sputum from healthy subjects and asthmatic patients, including five with exercise-induced bronchoconstriction [554]. Ten proteins were significantly differentially expressed in asthmatic patients, including SERPINA1 (α1-antitrypsin), a protein with a role in promotion of airway remodeling [555]. Within the exercise-induced patient group, complement component 3 (C3) was significantly upregulated [554]. C3 has many documented roles in asthma pathogenesis, including airway mucus secretion, smooth muscle contraction, vascular permeability, release of allergic mediators, and airway hyperresponsiveness [556, 557].

Proteomic profiles of neutrophilic asthma have also been investigated. In 2013, Lee et al. conducted a study on patients with controlled and uncontrolled neutrophilic asthma. Among 14 DEPs, S100A9 was identified as a specific marker for neutrophilic uncontrolled asthma [558]. Proteomic analysis has also been performed on CD4+ T lymphocytes from the blood of patients with uncontrolled asthma, revealing 14 DEPs, including vimentin [559]. Brasier et al. (2008) established IP-10, IL-7 and GM-CSF were different between high and low neutrophil asthmatics [560].

Metabolomics

Exhaled breath contains a complex gas mixture of volatile organic compounds (VOCs) and nonvolatile compounds derived from exhaled breath condensate (EBC) [561]. Samples are typically analyzed using metabolomic approaches based on mass spectrometry [562]. Both components of exhaled breath have been investigated in regards to asthma. Dallinga et al. (2010) examined breath samples analyzed for VOC by chromatography-mass spectrometry. They were able to use VOCs to distinguish between asthmatics and healthy controls [563]. Carraro et al. (2013) applied the metabolomic

approach to EBCs from children with severe or nonsevere asthma, successfully distinguishing between the two groups [564].

The systems approach to equine disease

One of the first attempts at mapping the genetic loci involved in RAO was carried out in 2009 by Swinburne et al. [565]. Using a genome-wide scanning approach, they located the chromosome regions responsible for inheritance in two half-sibling families of Warmblood sport horses. Those two chromosome regions were ECA13 and ECA15, with one family associated with ECA13, and the other family associated with ECA15. Located within those regions were several candidate genes, including interleukin 4 receptor (IL-4R), IL-21R, chemokine (C-C motif) ligand 24 (CCL24), IL-27, prostaglandin E receptor 4 (PTGER4), phosphodiesterase 4D (PDE4D), suppressor of cytokine signaling 5 (SOCS5), and IL-17R [565].

The candidate genes for RAO identified by Swinburne et al. have been further investigated by comparison to proteomics data generated from bronchoalveolar lavage fluid (BALF) collected from RAO-affected and unaffected herd mates [566]. Of the eight candidate genes investigated, those that had the greatest number of direct documented molecular actions with the BALF proteins were SOCS5, IL-7R, PTGER4, and PDE4D. IL-4R, IL-21R, and CCL24 had a large number of indirect interactions with the proteins present in BALF of RAO-affected horses. In addition, Racine et al. found that many proteins from the BALF in the IL-4R pathway interacted with the TNF-α family [566]. To this author's knowledge, this study reflects the one and only attempt to conduct a proteomic analysis of BALF from RAO-affected horses.

Similarly, only one gene expression analysis has been conducted on RAO-affected horses [567]. Lavoie et al. (2012) obtained peripheral lung biopsies via thoracoscopy in both control and affected horses during disease exacerbation and remission, and using suppression subtractive hybridization (SSH), only cDNAs that were unique to clinical exacerbation of RAO were sequenced. 195 genes were identified, including many that were associated with airway inflammation and remodeling. This same study suggested targeting RhoA, PPP3CB, EDN1, and IGF1 signaling pathways as potential targets for anti-remodeling therapies, particularly airway smooth muscle hypertrophy, while focusing on LTB4 and PGD2 pathways as targets for anti-inflammatory effects [567].

Summary

A growing understanding of asthma pathogenesis indicates that asthma is a complex disease that is best classified as a syndrome comprised of different pathophysiologic endotypes. Despite their differing pathophysiologic mechanisms, endotypes share a similar clinical picture that includes reversible airway obstruction, airway hyperresponsiveness, and chronic airway inflammation. [21]. Severe noneosinophilic asthma (neutrophilic), is the most costly form of disease, and often becomes refractory to treatment [87-89], creating a need for animal models to investigate pathophysiologic mechanisms and therapies for this asthma endotype [401]. Of all the animal models of asthma, the cat and the horse are the only two species with a naturally occurring asthma-like disease [120]. The cat is an excellent model for allergic asthma, due to its airway eosinophilia, while the horse is the optimal model for neutrophilic asthma, as its airway inflammation is comprised mostly of neutrophils. Although the

mouse model, and other animal models where disease is artificially induced have provided some valuable information about asthma, many findings have not been translated to understanding human disease or therapy [283].

There are two equine syndromes that are used to model human asthma: RAO and pasture heaves. They are clinically similar spontaneous asthma-like disease of horses, with RAO typically occurring in stabled horses in temperate climates [389], and pasture heaves in pastured horses in hot, humid climates [393]. There are many similarities between RAO/pasture heaves and asthma, although RAO has been described in more detail. Significantly, RAO models the clinical hallmarks of asthma: chronic airway inflammation, reversible airway obstruction, airway hyperresponsiveness, and airway remodeling [39, 40].

Systems biology has rapidly developed over the past two decades. The sequencing of the human genome led to increased availability to technology and created rapid advancements in the need to derive biologically relevant information from genome scale expression data including microarrays, proteomes and transcriptomes [512]. Since the herald of systems biology, countless proteomic and transcriptomic profiles of the asthmatic syndrome have been performed. Currently, most research is done via proteomics and next-generation sequencing technologies. A handful of studies have been performed on RAO; one on proteomics of BALF [566] and one where lung biopsy cDNA was sequenced [567]. At this juncture in the literature, no studies using -*omics* technologies have been performed on pasture heaves. The overarching goal of this dissertation work is to identify evidence of shared molecular events relevant to asthmatic airway neutrophilia in horses with pasture heaves. This has been accomplished through

the following steps: 1) assign functions to equine gene products in a manner that is computationally accessible, 2) identify conservation of gene functions in the lungs of normal horses, mice and humans, and 3) identify molecular events contributing to airway neutrophilic inflammation in pasture heaves lung lavage proteomes and their conservation in human asthma. This work is the first to use -*omics* technologies to investigate pasture heaves.

Table 2.1 Significant historical discoveries preceding systems biology

Year(s)	Event	Investigators
1944	Obtained conclusive evidence that DNA was the carrier of hereditary characteristics, with *Diplococcus pneumoniae* bacterium.	Oswald T. Avery, Collin M. MacLeod, Maclyn McCarty [500]
1953	Double helical structure of DNA described.(Received 1962 Nobel Prize in Physiology or Medicine.)	James Watson, Francis Crick [501]
1960s-1980s	Progress made in the understanding of the structure of genes, and mechanisms of replication, expression, and regulation in prokaryotes and viruses.	Various [502];
1963	Simulations of integrated molecular functions performed on analog computers	Brian Goodwin [503]
1969	The idea of recombinant DNA was first proposed.	Peter Lobban [504]
1972	First gene sequenced - Bacteriophage MS2, an RNA virus that infects *E. coli*	Walter Fiers [505]
1977	Chemical DNA sequencing developed; Maxam-Gilbert sequencing	Allan Maxam, Walter Gilbert [506]
1977	Chain-termination method DNA sequencing developed; Sanger sequencing. (Received 1980 Nobel Prize in Chemistry.)	Frederick Sanger [507]
1983	Polymerase chain reaction (PCR) invented. (Received 1993 Nobel Prize in Chemistry.)	Kary Mullis [289]
1987	First automated DNA sequencer invented, using the Sanger-sequencing method.	Lloyd M. Smith [508]
1990-2000	High throughput, genome scale, data-rich biology	Various [509]
1995	The first complete genome sequenced from a free-living organism, *Haemophilus influenzae.*	Robert D. Fleischmann et al. [510]
1999	Functional modules recognized as critical levels of biological organization; influenced the development of systems biology.	Leland H. Hartwell et al. [511]
2001	Human genome sequenced using Sanger-sequencing.	Eric S. Lander et al. [512]
2001	Systems biology first described in scientific literature.	Trey Ideker et al. [498]

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CHAPTER III

STRUCTURAL AND FUNCTIONAL ANNOTATION OF AN EQUINE WHOLE GENOME OLIGOARRAY

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Abstract

Background

The horse genome is sequenced, allowing equine researchers to use high-throughput functional genomics platforms such as microarrays; next-generation

sequencing for gene expression and proteomics. However, for researchers to derive value from these functional genomics datasets, they must be able to model this data in biologically relevant ways; to do so requires that the equine genome be more fully annotated. There are two interrelated types of genomic annotation: structural and functional. Structural annotation is delineating and demarcating the genomic elements (such as genes, promoters, and regulatory elements). Functional annotation is assigning function to structural elements. The Gene Ontology (GO) is the de facto standard for functional annotation, and is routinely used as a basis for modelling and hypothesis testing, large functional genomics datasets.

Results

An Equine Whole Genome Oligonucleotide (EWGO) array with 21,351 elements was developed at Texas A&M University. This 70-mer oligoarray was designed using the approximately 7x assembled and annotated sequence of the equine genome to be one of the most comprehensive arrays available for expressed equine sequences. To assist researchers in determining the biological meaning of data derived from this array, we have structurally annotated it by mapping the elements to multiple database accessions, including UniProtKB, Entrez Gene, NRPD (Non-Redundant Protein Database) and UniGene. We next provided GO functional annotations for the gene transcripts represented on this array. Overall, we GO annotated 14,531 gene products (68.1% of the gene products represented on the EWGO array) with 57,912 annotations. GAQ (GO Annotation Quality) scores were calculated for this array both before and after we added GO annotation. The additional annotations improved the *meanGAQ* score 16-fold. This data is publicly available at *AgBase* (http://www.agbase.msstate.edu/).

Conclusions

Providing additional information about the public databases which link to the gene products represented on the array allows users more flexibility when using gene expression modelling and hypothesis-testing computational tools. Moreover, since different databases provide different types of information, users have access to multiple data sources. In addition, our GO annotation underpins functional modelling for most gene expression analysis tools and enables equine researchers to model large lists of differentially expressed transcripts in biologically relevant ways.

Background

Although the availability of a completed horse genome sequence enables researchers to use genomic technologies in their research [1], deriving value from high throughout genomics datasets requires genomic annotation. Genomic annotation includes the demarcation of functional elements within the genomic sequence ("structural annotation") and associating functional data with these same elements ("functional annotation"). Structural annotation is initially provided during the final stages of genome sequence assembly using computational pipelines to predict open reading frames and other functional elements. For example, the National Center for Biotechnology Information (NCBI) Gnomon annotation pipeline (http://www.ncbi.nlm.nih.gov/genome/guide/gnomon.shtml) combines *ab initio* predictions with sequence homology based upon RefSeq transcript alignments of the known genes. This structural annotation pipeline currently identifies 21,842 horse genes, and of these, 82.4% are "predicted" based upon sequence similarity with known genes from other species (as of 10/04/08). This means that these 17,997 horse genes are only

listed because they are similar in sequence to genes that are already known to exist in other species.

In contrast to structural annotation, functional annotation is not generally done automatically as part of the genome sequencing process. Typically, functional annotation is done as a separate, focused effort and the *de facto* method for functional annotation in eukaryote genomes is the Gene Ontology [2]. The GO is a structured network consisting of defined terms and the relationships between them that describe three attributes of gene products: Molecular Function, Biological Process and Cellular Component [3]. Annotation to the GO involves providing information about the gene product being annotated, its attributed function and the evidence for associating the function with this gene product [4]. There are two broad types of GO evidence codes: direct experimental codes (the evidence codes used for biocuration of published literature) and indirect evidence codes. Indirect evidence codes include function prediction based on sequence such as "inferred from sequence orthology" (ISO), where functional conservation is inferred for predicted orthologs, and "inferred from electronic annotation" (IEA), which includes function predicted based on functional motifs and domains [5]. The European Bioinformatics Institute GOA Project (EBI GOA) provides IEA based GO annotations for all proteins in the UniProtKB database [5].

Analyzing microarray data using GO has provided new insights into agriculturally important areas of research, including reproduction [6], lactation [7], adipogenesis [8] and animal health [9, 10]. Moreover, GO annotation has become the accepted standard for functional annotation and its use is growing exponentially in species that have a history of dedicated GO annotation effort [2, 11]. GO annotations provided by GO

Consortium members are used by public databases (eg. Entrez Gene, UniProt), genome browsers (eg. Ensembl), commercial vendors (eg. Affymetrix, Ingenuity Pathways Analysis) and freely available analysis tools (eg. Onto-Tools [12], Cytoscape [13]). However, while there are many tools available for analyzing microarray data (http://www.geneontology.org/GO.tools.shtml#micro), researchers wishing to do functional analysis of their equine array results are hampered by the lack of GO for equine gene products represented on microarrays. For example although there are, 21,842 horse genes, only 1,582 equine proteins are represented in the UniProtKB database, so only 7.2% of horse gene products have any GO annotation. This is further complicated since different tools use different database accessions, and it is currently difficult to determine the equivalent database accessions for horse sequences found in different public databases.

If equine researchers are to translate functional genomics results into practical solutions for equine health and production, they need to be able to translate data provided by high throughput functional genomics platforms (such as microarrays) into relevant biological knowledge. The Texas A&M Equine Whole Genome-oligoarray is a 21,000 element 70-mer expression array designed from the assembled equine genome sequence in order to represent the majority of expressed equine sequences.

Briefly, the vast majority (97.5%) of the genes were documented by one or more transcript sequences (RNA, UniGene or EST) while the remaining (2.5%) were documented solely by a protein hit. The oligo design process searched for 70-mer long hybridization probes representing all genes with due consideration to probes reporting multi-copy genes and other more complex cases. The probe selection process resulted in

21,351 probes (20,461 addressing single-gene and 890 addressing multi-gene targets) representing 22,296 genes. Appropriate positive, distance, specificity and negative controls (total 321) were added. The probes were commercially synthesized (Invitrogen, USA) and spotted onto UltraGAPS aminosilane coated slides with barcodes (Corning, MA) using a Chip Writer Pro microarrayer (BioRad, CA) equipped with 24 Telechem SMP3 pins (TeleChem International, CA) [14, 15].

