5-4-2018

Funny Channel Signaling in Equine Airway Disease

Courtney Hunter

Funny channel signaling in equine airway disease

By

Courtney Hunter

A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Veterinary Medical Sciences
in the College of Veterinary Medicine

Mississippi State, Mississippi

May 2018

Funny channel signaling in equine airway disease

By

Courtney Hunter

Approved:

_____
Cyprianna E. Swiderski
(Major Professor)

_____
Avery James Cooley
(Committee Member)

_____
Lais Costa
(Committee Member)

_____
Bindu Nanduri
(Committee Member)

_____
Fiona McCarthy
(Committee Member)

_____
Shane C. Burgess
(Committee Member)

_____
Larry Hanson
(Graduate Coordinator)

_____
Kent H. Hoblet
Dean
College of Veterinary Medicine

Name: Courtney Hunter

Date of Degree: May 4, 2018

Institution: Mississippi State University

Major Field: Veterinary Medical Sciences

Major Professor: Dr. Cyprianna E. Swiderski

Title of Study: Funny channel signaling in equine airway disease

Pages in Study 137

Candidate for Degree of Doctor of Philosophy

Traditional animal models of severe asthma do not recapitulate defining asthma characteristics, including persistent airway hyper-responsiveness, and chronic neutrophilic inflammation. This is problematic because moderating airway hyper-responsiveness decreases asthma frequency and severity, making it a paramount pharmacological goal in asthma research. Employing a spontaneous equine asthma model (equine pasture asthma, EPA), we first confirmed reversible airway obstruction in eight diseased horses during asthma exacerbations in response to ß$_2$-adrenergic agonist stimulation. Next, non-specific airway hyper-responsiveness was confirmed using methacholine bronchoprovocation to identify the provocative concentration causing a 40% increase in baseline lung resistance ($PC_{40}R_L$)- a threshold similarly employed in evaluating human asthmatics unable to mount forced expiration. The $PC_{40}R_L$ of ten EPA horses was consistently ≤1mg/ml of methacholine, which is a cutoff that has been used to diagnose severe human asthma. Like non-asthmatic humans, ten control horses did not

respond to methacholine doses up to 16 mg/ml. Finally, persistence of AHR was documented during absence of seasonal aeroallergen triggers in five horses that were evaluated between 3 and 31 months following the initial methacholine bronchoprovocation.

This unique ability of EPA horses to model AHR attributes that are not addressed by other animal models points to the suitability of EPA horses to decipher the mechanistic basis of airway hyper-responsiveness. Building on knowledge that β_2-adrenergic receptor (AR) signaling is required to develop the asthma phenotype in a murine model, differentially expressed genes from serial lung biopsies of two EPA affected and two controls were filtered to identify genes that interact with the β_2-AR. Hyperpolarization Activated Cyclic Nucleotide-Gated Potassium Channel 4 (HCN4) was prioritized because of its interactions with the β_2-AR. Relative to control horses, HCN4 was constitutively expressed in airway smooth muscle of EPA horses during remission and increased during seasonal disease exacerbation. Agonism of airway contraction by HCN4 was proven using the specific HCN4 antagonist, ivabradine, which caused dose dependent decreases in carbachol induced contractile responses in both EPA and control bronchi *in vitro*. These findings highlight utility of EPA as a model of severe asthma and HCN4 as a mediator of airway contraction that warrants further investigation in severe human asthma.

DEDICATION

To all the boss females that have inspired me in my life, but particularly to two that have been there throughout the seven-year journey of completing this dissertation: my mother and Dr. Swiderski.

"Dreams are lovely. But they are just dreams. Fleeting, ephermal. Pretty. But dreams do not come true just because you dream them. It's hard work that makes things happen. It's hard work that creates change." -Shonda Rhimes

Thanks for not letting me just be a dreamer. Don't be dreamers ladies. Be do-ers.