The Equine Whole Genome array is presently being validated and will be available to the equine research community worldwide. To assist equine researchers with the functional modelling of data produced using this array, we provide information about public database accessions and functional annotations for elements represented on this array. The method of functional annotation that we use to provide GO annotation for this array is a combination of manual biocuration with computational analysis. We are continuing to add additional GO annotations based upon published literature and all GO annotations will be made publicly available at *AgBase* (http://www.agbase.msstate.edu/).

Results and discussion

Array annotation is useful because it facilitates integrating and interpreting large data sets that are produced when oligoarrays are used to evaluate complex biological processes. By annotating the equine whole genome array, researchers can step from lists of differentially regulated gene products to model-based clustering of gene expression data that advances the understanding of a biologic process. Further, accurate modelling requires up-to-date functional annotation, regardless of species, and is relevant to physiology, health, and disease.

The importance of integrating biological knowledge gleaned from gene expression profiles has been eloquently demonstrated by Chen and Wang. Using breast cancer microarrays, they demonstrated that prediction models constructed based on information from gene sets (pathways) outperformed the prediction models based on expression values of single genes, with improved prediction accuracy and interpretability [16]. This approach has also been applied to investigate the molecular basis of bone remodelling in osteoarthritis. The researchers conducted a microarray gene expression profile of the bone. Through this profile, researchers identified altered expression of two signalling pathways and target genes in osteoarthritic bone. Using an annotated array, these researchers were able to include genes with known or predicted roles in osteoblast, osteocyte, and osteoclast differentiation and function [17].

The Texas A&M Equine Whole Genome-oligoarray is a 21,351 element expression array that is presently being validated and will be available to the equine research community worldwide. To ensure that users are able to derive value from their array results we have provided information about the public database accessions represented on this array and provided GO annotations for these gene products.

Database accession mapping

So that users could access the information from multiple public databases, we provided multiple database accessions corresponding to each element on the array. To do this we used ArrayIDer [18], a tool that retrieves structural annotations for ESTs and provides 13 different identifiers for access to several publicly available databases (including UniProtKB, Ensembl, RefSeq, IPI and UniGene). An example of the ArrayIDer output is shown (Supplemental File 1) and the complete results will be made

publicly available both with the array and on the *AgBase* website (http://www.agbase.msstate.edu/). Until this data is available online, users can contact *AgBase* for this mapping table or to run ID mapping for datasets.

The presence or absence of these gene products in different databases (Figure 3.1) also provides biological clues about these gene products. For example, we found 337 elements that map to UniProt or Genbank RefSeq accession numbers. These are the equine gene products that were experimentally studied prior to gene sequencing and are likely to have published functional information available. A further 12,343 elements map to "XP" accessions from NCBI; these are proteins that are predicted based upon the NCBI structural annotation pipeline. These predicted gene products will not yet have experimental functional information available but they will have sequence homology to experimentally validated genes other species. The relatively large proportion of predicted gene products is typical of newly sequenced genomes such as horse. For example 84% of equine genes in NCBI are predicted, compared to 57.3% of the gene products represented on this array. Moreover there are 4,399 additional gene assemblies represented on this array that are not available from NCBI.

We found 2,164 Expressed Sequence Tags (ESTs) that did not map to any of the current equine genes. ESTs represent the transcriptionally expressed elements within a genome and since these do not align with any predicted genes, these may represent mRNAs that are unique to horse. Another feature of newly sequenced genomes is that there are rapid revisions and changes to the publicly available gene products as structural annotation proceeds. We found 2,108 elements mapping to NCBI database accessions that have been removed due to updates in structural annotation of the equine genome.

GO annotation results

Since the EBI GOA Project provides IEA annotation for UniProtKB proteins, we found that 208 equine UniProtKB entries already had existing GO. This represents 61.4% of the UniProtKB but only 1% of the elements on the array. To improve the amount of GO annotation for horse gene products represented on this array we did our own GO annotation for equine gene products. In total, we added 57,912 GO annotations for 14,531 gene products, representing 68.1% of the elements on the Equine Whole Genome-oligoarray. Using a similar approach, the Affymetrix chicken genome array was reannotated, increasing the number of probes associated with GO annotation by 45% and the quality of annotation by 14%. The large proportion of equine gene products associated with GO is partially due the improved ability to recognize equine: mammal orthologs (compared to chicken) and that 31.9% of these gene products were listed as "No Data" (ND), indicating that there is presently no functional information for these elements. This GO annotation is summarized into broad functional groups using the GOA and whole proteome GOSlim and the GOSlimViewer tool [19] (Figure 3.2). The GO annotation is divided into three groups: cellular component, molecular function, and biological process. The GO is evenly represented as 38% of the annotations are biological processes, 35% are molecular functions, and 27% are cellular components. Furthermore, there is information about membranes, cells, binding, regulation of biological processes, cell communication, cellular processes, and metabolic processes, along with much more. Thus annotation allows investigators to rapidly translate and integrate the full complement of array data into a bar code of structurally and functionally meaningful

changes at the protein level, changes which reflect the differential regulation of the experimental intervention.

Since UniProtKB and RefSeq accessions are likely to have literature that delineates protein function, we provided GO annotations by manual curation of existing literature. Since this process is necessarily time consuming this effort is continuing. To provide initial GO annotation we used known orthologs to human, mouse and rat genes that have existing GO. Orthologs were manually verified and GO annotation based on direct, experimental evidence transferred to the equine proteins. Fifty gene products were manually annotated using ISO annotation, producing 529 annotations. A further 43 cannot be annotated until confirmation of the existence or absence of any literature available.

While there is no experimental literature for any of the equine predicted proteins, many of these are likely to have orthologs amongst mammalian species. By transferring GO annotations from orthologous genes products that have experimental based GO annotation 48,887 annotations were added for 12,227 predicted proteins, representing 98.3% of all predicted proteins. The other elements on the array had no experimental literature or ortholog information available. Instead we provided GO annotations based upon functional motifs using an automated pipeline to assess functional motifs [20]. We added 6,466 annotations for 4,154 gene products, representing 23.6% of all gene products. Notably, 76.4% of the gene products were annotated as "no data." These are summarized in Table 3.1.

Currently there are no commercial equine arrays, so there is no GO associated with any of the other equine arrays. However we do have information about the GO

provided for commercial arrays in other livestock species. Notably, although Affymetrix provides GO annotation with their array annotation files, re-annotation of GeneChip Chicken Genome Array resulted in a 37% increase in the number of array elements with GO annotations and a 14% increase in the GO annotation quality [21]. For the more closely related pig, only 11% of gene products on the Affymetrix array have GO annotation.

The GO annotations that we have provided will be made publicly available via the *AgBase* database. Since GO annotations change as new data becomes available and new GO terms are added, this information will be updated periodically. Providing GO annotations for 68% of the elements on the equine array is a significant achievement and work is continuing to provide more detailed GO annotations and make these publically available. Array users are encouraged to contact *AgBase* (agbase@cse.msstate.edu) with specific questions about this data or to request further GO annotation.

GAQ score results

To determine the overall quality of the GO annotations added to the array, we evaluated the GO Annotation Quality Score[22] for gene products associated with this array both before and after we added our GO annotations. Briefly, *GAQ* score quantitatively measures GO quality, which includes breadth of GO annotation, the level of detail of annotation (depth), and the type of evidence used to make the annotation. *GAQ* Scores are calculated exactly as described previously [22] and the meanGAQ Score, the average GAQ Score for the dataset reported (Figure 3.3). Our GO annotations improved the *meanGAQ* score 16-fold for the array, from 1.6 for the pre-existing GO to 26.7 for the completed or additional GO. The *meanGAQ* score was also reported and as

expected there was an increase for each of the three ontologies. Cellular component increased 11-fold, from 0.4 to 4.5, biological process increased 16-fold, from 0.5 to 8.1, and molecular function increased 18-fold, or from 0.7 to 13.2.

Conclusion

This work is an initial computational based survey to provide GO annotation for a broad range of equine gene products. However detailed, species specific function can only be derived from manual curation of experimental literature and necessarily requires a focused biocuration effort which is currently lacking for horse. Nevertheless, this GO annotation provides the overview required to facilitate functional modelling of equine datasets based upon this array. Moreover, the GO annotations are made publicly available and will assist all equine researchers wishing to use the GO to model their data.

Methods

Accession mapping

Accession mapping was done using the standalone version of ArrayIDer from *AgBase*[18]. ArrayIDer accepts data from any microarray containing expressed sequence tag (EST) identifiers compatible with the NCBI UniGene database. ArrayIDer generates a list of gene and protein accessions from the latest databases (NCBI UniGene and the International Protein Index) and retrieves identifiers that match the EST input list. ArrayIDer will be activated for the horse dataset but until this is available online, users may contact *AgBase* to retrieve the mapping table or to run accession mapping for their own datasets. ArrayIDer is available from *AgBase* (http://www.agbase.msstate.edu/arrayider.html).

GO annotation

Our strategy for providing GO annotations for gene products represented on this array is summarized in Figure 3.4. We initially used GORetriever [19] to determine which UniProtKB or RefSeq accessions already had existing GO annotations. The remaining UniProtKB and RefSeq accessions were manually GO annotated based upon functional literature and mapped to orthologous mammalian gene products with experimentally based GO. Orthologs were determined using Ensembl version 53 and only 1:1 orthologs from human, rat, or mouse were returned. This type of GO annotation was assigned "inferred from sequence orthology" (ISO) GO evidence code, based upon standard GO Consortium procedures [4].

The NCBI predicted proteins do not have direct experimental evidence, and are unlikely to have any orthologs. These were first annotated by ISO annotation, or if there was no 1:1 ortholog available, we used known functional motifs to provide GO annotation. This is an automated process and is referred to as "inferred from electronic annotation" (IEA). Other gene products represented on the array were also GO annotated using the IEA method.

The results of these GO annotations were summarized using GOSlimViewer [19] with the GOA and whole proteome GOSlim Set.

List of abbreviations

EBI-GOA - European Bioinformatics Institute GOA Project

ESTs – Expressed Sequence Tags

EWGO – Equine Whole Genome Oligoarray

GAQ – GO Annotation Quality

GO – Gene Ontology

IEA – Inferred from Electronic Annotation

ISO – Inferred from Sequence Orthology

NCBI – National Center for Biotechnology Information

ND – No Data

NRPD – Non Redundant Protein Database

Competing interests

The authors declare that they have no competing interests.

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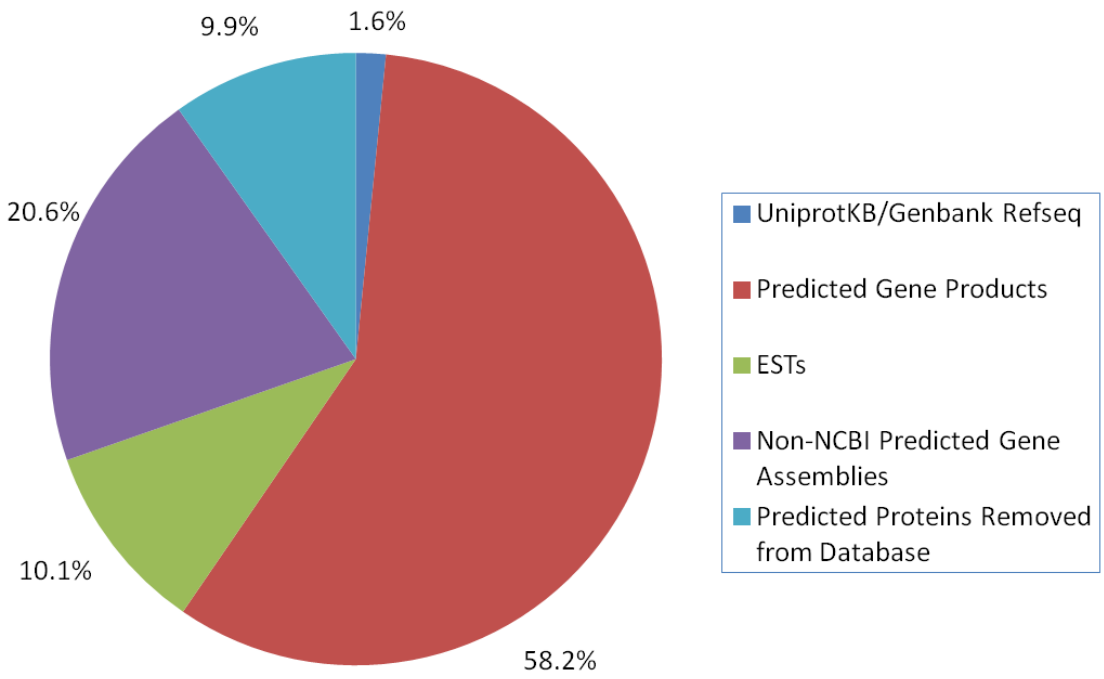

Figure 3.1 Gene products represented on the equine whole genome array

Array gene products were linked to public databases to facilitate functional modelling. 1.6% of the elements represent experimentally validated products found in UniProtKB or the RefSeq databases while 58.2% are predicted based upon computational structural annotation of the horse genome. 20.6% are predicted genes not available from NCBI and 10.1% are ESTs that are not linked to known or predicted horse genes. A further 9.9% have been removed from the NCBI databases due to structural reannotation.