ACKNOWLEDGEMENTS

This project was supported by the Agriculture and Food Research Initiative (AFRI) Animal Health Program competitive grant no. 2015-67016-23172 from the USDA - National Institute of Food and Agriculture, the MAFES Special Research Initiative, and the Mississippi State College of Veterinary Medicine Office of Graduate Studies. I would also like to acknowledge the many student workers and summer interns who helped over the seven-year span of this project. Additionally, I would like to thank Dr. Lauren Bright, Dr. Nisma Mujahid, Stephany Mays, Dr. Ayfer Akgul, Dr. Jacquelyn Bowser, Dr. Amy Lack, Cailtin Wenzel, and Santosh Kumar for all of their help throughout this journey.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

LITERATURE REVIEW

Introduction

Asthma is a chronic respiratory disease affecting 7.1 million children and 18.9 million adults throughout the United States (1). It is characterized by cyclic bouts of airway obstruction, with persistent airway hyper-responsiveness (AHR) and airway inflammation (2). "Asthma" is a Greek word meaning "short of breath" (3). The first written account of asthma is in Homer's Iliad that refers to a warrior who died following a battle with "asthma and perspiration" (4). The first time the term "asthma" appeared medically was during the time of Hippocrates (460-360 B.C.) (4). In the early 19th century, Henry Hyde Salter defined asthma as a "paroxysmal dyspnoea of a peculiar character, generally periodic with intervals and healthy respiration between the attacks," in his publication "On Asthma: Its Pathology and Treatment"(5). Despite its long history and world-wide prevalence, understanding of the pathophysiologic basis of asthma is incomplete.

This dissertation focuses on a naturally occurring animal model of asthma, Equine Pasture Asthma (EPA), and establishes its translational relevance to severe adult neutrophilic asthma. Building on shared clinical and pathologic features of EPA and severe human asthma previously documented by our laboratory (6, 7), the present work first demonstrates that airway hyper-responsiveness (AHR) is a hallmark of EPA,

confirming EPA's homology with a key diagnostic criterion of severe human asthma. Then, building on the strong translational unity of EPA and severe human asthma, a self-controlled transcriptomics approach was employed to identify a novel mechanistic basis of AHR. We hypothesize that this mechanistic basis of AHR is relevant to AHR in severe human asthma and demonstrate that alternate use of an approved human therapeutic agent moderates AHR in the EPA model.

Equine Pasture Asthma (EPA)

EPA is an inflammatory small airway disease that is a part of the broader range of respiratory conditions now termed "equine asthma" (8). Equine asthma ranges from a mild-moderate condition, previously referred to as inflammatory airway disease (IAD), to a severe condition, which is referred to as severe equine asthma (SEA) that includes a pasture associated form (EPA) and an indoor or barn associated form "barn-dust asthma" (BDA), previously referred to as Recurrent Airway Obstruction or RAO) (8). The term equine asthma has only recently been adopted, despite the fact that these conditions have been described for several decades. Over time these conditions affecting the airways of horses have been referred to by different names. For instance, BDA has been previously referred to as Recurrent Airway Obstruction or (RAO), heaves, and equine chronic obstructive pulmonary disease (COPD) (8). Similarly, EPA has been previously referred to as summer-pasture associated RAO (SPARAO) and summer-pasture associated obstructive pulmonary disease (SPAOPD) (9, 10). The change in nomenclature over the years has been in efforts to avoid comparisons with similar, yet different human diseases. For example, the

term ‘chronic obstructive pulmonary disease’ or ‘COPD’ was commonly used to name equine conditions during the 1980s and 1990s, but this was discontinued to acknowledge the many differences from the human COPD, for instance, emphysema is not a component of the equine conditions, but it is a hallmark of human COPD. Due to recent changes in the equine nomenclature to characterize the disease more appropriately, EPA will be used for the duration of this work.

BDA has been estimated to affect roughly 14% of horses housed in temperate climates (11). It is a disease of mature, often middle aged, horses that have been found to be responsive to bronchodilators or a change in environment (12). EPA is typically seen in horses seven years of age and older. Both BDA and EPA share many similarities with respect of clinical signs, which are characterized by coughing, reluctance to exercise, labored breathing, and wheezing. These signs improve in response to bronchodilators and decreasing environmental antigen exposure (8, 13). In contrast, mild-moderate equine asthma affects younger horses, and is characterized by horses that appear clinically normal at rest, but exhibit exercise intolerance and exercise induced coughing. (8).