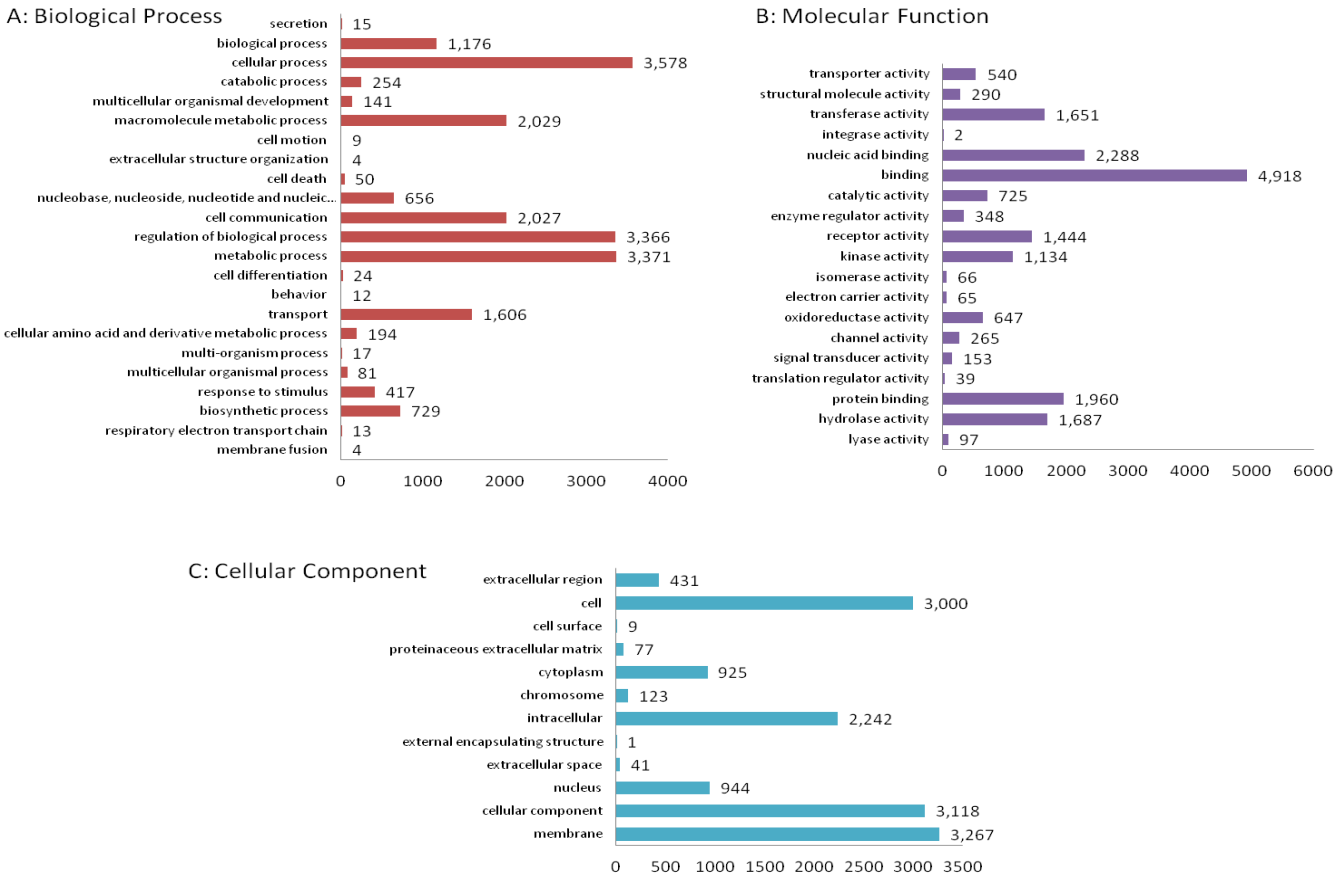

Figure 3.2 Functional grouping of equine array gene products using GOSlimViewer.

The GO annotation is divided into three broad functional groups using the GOA and whole proteome GOSlim and the GOSlimViewer tool: A. Biological Process, B. Molecular Function, and C. Cellular Component. Further subcategories within functional groups A-C are listed on the y-axis and the frequency of this function within the array is represented on the x-axis. The functional group, "biological process" had the most GO IDs represented, followed by "molecular function," and finally "cellular component." In A, the largest three subcategories were: cellular process, regulation of biological process, and metabolic process. In B, binding was the most annotated function. For C, the top three cell component subcategories were e cell, cell membrane, and cellular component. Particularly significant is the wide display of GO IDs shown, suggesting the equine whole genome array is fairly comprehensive.

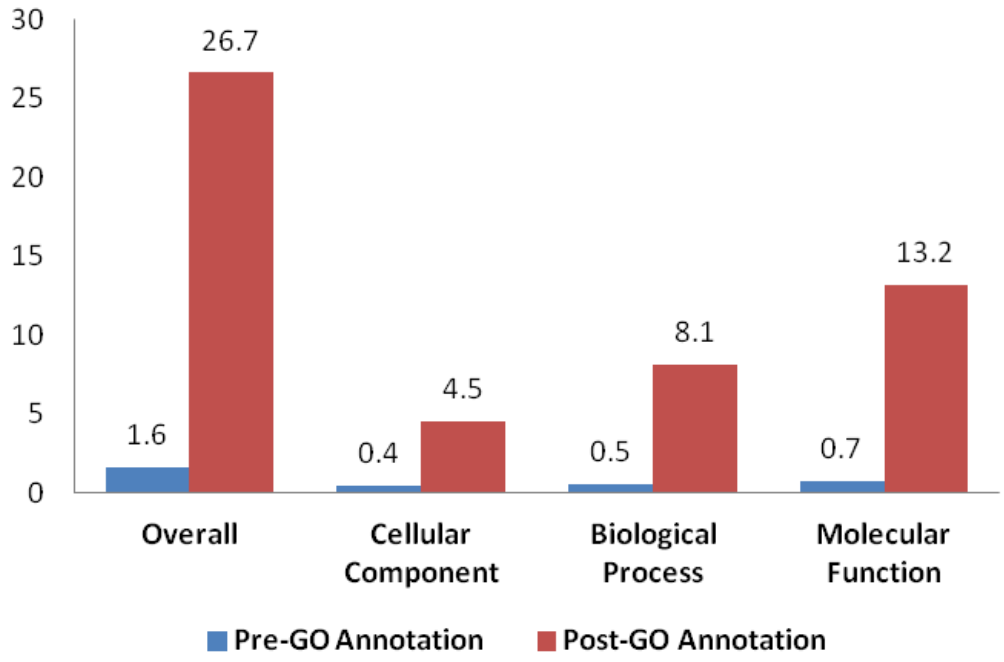

Figure 3.3 GO Annotation Quality (*GAQ*) score

GAQ Scores were calculated for the existing GO annotation on the array and the GO annotation available after we added the additional annotations described in this paper. *GAQ* Scores are calculated exactly as described previously [22]. Briefly, *GAQ* score quantitatively measures GO quality, which includes breadth of GO annotation, the level of detail of annotation (depth), and the type of evidence used to make the annotation. Additional GO improved the *meanGAQ* score 16-fold, from 1.6 for the pre-existing GO to 26.7 for the completed or additional GO. *meanGAQ* score for each ontology is shown as well. Cellular component increased 11-fold, from 0.4 to 4.5, biological process increased 16-fold, from 0.5 to 8.1, and molecular function increased 18-fold, or from 0.7 to 13.2.

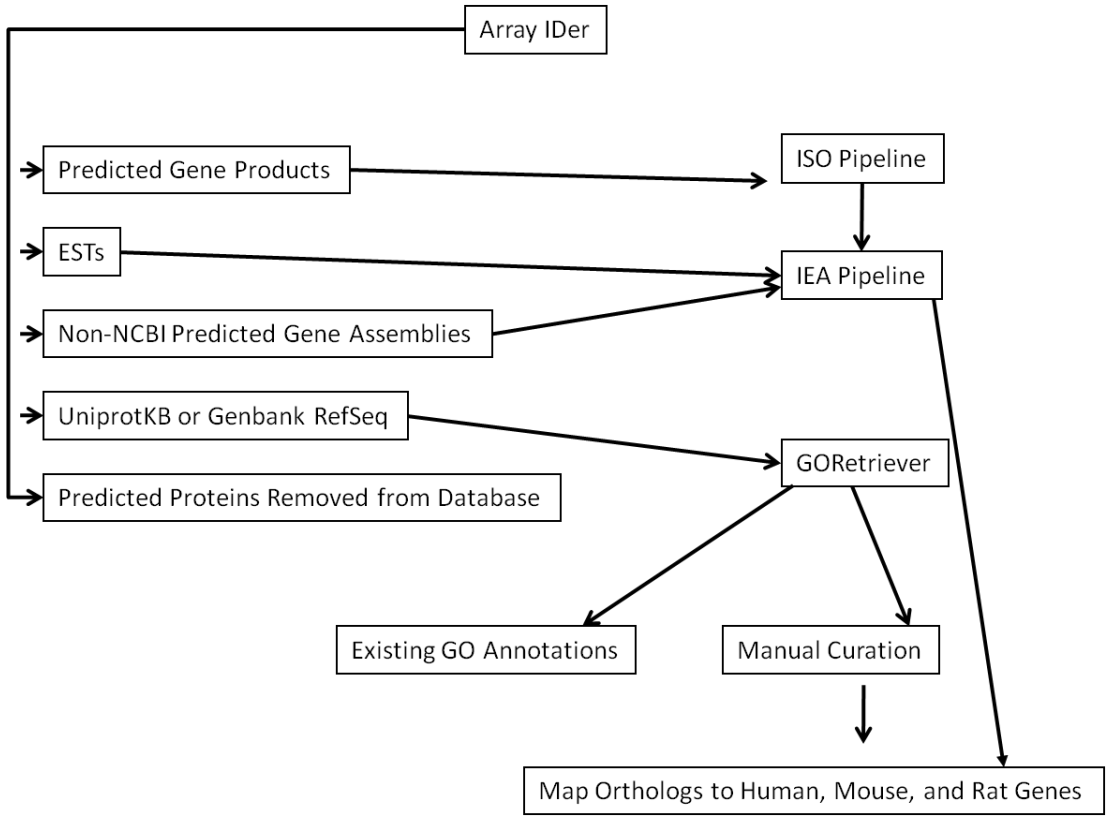

Figure 3.4 Flow chart demonstrating the functional annotation process

Functional annotation begins by accession mapping through ArrayIDer. ArrayIDer divides the input file into broad categories: predicted gene products, ESTs, non-NCBI predicted gene assemblies, and UniProtKB or Genbank RefSeq, as well as predicted proteins that were removed from the database. Predicted gene products go down the ISO pipeline, and the rest go through IEA pipelines, with the exception of UniProtKB or RefSeq, which are sent to GORetriever. GORetriever pulls out the genes which already have existing GO annotations, and the rest are manually curated by mapping orthologs to human, mouse, and rat genes.

Table 3.1 GO Annotation of the equine whole genome oligoarray

Database Category	No. Gene Products	Number of GO annotations added
UniProtKB/Genbank RefSeq	337	2,559
Predicted Gene Products	12,434	48,887
ESTs	2,164	4,546
Non-NCBI Predicted Gene Assemblies	4,399	1,920
Predicted Proteins Removed from Database	2,108	--
TOTAL	21,351	57,912

A total of 57,912 annotations were derived from the Equine Whole Genome Oligoarray. 2,559 annotations were derived from the 337 UniProtKB and RefSeq accessions. 48,887 annotations were derived from 12,434 predicted gene products. 4,546 annotations were derived from 2,164 ESTs. Finally, 2,108 predicted proteins removed from the database were not annotated. Thus 21,351 gene products yielded 57,912 new annotations.

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CHAPTER IV

FUNCTIONAL MODELLING OF AN EQUINE BRONCHOALVEOLAR LAVAGE FLUID PROTEOME PROVIDES EXPERIMENTAL CONFIRMATION AND FUNCTIONAL ANNOTATION OF EQUINE GENOME SEQUENCES

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Summary

The equine genome sequence enables the use of high-throughput genomic technologies in equine research, but accurate identification of expressed gene products

and interpreting their biological relevance requires additional structural and functional genome annotation. Here, we employ the equine genome sequence to identify predicted and known proteins using proteomics and model these proteins into biological pathways, identifying 582 proteins in normal cell-free equine bronchoalveolar lavage fluid (BALF). We improved structural and functional annotation by directly confirming the *in vivo* expression of 558 (96%) proteins, which were computationally predicted previously, and adding Gene Ontology (GO) annotations for 174 proteins, 108 of which lacked functional annotation. Bronchoalveolar lavage is commonly used to investigate equine respiratory disease, leading us to model the associated proteome and its biological functions. Modelling of protein functions using Ingenuity Pathway Analysis identified carbohydrate metabolism, cell-to-cell signalling, cellular function, inflammatory response, organ morphology, lipid metabolism and cellular movement as key biological processes in normal equine BALF. Comparative modelling of protein functions in normal cell-free bronchoalveolar lavage proteomes from horse, human, and mouse, performed by grouping GO terms sharing common ancestor terms, confirms conservation of functions across species. Ninety-one of 92 human GO categories and 105 of 109 mouse GO categories were conserved in the horse. Our approach confirms the utility of the equine genome sequence to characterize protein networks without antibodies or mRNA quantification, highlights the need for continued structural and functional annotation of the equine genome and provides a framework for equine researchers to aid in the annotation effort.

Keywords: Bronchoalveolar lavage, equine, genome annotation, proteomics

Introduction

The completion of the equine genome sequence is a pivotal event for equine researchers, as it enables the use of high-throughput whole genome technologies to investigate clinically relevant hypotheses [1]. With the application of these technologies to all species, including the horse, the major challenge facing researchers is now to interpret the biological relevance of hundreds or even thousands of differentially expressed genes that typify high-throughput data sets. The path to recognize the biological relevance of a genome sequence begins with structural annotation, where functional elements including open reading frames, genes, coding and non-coding regions, and regulatory elements are demarcated. Refined structural annotation is a fundamental requirement for accurately identifying gene products using high-throughput functional genomics (microarrays, proteomics). Following this, biological functions are assigned to the identified structural elements (functional annotation). For the horse, the primary impediments to recognizing biological relevance from high-throughput data sets are a lack of refinement in structural annotation of the equine genome and an absence of robust functional annotation of the structural elements, particularly gene products.

The Gene Ontology (GO) is a species-unifying structured hierarchical vocabulary for the annotation of biological knowledge relevant to gene products. Biological functions of a gene product are identified by the European Bioinformatics Institute Gene Ontology Annotation (EBI-GOA) Project [2] as 'cellular component,' 'molecular function' or 'biological process' and are assigned GO terms with corresponding unique numerical identifiers. The structured order of these ontologies, as well as their numerical identifiers, makes GO amenable to computational analysis and able to be used to streamline

identification of functions associated with gene products. As such, GO facilitates modelling of large high-throughput functional genomic technology, turning data into knowledge. The utility of the GO for modelling functional genomics data sets has made it a de facto standard for gene product functional annotation [3]. Annotations from EBI-GOA are accessible through the UniProt Knowledgebase (Uni-ProtKB) database [4]. In addition, Ensembl [5], Entrez Gene [6] and Affymetrix [7] utilize these GO sources for their functional annotation. However, the GO databases do not include annotations for 'predicted' gene products i.e. those without experimental evidence for their *in vivo* expression. For example, of the nearly 20,000 protein coding genes predicted for horse, only 1537 are represented in UniProtKB and can receive automatic GO annotation via the EBI-GOA Project [2].

In the absence of experimental evidence for *in vivo* expression of a gene product, other indirect lines of evidence are used to assign function, including electronic annotation based upon functional motifs and sequences (inferred from electronic annotation or IEA). The most rigorous of these is based upon the determination of orthology relationships between species. Orthologous genes/proteins are those that exist in multiple species that have diverged from a common ancestral gene that existed in the last common ancestor [8-12]. Presuming the absence of evolutionary bottlenecks, minimal evolutionary separation between orthologous pairs results in significant structural similarity, increasing the likelihood of conserved function. Commonly employed algorithms for assigning orthology include Homologene [13], Ensembl [5], Inparanoid [8] and Treefam [14]. Differences between these algorithms are responsible

for variations in the composition of an orthologous group between algorithms. Furthermore, these are only predictions and should be validated experimentally.

The most rigorous annotation of protein function requires time consuming manual biocuration of published literature. This is why, even in species such as human and mouse, where the greatest efforts in GO annotation have been expended, manual curation of literature to the GO lags behind the understanding of gene product functions represented in contemporaneous peer-reviewed literature. Accordingly, most newly sequenced genomes without funded GO biocuration, such as the horse, lack even preliminary functional annotation. For the horse, this problem is compounded because the breadth and depth of peer-reviewed literature provide limited experimental evidence for in vivo existence of equine gene products. To begin to address this problem, we have previously improved the breadth of available equine GO annotation by focusing on gene products represented on the TAMU equine whole genome oligoarray and by providing primarily computationally derived GO annotations for these gene products [15]. This includes both predicted and known gene products accessible at *AgBase* [16] (http://www.agbase.msstate.edu), our curated, open-source, web-accessible resource for functional analysis of agricultural plant and animal gene products.

In addition to being a repository for GO annotations in nontraditional species, *AgBase* provides tools to deliver GO annotations for large numbers of gene products. Two such tools, GORetriever and GOanna [16], accept nucleotide or amino acid sequences or accessions in various formats and return the associated GO annotations. This is an essential first step in modelling large high-throughput equine data sets (transcriptomes or proteomes) to identify their functional significance. Complementary to

the information obtained from GO annotations are tools that model the gene products of high-throughput data sets within the context of the complex interactions that characterize biological systems. Examples of these tools, which facilitate identification of relationships among genes, proteins, cell processes and diseases, include Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems®, http://www.ingenuity.com) and Pathways Studio (Pathways Studio®, http://www.ariadnegenomics.com/products/pathway-studio).

Nuances in both structural and functional annotation of the equine genome influence the information obtained from high-throughput functional genomic technologies, and tools that bridge these gaps are critical to realizing clinically applicable discoveries. Our interest in equine respiratory disease, coupled with the central dependence of equine athletic performance on respiratory function, were the stimuli for our desire to understand the physiology represented by proteins in the fluid secretions of normal equine lung. We used cell-free bronchoalveolar lavage fluid (BALF) from the lungs of normal horses, followed by quantitative shotgun proteomics and computational biology to provide experimental evidence for the expression of both known and predicted proteins from the equine genome sequence. Knowledge of the biological functions of these proteins was improved by assigning standardized nomenclature and GO annotations based on orthology. Finally, the biological information associated with these proteins was modelled using Ingenuity Pathways Analysis (IPA, Version 8.5; Ingenuity Systems) and compared to normal cell-free human and mouse BALF proteins using GO Modelling [16].

Methods and materials

Cell-free bronchoalveolar lavage fluid samples

Bronchoalveolar lavage fluid was collected from six clinically normal horses as previously described [17], placed immediately on ice, transported to the laboratory and centrifuged (600 g 10 min 4 °C). Aliquots of cell-free BALF supernatant were frozen at -80 °C for subsequent proteomic analysis.

Protein isolation, digestion, and peptide detection

The workflow for sample analysis and modelling is summarized in Fig 4.1. Detailed methods are provided as Supplemental File 2. Proteomic analysis was performed twice using a composite sample consisting of 100 µg of BALF protein from each of three horses. Triplicate aliquots of the composite (75 µg of protein each) were subjected to acid precipitation and trypsin digestion and analysed using liquid chromatography tandem mass spectrometry as previously described [18]. However, we did not perform differential detergent fractionation, and we used one dimensional liquid chromatography (1D LC) nanospray ionization and not electrospray ionization. 1D LC peptide separation was achieved using a Thermo Surveyor MS pump and 5–50% acetonitrile (ACN) gradient over 180 min followed by 95% ACN wash and 5% ACN equilibration with 0.1% formic acid as a proton source. Nanospray ionization of peptides was performed using a Thermo Finnigan nanospray type I source operated at 1.9 kV with 8-µm internal diameter silica tips at 500 nl/minute. Peptides were identified using a Thermo LCQ DECA XP Plus ion trap mass spectrometer for 225 min per sample. Precursor mass scans were performed using repetitive MS scans followed by three MS/MS scans of the three most intense MS peaks. Dynamic exclusion was enabled with a duration of 2 min and a

repeat count of two. Spectra were measured with an overall mass/charge (m/z) ratio range of 300–1700.

Protein identification

Proteins were identified in the manner previously described [19]. Briefly, mass spectra and tandem mass spectra were searched using TurboSEQUEST™ (Bioworks Browser 3.3; ThermoElectron, Florida, USA) against an in silico trypsin-digested database of equine non-redundant RefSeq proteins and filtered using a decoy search-based probabilistic method in which only peptides with a probability ≤0.05 were considered to be correct. The equine non-redundant protein database, which was downloaded from the National Center for Biotechnology Institute on 30 October 2009, contained 17,757 proteins and 1,347,511 unique peptides. Protein identifications and their associated MS/MS data have been submitted to the PRoteomics IDEntifications database (PRIDE; accessions 13712–13717) [20].

Functional analysis

To determine the biological relevance of proteins in the BALF, we performed functional modelling of the proteins using GO analysis and also used Ingenuity Pathways software to identify the biological functions that were significantly represented in the BALF proteome. Briefly, we used *GORetriever* [16] to retrieve associated GO annotations, and proteins that initially had no GO were annotated first based upon orthology to annotated mammalian genomes (GO evidence code: ISO, or inferred from sequence orthology) and second by sequence analysis to detect functional domains and motifs (GO evidence code: IEA, or inferred from electronic annotation). Orthology to

GO-annotated human, mouse and rat proteins was determined using Ensembl orthology predictions, and analysis of functional domains was carried out using InterProScan; both of these methods are routinely used by the *AgBase* database to provide first-pass GO annotations for gene products.

Existing and newly assigned GO annotations were combined to provide a comprehensive GO annotation set for our identified proteins. These data were summarized using GOSlimViewer [16] with the generic slim set, which groups the retrieved functional annotation information for the protein data set using common ancestor GO terms in each of the three gene ontologies (cellular component, molecular function and biological process). The resulting analysis summarizes the known biological information for the healthy cell-free equine BALF proteome. We also compared the biological information from our normal cell-free horse BALF proteome to that of human [21] and mouse [22] cell-free BALF proteomes by retrieving the associated GO annotations for the published human and mouse proteomes and modelling them in an identical fashion using GOSlimViewer. To determine how the biological information in the normal equine BALF proteome compares to biological information for all horse gene products, we also obtained the complete GO annotation file for horse from *AgBase* and summarized it using GOSlimViewer for comparison.

Finally, the normal cell-free BALF proteome was also modelled using Ingenuity Pathways Analysis software (IPA, Version 8.5; Ingenuity Systems) to identify significantly represented biological functions, pathways, and diseases, based on canonical networks generated from rat, mouse and human data. Because IPA accepts only mouse, rat, canine and human orthologs for upload and analysis, the human, mouse and rat

orthologs were identified for each equine protein using Ensembl BioMart (Version 0.7, Ensembl 56 set) [23] and uploaded to IPA. IPA selects 'focus genes' to be used for generating biological networks. Focus genes are based on proteins from the input data set that are mapped to corresponding gene objects in the Ingenuity Pathways Knowledgebase (IPKB) and are known to interact with other genes based on published peer-reviewed content in the IPKB. Based on these interactions, IPA builds networks with a size of no more than 35 genes or proteins. A p-value for each network and canonical pathway is calculated according to the fit of the user's set of significant genes/proteins. IPA computes a score for each network from this p-value that indicates the likelihood of the focus genes in a network being found together by chance. We selected networks scoring ≥2, which have >99% confidence of not being generated by chance [24, 25]. IPA assigns biological functions to these networks using annotations from the scientific literature that are stored in the IPKB, and a Fisher's exact test is used to calculate a p-value associated with the probability that each biological function/disease or pathway is assigned by chance. We used $P \leq 0.05$ to select significant biological functions and pathways represented in our proteomic data sets [24].

Results and discussion

Protein identification

We identified 582 unique proteins from the cell-free BALF of normal horses. A list of identified proteins is accessible in Supplemental File 3, and the spectra for identification of these peptides have been submitted to the PRIDE database. During manuscript preparation, 17,859 of the 18,448 (96.8%) in the NCBI Reference Sequence (RefSeq) non-redundant protein database carried the 'XP' designation in their RefSeq

number, which signifies that there is not yet experimental evidence of in vivo expression for these proteins. Proteins with documented in vivo evidence carry the designation 'NP' in their RefSeq number. Of the 582 proteins identified in our normal cell-free BALF proteome, 24 (4%) carried the NP designation and 558 (95.9%) carried the XP designation. This result is in line with the overall composition of NCBI equine proteins. The XP proteins within this equine BAL proteome data set constitute 3% of the total proteins (both XP and NP) predicted by the equine genome sequence (and 3.4% of proteins with the predicted (XP) designation). Identification of proteins with an XP designation in equine samples is important to the annotation effort of the equine genome sequence, because it provides experimental evidence for the in vivo expression of these proteins, improving both structural and functional annotation of the genome sequence. The 558 experimentally validated proteins were given standard nomenclature based upon recommendations for standardized nomenclature for human genes [26], and we are making this data available on the NCBI database.

GO annotation of identified proteins

Our initial GO survey of the 582 BALF proteins indicated that 408 proteins (70%) had 2229 existing GO annotations, the majority of which were provided by our previous equine annotation effort [15]. The remaining 174 proteins were annotated as previously described [15], and as outlined in the methods section, yielding 870 annotations. Ortholog-derived GO annotations (GO evidence code 'ISO,' inferred by sequence orthology) added GO annotations for 72 proteins (12.4%), and GO annotations for the remaining 102 proteins (17.5%) are based on functional motif analysis (GO evidence code 'IEA,' inferred by electronic annotation). Predicted proteins were annotated 'no data'

(GO evidence code 'ND'), as these proteins are computationally predicted and thus do not have published functional data available. While GO was returned for all BALF proteins, this is somewhat misleading, because 346 (59%) of the BALF proteins had 'ND' evidence codes in at least one of the three GO ontologies (cellular component, biological process, molecular function), signifying that the protein has been annotated but lacks functional information in that ontology. The absence of GO annotation may affect any or all of the three ontologies, and in our protein set, 108 (19%) proteins lacked GO annotation in all three ontologies. These results, summarized in Table 4.1, highlight the need for a focused effort to provide functional annotation for the equine genome sequence.

GO modelling

GOSlimViewer [16] was used to model the biological information associated with GO annotations for the healthy cell-free equine BALF proteome by mapping GO terms to the Generic GOSlim sets within each of the three ontologies (Generic GOSlim Set, 2009; Figs 4.2–4.4). The most common GO cellular component annotations were 'intracellular ' (12.6%), 'cell' (20.6%) and 'component unknown' (22.1%; i.e. GO terms determined to have a cellular component but not further categorized). In the GO molecular function ontology, 19% of the GO annotations mapped to 'binding' (GO:0005488). 'Protein binding' and 'catalytic activity' were highly represented, at 12.5% and 8.4%, respectively. Finally, the most represented GO biological processes were 'unknown' (24.2%), 'metabolic process' (13.3%) and 'regulation of biological processes' (9.2%). The finding that approximately 46% of the GO annotations for our 582 protein normal BALF proteome map to 'component unknown' and 'biological process unknown' further underscores the current challenges facing researchers in interpreting how lists of

differentially expressed equine gene products support or refute an experimental hypothesis.

To compare the functions of proteins in normal BALF to what is known about proteins in the equine genome, a reference GOSlim data set was created from the equine gene association file, which represents all GO annotations previously assigned to the horse. Comparisons of the reference and BALF GOSlim sets support our approach, demonstrating that the percentages of proteins within the top five groups of each ontology in the BALF proteome align to the top five groups of each ontology within the horse genome association file (Figs. 4.5-4.7). Within the 'cellular component ' ontology, we identified a higher percentage of 'component unknown' and 'cell' GO terms in the whole genome than the BALF normal proteome data; this is expected, as our BALF sample is a lung lining fluid and cells are removed during processing. A higher percentage of intracellular GO terms was identified in the BAL proteome, which may initially seem counterintuitive to the absence of cell terms. However, it may reflect the nature of the granularity of GO terms where, for example, proteins derived from ribosomes or bound to DNA are within the 'intracellular ' category and thus constitute a major portion of BALF protein associated with normal cell turnover and lysis within the airways. In the molecular function ontology, 'protein-binding' activity was over-represented in the BALF proteome, and this enabled us to rapidly identify protein-binding pathways of interest. Within the biological process ontology, the only differences detected between the BALF proteome and genome were small decreases in GO functions associated with 'metabolic process,' 'regulation of biologic process' and 'signal transduction;' again this most likely reflects the absence of cells.

Pathway analysis

As there are currently no tools that allow us to do direct analysis of horse pathways and networks, we relied on identifying orthologs from human and mouse. Using these two species, we identified orthologs for 494 of the 582 total proteins (84.4%). We note that the exclusion of 88 proteins could prevent the identification of biologically relevant pathways and metabolic processes that address a research hypothesis (included in Supplemental File 3). These omissions further highlight the need for organized annotation and gene nomenclature efforts on behalf of the equine genome. Regardless, the top biological pathways identified in descending order were carbohydrate metabolism, cell-to-cell signalling and interaction, cellular function and maintenance, inflammatory response, organ morphology, lipid metabolism and cellular movement, which are all homoeostatic pathways that would be expected in healthy lung samples. The top 15 pathways identified in normal equine BALF are represented in Fig. 4.8.

Comparative analysis of BALF proteomes using GO modelling

To compare the distribution of biological functions of proteins in normal equine BALF to those in normal human and mouse BALF, we modelled published normal cell-free BALF proteomes containing 167 proteins (human) [21] and 297 proteins (mouse) [22] by mapping the associated GO terms to the Generic GOSlim sets within each of the three ontologies (Generic GOSlim Set, 2009). The horse BALF proteome contained 91 of 92 GOSlim categories identified in the human BALF proteome, corresponding to 2718 horse and 2972 human GO annotations. One hundred and five of 109 GOSlim categories identified in the mouse BALF proteome were identified in horse, corresponding to 2718 horse and 5287 mouse GO annotations. Human and mouse GOSlim categories that were

not represented in the equine proteome contained only a single annotation and were mitochondrial organization for human, and mitochondrial organization, cellular recognition, epigenetic regulation, molecular function and vacuole for mouse. Comparisons of the top ten GOSlim categories within each ontology indicate that horse and human BALF proteomes share 9 cellular component categories (unknown, cell, intracellular, cytoplasm, protein complex, organelle, extracellular, cytoskeleton, plasma membrane), eight molecular function categories (binding, protein binding, catalytic activity, nucleotide binding, hydrolase activity, receptor activity and binding, peptidase activity, enzyme regulator activity) and five biological process categories (process unknown, metabolic process, regulation of biological process, multicellular organismal development, transport).

Despite the mouse BALF proteome having approximately twice the number of annotations of the horse BALF proteome, comparisons of the top ten GOSlim categories of horse and mouse BALF proteomes were in direct agreement with cellular component categories, which included organelle categories in addition to the nine categories previously detailed as common to human and horse. The eight molecular function GO categories and five biological process GO categories that are conserved in horse and human are identically conserved in mouse. Top GOSlim categories in the BALF proteome of horse, human and mouse are summarized in Figs. 4.9-4.11. These conservations in the distribution of biological functions of normal BALF proteome across horse, human and mouse support not only the utility and validity of GO modelling of functional genomic data, but also speak to the comparative utility of the horse as a model

in respiratory investigations, particularly in light of the ease of attaining large volumes of BALF from horses when compared to human and mouse.

Conclusion

Genomic technologies, coupled with the equine genome sequence, provide the potential to identify thousands of differentially expressed genes that have not been previously identified in the horse. We illustrate this by employing proteomics to identify 582 proteins in equine BALF, 558 of which were previously only computationally predicted. However, the ability to identify the full complement of gene products coded within the equine genome and to develop system models that allow researchers to recognize changes within large functional genomics data sets that are clinically relevant to equine disease necessitates a concerted effort towards both structural and functional genome annotation. To identify clinically relevant biological functions represented in normal equine BALF, a sample commonly used to diagnose and investigate inflammatory lower airway disease, which affects 12% of performing horses [27], we modelled our BALF proteome using both IPA and GO. These efforts highlighted challenges to system modelling of equine samples that extend beyond rudimentary structural and functional annotation, such as assignment of standard gene nomenclature, limited experimental evidence for *in vivo* expression of equine gene products and temporal delay between peer-reviewed literature and annotation to GO. Substantiating the validity of our BALF proteome and modelling system, Ingenuity Pathways Analysis identified key homoeostatic processes as predominating in the BALF proteome, including carbohydrate metabolism, cell–cell signalling and interaction, and cell function and metabolism. Furthermore, GO modelling of normal equine BALF proteome identified seven known

cellular component GO categories (cell, intracellular, cytoplasm, protein complex, organelle, extracellular, cytoskeleton, plasma membrane); eight molecular function categories (binding, protein binding, catalytic activity, nucleotide binding, hydrolase activity, receptor activity and binding, peptidase activity, enzyme regulator activity); and four known biological process categories (metabolic process, regulation of biological process, multicellular organismal development, transport) as conserved among the top 10 categories in each ontology between the normal BALF proteomes of human, mouse and horse. Our presentation of the functional characterization of the BAL proteome using GO and pathways analysis modelling is novel and important in equine research, because it demonstrates a framework for approaching large functional genomic data sets to realize biologically relevant information that addresses research hypotheses and enables comparisons of results from disease models across species.

Using our BAL proteomics analysis, we have highlighted some impediments to effectively employing the equine genome sequence and employing genomic technologies to investigate clinical conditions of relevance to the equine industry. In addition, we present the first BAL proteome and an initial characterization of this proteome using GO modelling and IPA analysis.

Acknowledgements

The authors gratefully acknowledge Ken Pendarvis, Allen Shack and Amanda Cooksey for their technical assistance. This investigation was supported by the College of Veterinary Medicine, Office of Graduate Research, Mississippi State University.

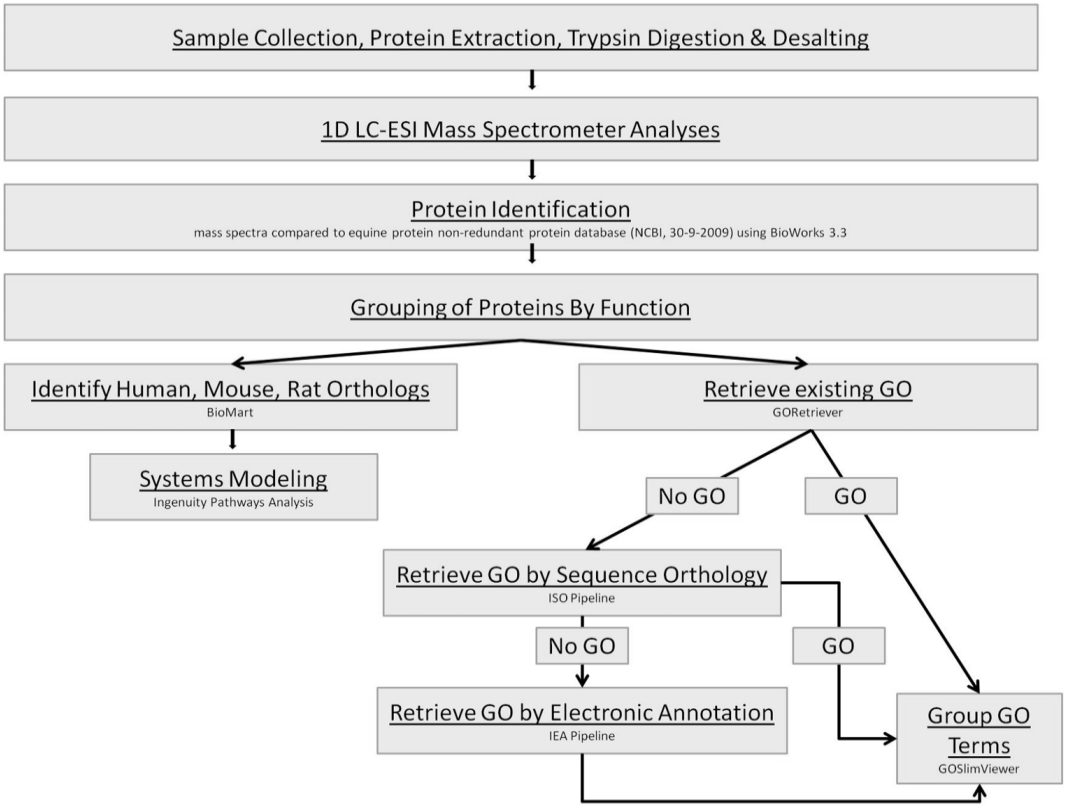

Figure 4.1 Strategy for identifying biological relevance of high-throughput data sets from horses.

Proteins identified by mass spectrometry are mapped to existing orthologs using BioMart and then analysed using Ingenuity Pathways Analysis (IPA). Proteins are also submitted to GORetriever to identify existing Gene Ontology (GO) annotations. Proteins without GO in GORetriever move through sequence orthology and electronic annotation to identify GO. All GO annotations for the protein data set are categorized using GOSlimViewer.

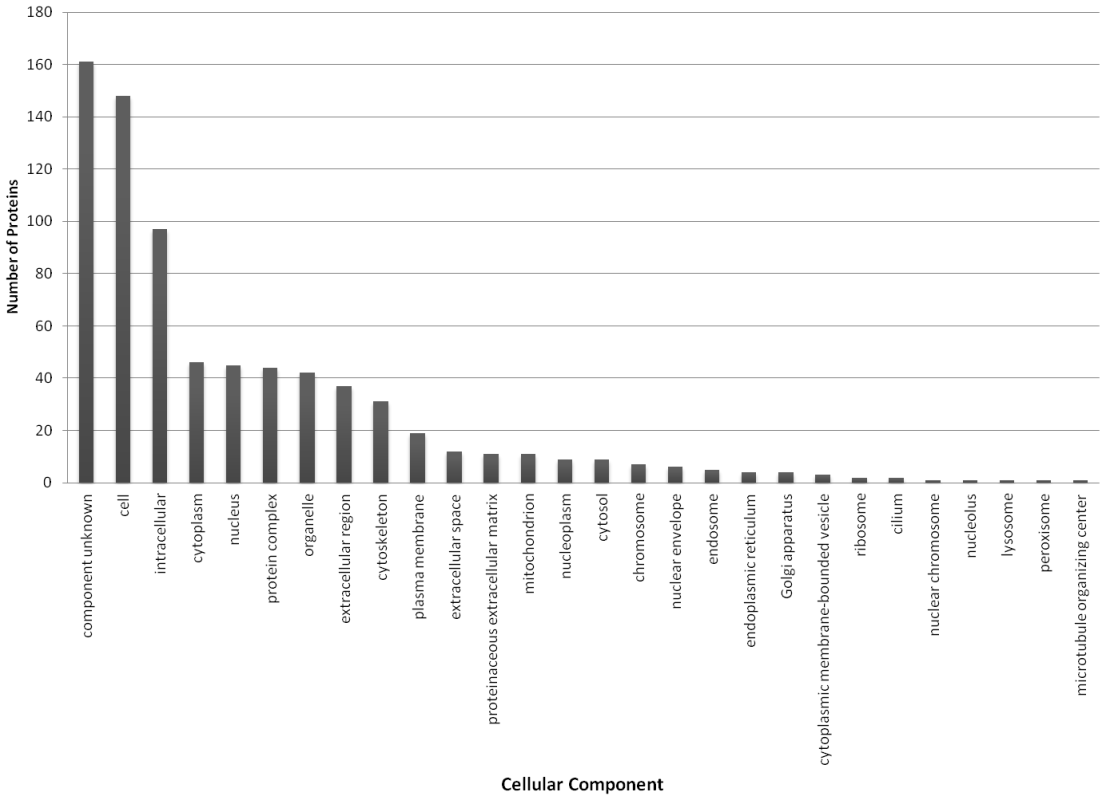

Figure 4.2 Cellular components transferred to orthologous proteins in equine bronchoalveolar lavage fluid.

The Gene Ontology (GO) annotations are summarized in broad terms of cellular component. These GO annotations are publicly available on *AgBase* [16].

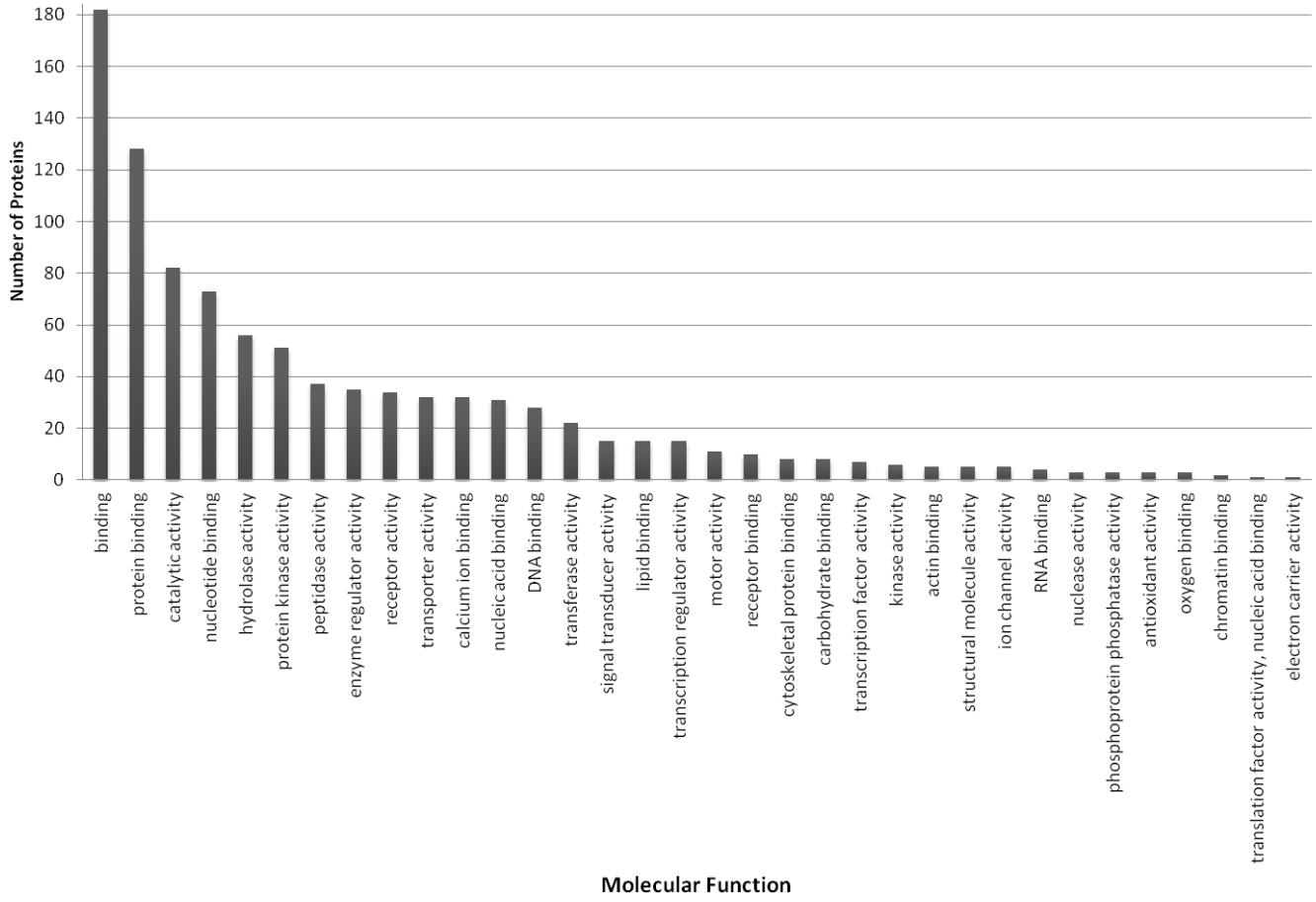

Figure 4.3 Molecular functions transferred to orthologous proteins in equine bronchoalveolar lavage fluid.

The Gene Ontology (GO) annotations are summarized in broad terms of molecular function. These GO annotations are publicly available via the *AgBase* database [16].

Figure 4.4 Biological processes transferred to orthologous proteins in equine bronchoalveolar lavage fluid.

The Gene Ontology (GO) annotations are summarized in broad terms of biological process. These GO annotations are publicly available via the *AgBase* database [16].

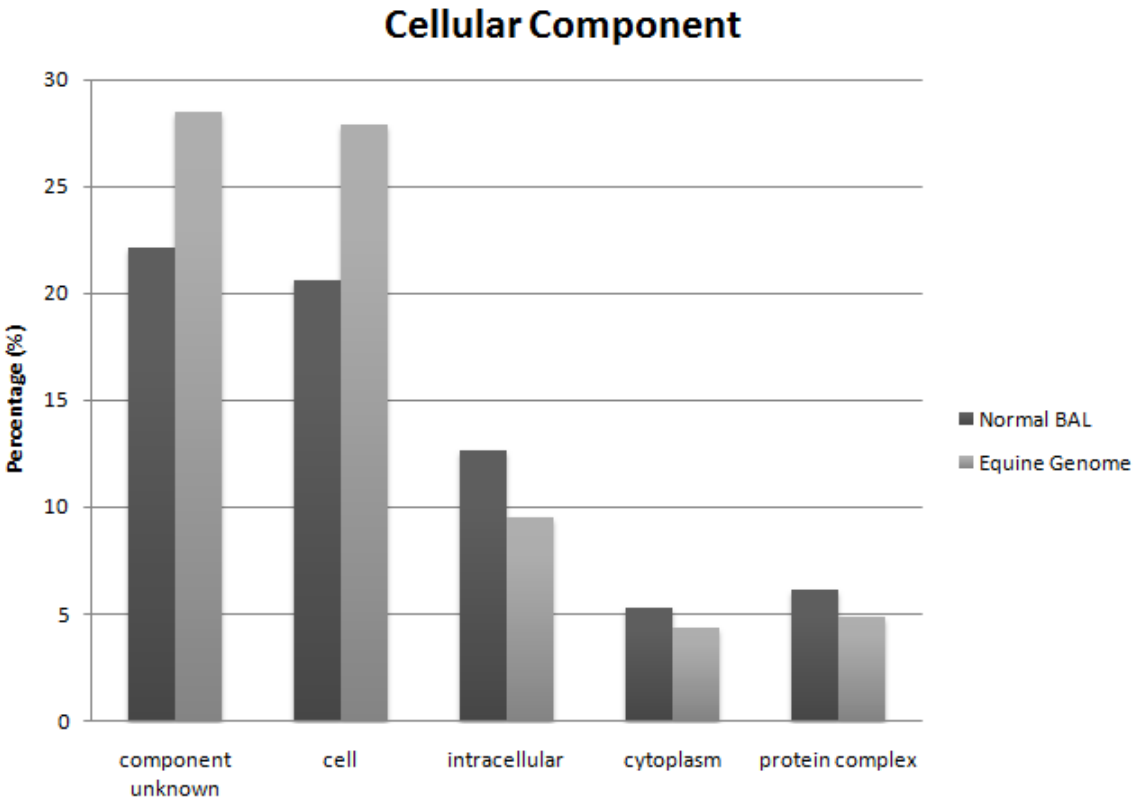

Figure 4.5 Top five Gene Ontology (GO) categories in normal bronchoalveolar lavage fluid vs. all equine GO annotations for the cellular component gene ontology.

Proteins are represented as a percentage of the total.

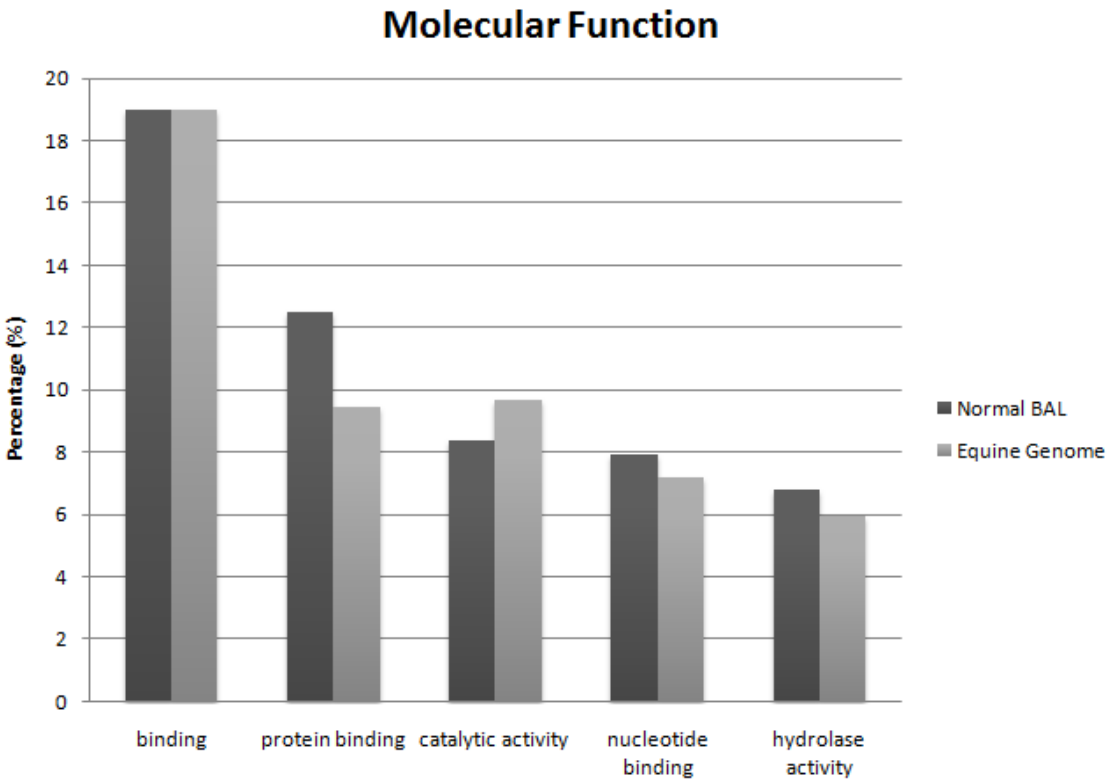

Figure 4.6 Top five Gene Ontology (GO) categories in normal bronchoalveolar lavage fluid vs. all equine GO annotations for the molecular function gene ontology.

Proteins are represented as a percentage of the total.

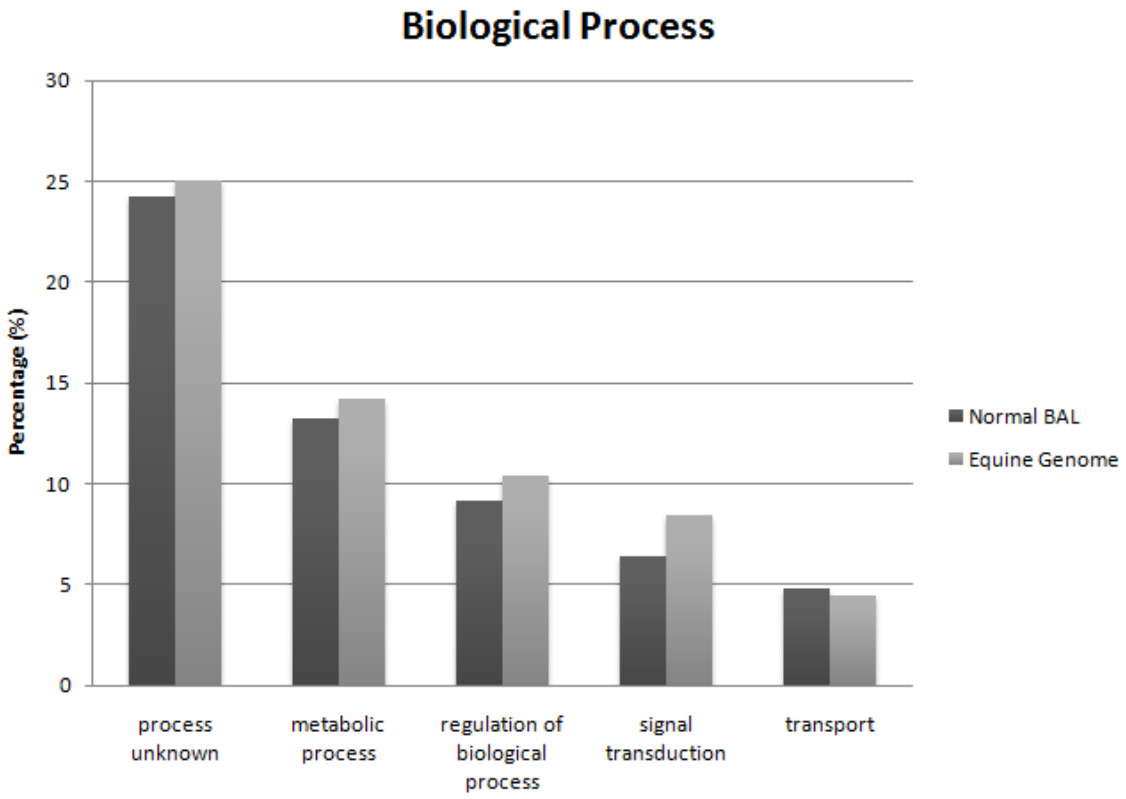

Figure 4.7 Top five Gene Ontology (GO) categories in normal bronchoalveolar lavage fluid vs. all equine GO annotations for the biological process gene ontology.

Proteins are represented as a percentage of the total.

Figure 4.8 Top 15 biological functions represented in the normal equine bronchoalveolar lavage fluid proteome.

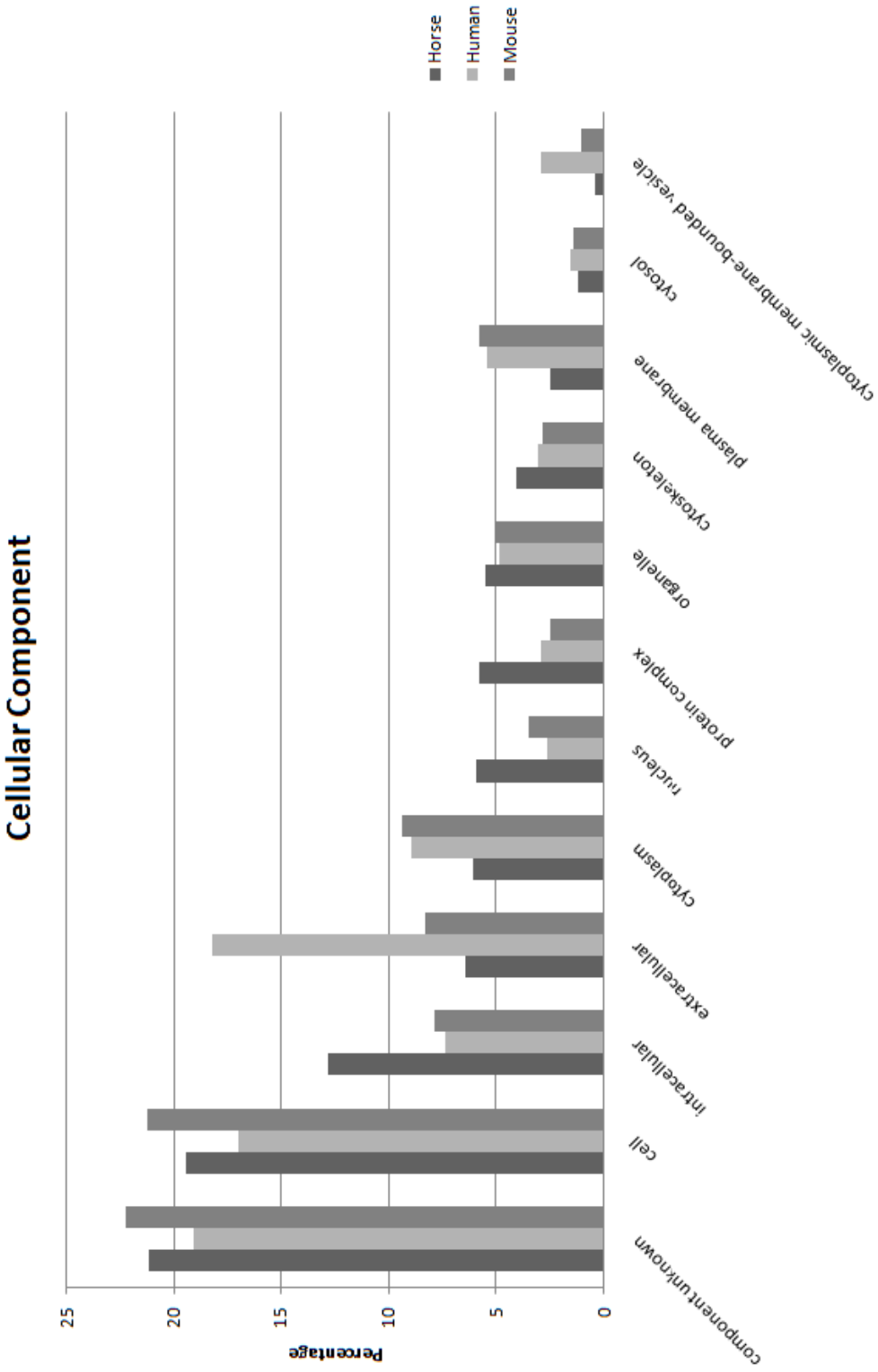

Figure 4.9 Top Gene Ontology categories in normal bronchoalveolar lavage fluid from horse, human and mouse for the cellular component gene ontology.

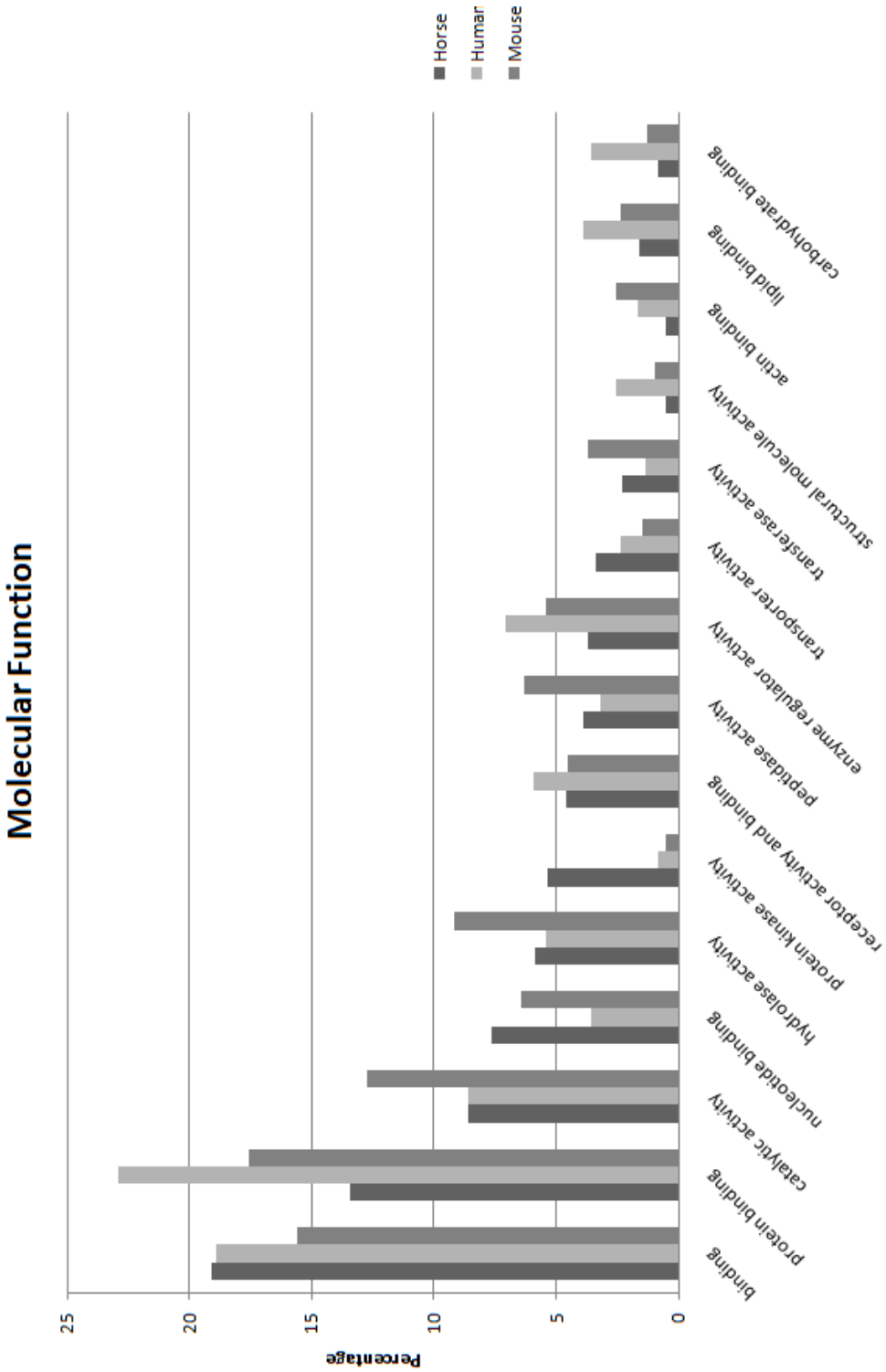

Figure 4.10 Top Gene Ontology categories in normal bronchoalveolar lavage fluid from horse, human and mouse for the molecular function gene ontology.

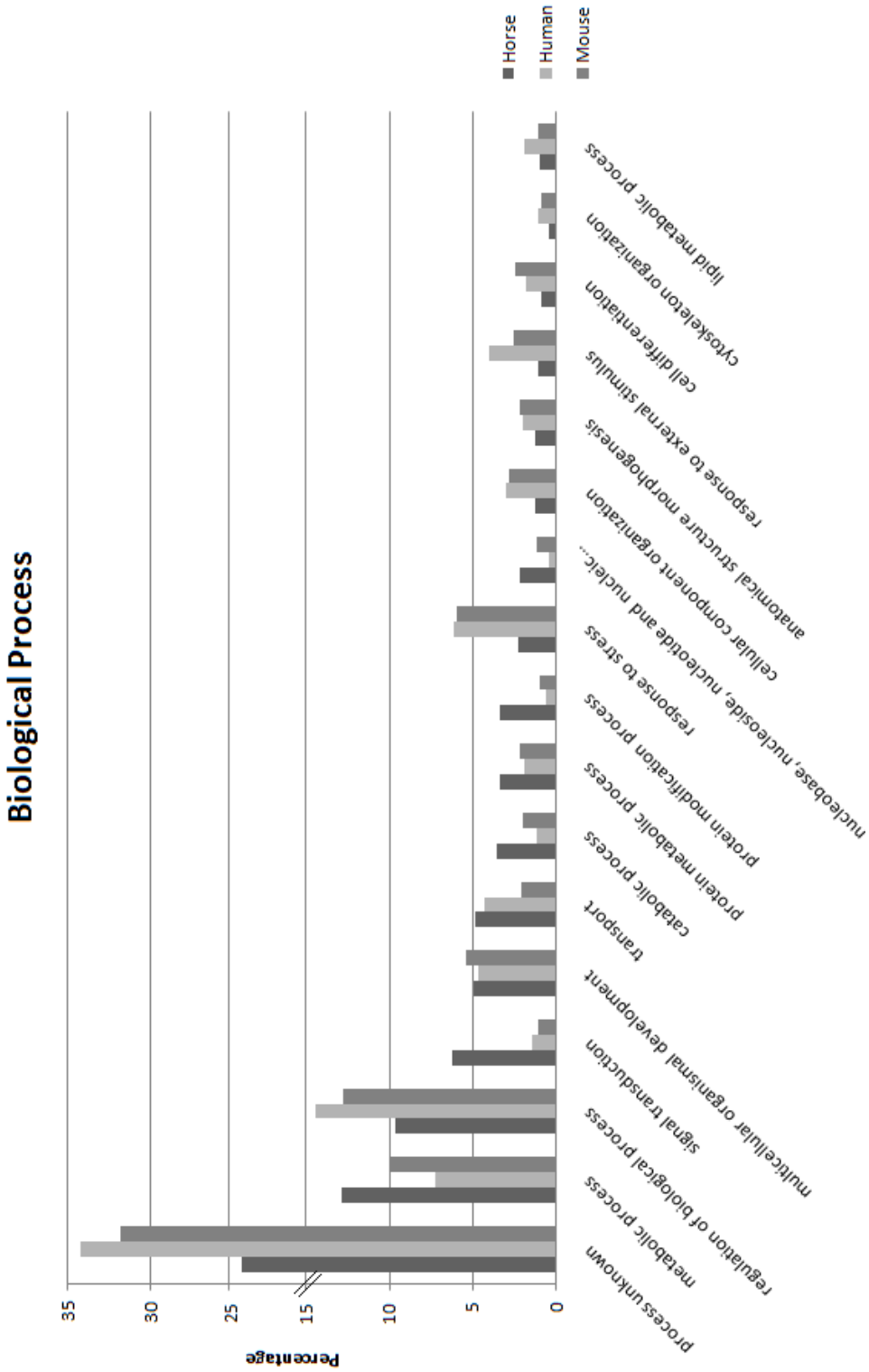

Figure 4.11 Top Gene Ontology categories in normal bronchoalveolar lavage fluid from horse, human and mouse for the biological process gene ontology.

Table 4.1 Comparison of functional annotation of the horse genome and its impact on the annotation of a normal bronchoalveolar lavage fluid (BALF) proteome.

	Horse genome	Normal BALF data set
(Non-redundant) Proteins	21,335 (18,848)	582
Predicted Proteins (XP RefSeq identifier)	85.6% (18,259)	95.9% (558)
Proteins with Gene Ontology Annotation	99.3% (21,185)	100% (582)
Proteins with NO in at least one ontology	39.6% (8449)	59.5% (346)
Proteins with ND in all 3 ontologies	15.1% (3214)	18.6% (108)

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CHAPTER V

MODELING OF AN EQUINE PASTURE HEAVES BAL PROTEOME IDENTIFIES MOLECULAR EVENTS MEDIATING AIRWAY NEUTROPHILIA

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Abstract

Pasture heaves, also termed summer pasture-associated recurrent airway obstruction (SPARAO), is an environmentally induced disease affecting horses maintained on pasture during conditions of high heat and humidity and is characterized

by seasonal exacerbation of asthma-like signs with neutrophilic airway inflammation. A similar syndrome commonly termed 'heaves' or recurrent airway obstruction (RAO), affects horses maintained indoors in temperate regions and is recognized as a spontaneous asthma model. During exacerbation, signs vary from mild to life-threatening episodes of wheezing, coughing, and chronic debilitating labored breathing. Among spontaneous animal models of asthma, both stall (RAO) and pasture-associated forms of 'heaves' have the distinction of airway inflammation that is predominantly neutrophilic - a characteristic that is now increasingly recognized in more severe and chronic forms of asthma. Building upon our prior evidence that protein functions in normal lung fluids are conserved across horse, human, and mouse, this investigation identifies proteins in lung fluid from horses with pasture heaves that contribute to neutrophilic airway inflammation and identifies conservation of these protein signatures in asthma.

Shotgun proteomic analysis using 1D liquid chromatography nanospray ionization tandem mass spectrometry was employed to identify proteins in the cell-free bronchoalveolar lavage fluid (BALF) from 6 pasture heaves-affected and 6 nonaffected herdmates during seasonal clinical exacerbation. Peptides were identified and quantified using the SEQUEST and PROTQUANT algorithms, respectively. 1003 proteins were identified ($P<0.05$; 472 unique to nonaffected, 417 unique to affected, and 114 proteins common to both groups). The contributions of each protein to 13 neutrophil functions described in the Ingenuity Knowledge Base ® were modeled as the product of magnitude of expression multiplied by its effect (i.e. -1, 0, +1). When compared to proteins in the BALF of control horses, proteins in BALF from diseased favor airway neutrophilic inflammation by increasing migration, chemotaxis, adhesion, detachment, transmigration,

and degranulation, while reducing activation, cell spreading, infiltration, phagocytosis, respiratory burst, apoptosis, and clearance. This approach demonstrates the utility of systems modeling to organize functional genomic datasets in order to characterize complex molecular events associated with clinically relevant equine disease.

Key words: heaves, *Equus caballus*, horse, proteomics, neutrophil functional genomics Platforms such as microarrays; next-generation

Introduction

Human asthma has been traditionally regarded as an allergic airway disease mediated by Th2 cells and characterized by eosinophilic inflammation [1, 2]. However, it is now well recognized that airway eosinophilia is present in as few as 50% of asthmatic subjects [3] and that a sizeable subgroup of asthmatic patients have neutrophilic airway inflammation. Though neutrophilic asthma and eosinophilic asthma are not mutually exclusive, an association has been established between airway neutrophilic inflammation and increased severity of signs in severe asthma [4-6], corticosteroid resistant asthma [7-9], asthma exacerbations [10], nocturnal asthma [11], ‘‘asthma in smokers’’ [12], occupational asthma [13], and ‘‘sudden onset’’ fatal asthma [14].

Two naturally occurring environmentally induced asthma-like syndromes affect out bred populations of animals: equine heaves and feline asthma [15]. Two forms of equine heaves are recognized: a stable-associated form exacerbated by mold and dust when horses are maintained in stalls [16], and a summer pasture-associated form affecting grazing horses [17]. Airway inflammation in both barn-associated, and in pasture heaves (aka Recurrent Airway Obstruction, SPARAO), may be devoid of eosinophils, and is consistently and predominantly neutrophilic. [16, 18-21]. Other

features shared with human asthma include genetic linkages [19], reversible bronchoconstriction triggered by environmental aeroallergen challenge, airway remodeling, bronchial hyperresponsiveness, chronic airway inflammation and responsiveness to β_2-adrenoceptor agonists and corticosteroids. These clinical parallels have lead to the recognition of heaves as a unique spontaneous model of neutrophilic asthma [16, 18-21].

Neutrophilic inflammation has been shown to correlate to poor outcome in other chronic lung diseases in addition to asthma, including bronchiolitis obliterans in lung transplant recipients [22], and COPD [23] where the correlation is independent of viral or bacterial infection. Neutrophil elastase also correlates to declining lung function in cystic fibrosis [24]. Neutrophilic inflammation has been linked to worsening prognosis in other organs, for example, with intracranial hemorrhage [25], alcoholic hepatitis [26], myocardial infarction [27-31], pulmonary embolism [32], peripheral vascular disease following angioplasty, tumor recurrence in hepatocellular carcinoma [33, 34], colorectal tumors [35], and lung cancer [36]. Accordingly, identifying proteins that contribute to neutrophilic airway inflammation in horses with pasture heaves contributes to the identification of molecular signatures that herald advancing disease, and identifies targets for investigating directed therapies to moderate neutrophilic inflammation.

The concept has been advanced that a state of homeostasis within a network of eukaryotic proteins (proteostasis) "enables healthy cell and organismal development and aging, and protects against disease [37]." 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. An emergent area of research is the

relationship between airways stress diseases (ASDs), including chronic obstructive pulmonary disease (COPD), emphysema and asthma, and disorders of proteostasis [38]. Congruent with the concept of disordered proteostasis, we hypothesize that changes in the protein composition of fluid in the airways of horses with pasture heaves alter functional attributes of neutrophils in ways that contribute to airway neutrophil accumulation that characterizes pasture heaves.

Identifying how changes in the BALF proteome associated with disease contribute to increased airway neutrophilic inflammation enables recognition of shared regulatory events in asthma and pasture heaves, and also presents the ability to inform understanding of the similar roles of neutrophils in human asthma and pasture heaves. Here we employ systems modeling of the BAL proteome of horses experiencing exacerbation of summer heaves, and that of asymptomatic controls exposed to identical aeroallergen challenge, to evaluate differences in the regulation of thirteen neutrophil functions: migration, activation, chemotaxis, adhesion, cell spreading, detachment, transmigration, infiltration, phagocytosis, respiratory burst, degranulation, apoptosis, and clearance.

Methods

Cell free bronchoalveolar lavage fluid (BALF) samples:

BALF was collected from six horses with pasture heaves during clinical disease exacerbation, and six clinically normal control horses by manual aspiration as has been previously described [39, 40]. Samples were placed immediately on ice, transported to the laboratory, centrifuged (N x g for 10 minutes) to derive cell free BALF, and aliquots of supernate were frozen at -80°C for subsequent proteomic analysis.

Protein isolation, tryptic digestion, and LC MS/MS

Composite samples containing 100 ug of cell free BALF protein from the 6 control and 6 pasture heaves affected horses were created. Aliquots containing 75 ug of protein were subjected to proteomic analysis using one dimensional liquid chromatography (1D LC) nanospray ionization in triplicate as described [41] 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^{TM} (Bioworks Browser 3.3; ThermoElectron). Mass spectra and tandem mass spectra were searched against an *in silico* trypsin-digested database of equine nonredundant RefSeq proteins downloaded from the National Center for Biotechnology Institute.

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 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 [42].

GO-based quantitative modeling: effects of BALF proteins on neutrophil function

Systems modeling was performed to test the hypothesis that proteins in the airways of horses with pasture heaves have the potential to modify neutrophil functions

in a manner that contributes to neutrophilic airway inflammation. Gene ontology (GO) is the *de facto* method for assigning molecular function to proteins [43]. Accordingly, proteins identified in BALF from affected and control horses were characterized for their contributions to eleven Gene Ontology terms that describe neutrophil functions: migration, activation, chemotaxis, adhesion, cell spreading, detachment, transmigration, infiltration, phagocytosis, respiratory burst, and apoptosis. The effects of BALF proteins on degranulation and neutrophil clearance, functions of neutrophils in the Ingenuity Knowledge Base, but not in the Gene Ontology, were also evaluated. Proteins were individually biocurated according to the principles of the GO consortium [44] using manual literature searches in PubMed and with Ingenuity Pathway's Analysis® (IPA) software to identify their effects on the 13 neutrophil functions. Because IPA does not accept equine gene products for upload and analysis, the human, mouse, and rat orthologs were identified for each equine protein using Ensembl BioMart (Version 0.7, Ensembl 59 set) [45] and uploaded to IPA. Peer reviewed manuscripts identified from both the IPA and PubMed were read and the effect of the protein on each of the thirteen neutrophil functions was scored as pro (+1), anti (-1), no effect (0), or no data. The potential of each protein to modify each of the thirteen neutrophil functions was approximated as the product of magnitude of expression multiplied by its effect (i.e. -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 [46]. These net effects are represented graphically for pasture heaves and control horses in Figure 5.1.

Results

Neutrophil relevant BAL proteome

1003 total proteins were identified in BALF from diseased and control horses: 417 proteins were unique to diseased BALF, 472 proteins were unique to control BALF, and 114 proteins were identified in BALF from diseased and control horses. 108 proteins in BALF had sufficient criteria to allow statistical determination of significance to differential expression between diseased and control groups. 64 of these 108 proteins were differentially expressed between diseased and control groups (Supplemental File 4 and 5), and 44 had no difference in expression (Supplemental File 6). Of the 64 proteins with significant differences in differential expression between groups, 12 proteins were unique to diseased BALF, 27 proteins were unique to control BALF, and 26 were identified in both diseased and control BALF. 5 of these 26 proteins were significantly over-expressed in diseased BALF. The remaining 21 proteins had significantly greater expression in control BALF. Accordingly, 17 proteins had significantly greater expression in diseased BALF (Supplemental File 4) and 48 proteins had significantly greater expression in control BALF (Supplemental File 5).

Evidence of biological activity in one or more of the thirteen neutrophil functions was identified for 106 of the 1003 identified proteins. BALF proteins from horses with pasture heaves and control horses are summarized according to their ability to impact neutrophil functions in (Supplemental File 7). 34 of these 106 proteins were unique to diseased BALF, 48 were unique to control BALF and 24 were identified in both diseased and control BALF. 27 of the 106 proteins known to modify neutrophil function were significantly differentially expressed between control and diseased BALF. 4 were unique

to diseased BALF, 11 were unique to control BALF, and 12 proteins identified in both diseased and control BALF had significantly greater expression in control BALF. Accordingly, 4 proteins modifying neutrophil function had significantly greater expression in diseased BALF and 23 proteins had significantly greater expression in control BALF (Supplemental 7).

Bioinformatic modeling: effects of BALF proteins on neutrophil function

Expression modeling of diseased and control BALF proteomes (Figure 5.1) demonstrates a net increase in proteins that induce neutrophil migration, chemotaxis, adhesion, detachment, transmigration, and degranulation in the BALF of diseased relative to control horses. This reflects a net negative regulation of migration, chemotaxis, adhesion, detachment, transmigration, and degranulation by proteins in control BALF, with proteins in diseased BALF facilitating a partial release of the net negative regulation of these neutrophil functions. The release of negative regulation by the diseased BALF proteome was negligible for neutrophil detachment and degranulation relative to other neutrophil functions. By contrast, a net decrease in proteins responsible for neutrophil activation, cell spreading, phagocytosis, apoptosis, and clearance was identified in the BALF of diseased relative to control horses. This reflects a net positive regulation of activation, cell spreading, phagocytosis, apoptosis, and clearance by proteins in control BALF, with proteins in diseased BALF facilitating partial decrease in the net positive modulation that characterizes homostasis. Respiratory burst is a specific facet of neutrophil activation that was neutralized by proteins in the BALF proteome of both controls and diseased horses. Neutrophil infiltration was positively regulated by proteins in control BALF, an effect reversed by the diseased BALF proteome.

Discussion

Though at least 50% of human asthmatics demonstrate neutrophilic inflammation in BALF, which correlates to increased clinical severity and resistance to corticosteroid therapy [3, 47, 48], regulatory mechanisms responsible for neutrophilic asthma are poorly characterized. Complicating this effort is the difficulty in attaining purified populations of airway neutrophils, resulting in a predominance of investigations that employ peripheral blood neutrophils, for which there is growing evidence that these cells do not behave in manners that mirror their airway counterparts [49, 50]. Deciphering regulatory mechanisms associated with airway neutrophilia and the major protein effectors responsible for these changes has the potential to identify molecular events that herald advancing airway 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 microarrays, proteomics, and transcriptomics, into a better understanding of complex biological phenomena.

In this work, systems modeling of the BALF proteome from horses with summer heaves was used to provide insight into the molecular events that contribute to neutrophilic airway inflammation, a characteristic of both heaves and asthma that is not addressed by rodent models. Relative to the control BALF proteome, proteins in BALF from summer heaves-affected horses increase neutrophil migration, chemotaxis, adhesion, and transmigration, and to a lesser extent, also increase detachment and degranulation. An increase in neutrophil migration, chemotaxis, transmigration, and detachment clearly favor the movement of neutrophils into the airways, congruent with airway neutrophilic inflammation that is characteristic of pasture heaves. Increased

neutrophil migration [51], chemotaxis [52, 53], adhesion [54], and degranulation have previously been reported in asthma, with degranulation increases corresponding to disease severity [55]. Our findings are also consistent with increased neutrophil migration [56] and chemotaxis [57] reported in barn associated heaves. Despite expanding knowledge of how individual proteins modify neutrophil movement in asthma, changes in neutrophil detachment and transmigration that occur during neutrophilic forms of asthma are not documented. Rather, increased airway neutrophils are considered a priori evidence of increased neutrophil detachment and transmigration [14, 58, 59]. Increased transmigration of neutrophils into the airway is a feature of several inflammatory diseases, including bronchitis and chronic obstructive pulmonary disease [58].

Proteins in the BALF proteome of diseased horses decreased neutrophil activation, spreading, infiltration, phagocytosis, apoptosis, and clearance. Similar decreases in neutrophil phagocytosis, clearance, and apoptosis are identified in asthmatic patients [60-63], and decreased apoptosis of airway neutrophils [64, 65] has been identified in horses with the barn-associated form of heaves. Decreased apoptosis contributes to airway neutrophilic inflammation that characterizes asthma and RAO by increasing neutrophil persistence in the airway. Similarly, neutrophil activation and phagocytosis program neutrophils for death [66-68], such that decreasing these activities contributes to neutrophil persistence and airway neutrophilic inflammation. We also identified an overall decrease in proteins that favor neutrophil clearance from the airways, again consistent as a contributing factor to airway neutrophilic inflammation [69].

While proteins in BALF of diseased horses decreased neutrophil activation, isolated measures of neutrophil activation are reported to increase in asthma [70-74]. However, neutrophil activation is a complex and multifaceted neutrophil function. Respiratory burst is a specific component of neutrophil activation. In this investigation, BALF proteins that increase and those that decrease respiratory burst yielded a net null effect on this neutrophil function in both control and diseased horses. Literature demonstrates that respiratory burst of blood derived neutrophils from asthmatics exceeds nonasthmatics [49, 75, 76]. 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 incubations periods of 25 minutes [49]. Further, the respiratory burst of *airway* neutrophils, which is congruent with our model, has been poorly characterized. Lacy et al. (2003) identified decreased respiratory burst activity in neutrophils from induced sputum of asthmatics, relative to controls [50]. However, this finding must be considered with the knowledge that beta agonist and corticosteroid therapy were not withheld from the asthmatic group. Despite evidence that selected measures of neutrophil activation are increased in asthma [70-74], our modeling indicates that the proteome favors decreased neutrophil activation which would be fully consistent with neutrophil persistence in the airways and the airway neutrophilic inflammation that characterizes pasture heaves.

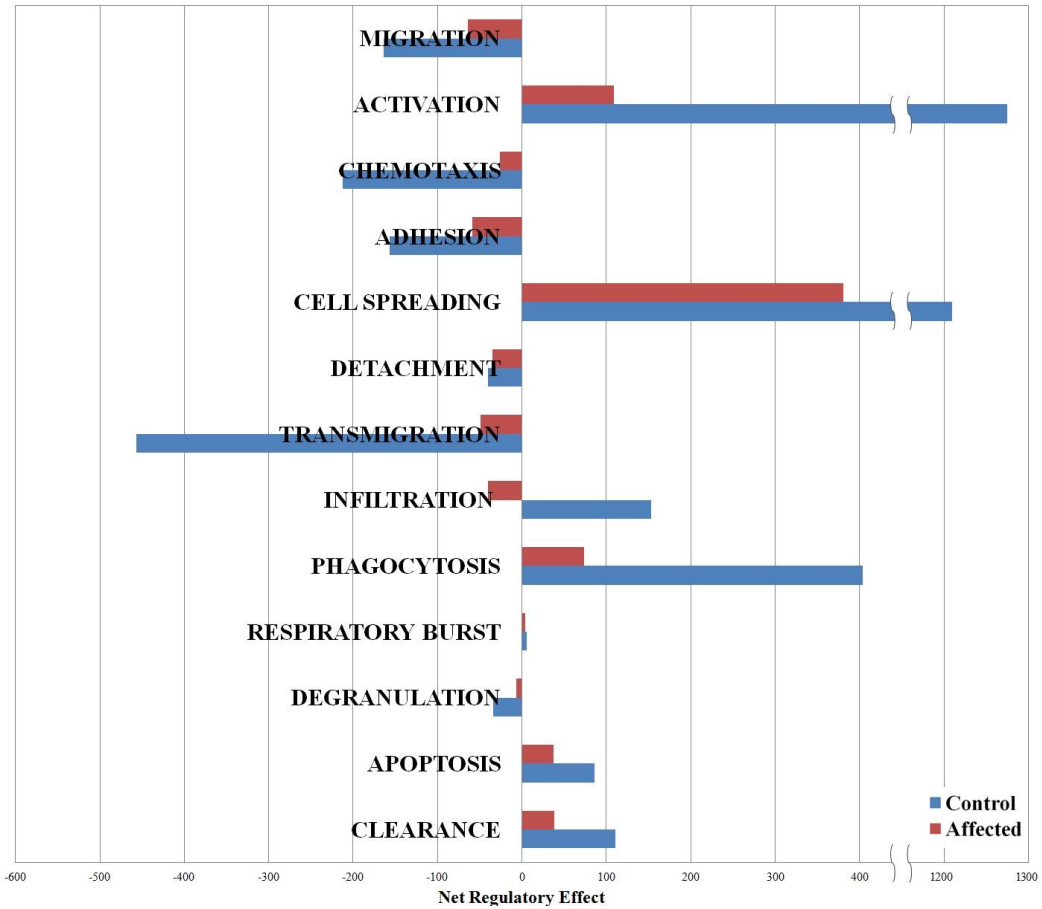

Figure 5.1 Effect of BALF proteins on neutrophil function.

Effects of proteins from pasture heaves-affected and control horses on neutrophil functions are scored as pro (+1), anti (-1), no effect (0), or no data. The potential of each protein to modify each of the neutrophil functions was approximated as the product of magnitude of expression multiplied by its effect (i.e. -1, 0, +1) and the net regulatory effect of BALF proteins within each functional category was tabulated.

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APPENDIX A

SUPPLEMENTAL FILE INFORMATION

The following supplemental files were created using Microsoft Office Excel 2007 and saved as PDF files. One exception is Supplemental File 2, which was created using Microsoft Office Word 2007. The PDF files were compressed into a .rar file using WinRAR. They can be accessed on the Mississippi State University Electronic Thesis and Dissertation Database, available at http://sun.library.msstate.edu/ETD-db/. Once downloaded, the files can be extracted using an unzipping program such as WinRAR or WinZip. Files can be viewed using Adobe Reader software or other software for viewing PDF files.

Supplemental File 1: Example of ArrayIDer Output

To facilitate linking array data to information in multiple public databases, ArrayIDer retrieves structural annotations for array elements and provides corresponding identifiers used in public databases (including UniProtKB, Ensembl, RefSeq, IPI and UniGene). The identifiers are: probe name, horse gene ID, the public accession number, the Unigene ID, any gene symbols it has, the Entrez Gene ID, its RefSeq accession number, and its UniProtKB ID. This is only an example, and the rest of the equine array data from ArrayIDer will be made publicly available via *AgBase*.

Supplemental File 2: Detailed methods for Chapter IV

Detailed methods for Chapter IV: Functional Modelling of an Equine Bronchoalveolar Lavage Fluid Proteome Provides Experimental Confirmation and Functional Annotation of Equine Genome Sequences.

Supplemental File 3: List of Proteins Identified in Control Equine BALF

A list of all 582 unique proteins from the cell-free BALF of normal horses, and lists the 88 proteins that did not have orthologous proteins and were therefore not submitted for Ingenuity Pathways Analysis.

Supplemental File 4: List of Proteins with Significantly Increased Expression in Pasture Heaves BALF

A list of the 17 proteins with significantly greater expression in diseased BALF. RefSeq ID, gene symbol, protein description, peptide counts, and corrected P values are listed for each.

Supplemental File 5: List of Proteins with Significantly Increased Expression in Control BALF

A list of the 48 proteins with significantly greater expression in control BALF. RefSeq ID, gene symbol, protein description, peptide counts, and corrected P values are listed for each.

Supplemental File 6: List of Proteins with No Difference in Expression Between Pasture Heaves and Control BALF

A list of the 44 statistically relevant proteins with expression values but with no difference in expression between pasture heaves and control horse BALF. RefSeq ID, gene symbol, protein description, peptide counts, and corrected P values are listed for each.

Supplemental File 7: Proteins Influencing Neutrophil Function

106 BALF proteins from horses with pasture heaves and control horses are summarized according to their ability to impact neutrophil functions. 4 proteins

modifying neutrophil function had significantly greater expression in diseased BALF and 23 proteins had significantly greater expression in control BALF. The remaining 79 proteins had no significant difference in expression between disease and control BALF. RefSeq ID, gene symbol, protein description, peptide counts, and corrected P values are listed for each, as well as their involvement in the thirteen neutrophil categories.