Unlike the similar phenotype of BDA, EPA is associated with a history of exposure to increased fungi and pollen counts while being kept on pasture during months of increased ambient temperature (9, 10, 13). BDA (also previously called COPD or ‘heaves’) is triggered by inhalation of dust particles and molds while in barns, especially particles associated with feeding moldy hay (14–16). The change in nomenclature over the years has been in efforts to avoid comparisons with similar, yet different human diseases. For example, the term ‘chronic obstructive pulmonary

disease' or 'COPD' was commonly used to name these conditions in the 1980s and 1990s, but was discontinued to acknowledge the differences (i.e. causes, variations in severity, and clinical presentation) between the human disease of the same name when compared to the equine disease (12, 14). In COPD the airway obstruction is not reversible while asthma is specifically defined as a condition in which airway obstruction is reversible. Generally, the previously mentioned conditions characterizing EPA are observed in the summer months, hence the previous nomenclature of EPA, summer pasture associated recurrent airway obstruction (SPARAO). However, due to recent changes in the equine nomenclature to characterize the disease more appropriately, EPA will be used for the duration of this work.

Unlike BDA, which is a disease associated with domestication (14), EPA is caused by antigens that are naturally occurring in the horse's environment. EPA is spontaneous and naturally occurring in a region of the United States seeing a steady increase in asthma prevalence (i.e. Mississippi, Alabama, Tennessee) (17) and in the state of Mississippi which the capital (Jackson, MS) has recently been named number one for spring allergies (18). The following sections discuss specific aspects of EPA, make comparisons between EPA and the barn-dust form of equine asthma, and also highlight similarities between these two equine diseases and human asthma.

Diagnosis of EPA

Diagnosis of EPA begins with a thorough history of the patient's clinical signs. Affected horses are adults, usually over seven years or age, that are housed on pasture when signs are noted (13, 19). No breed or sex predilection has been identified (20, 21). Owners may note an improvement in clinical signs when the animal is taken off pasture and moved into a barn, which is attributed to a decreased exposure to the inciting environmental triggers. By contrast, animals affected with BDA have historically been kept indoors, where they are exposed to barn dust and various hay molds.

Signs noted by owners may include coughing, increased respiratory effort and respiratory rate (tachypnea), exercise intolerance, nasal discharge, and nostril flare (13, 22). Physical exam findings may include increased lung sounds and wheezes, which are classically end expiratory, an increased contraction of the external abdominal oblique muscles (known as a 'heave line'), and increased tracheal sounds. Increased respiratory effort, as determined by the formula (medial nostril flare score + lateral nostril flare score)/2 + abdominal lift score, has been demonstrated to correlate to increased intrathoracic pressures (i.e. maximal change in pleural pressure, ΔPpl_{max}) that drive respiration (22, 23). Cytology results in SEA, often from bronchoalveolar lavage fluid (BALF), must include a predominance and increase in neutrophils above normal cell counts (22). Cytologic findings of horses with mild-moderate asthma, by contrast, are mildly neutrophilic and have monocytosis and lymphocytosis (24).

If the patient is compliant, non-specific bronchoprovocation testing may be performed for a definitive diagnosis (25). In this testing, horses are exposed to a non-specific airway irritant, most commonly methacholine or histamine, in order to identify increased

sensitivity that is characterized by airway constriction in response to irritant doses that do not cause airway constriction in normal horses. Increase in maximum pleural pressure (ΔPpl_{max}), increase in lung resistance (R_L), and decrease in dynamic compliance (C_{dyn}) of the lung occur in affected horses in response to serially increasing doses of irritant (24). Horses affected with EPA should be hyper-responsive to inhaled constricting agents compared with clinically normal horses (7). In humans with asthma, bronchoprovocation is not performed in patients with less then 30% of their predicted expiratory volume (27) and should also not be performed in horses experiencing clinical asthma exacerbation. In such cases, conventional pulmonary function testing has been shown to yield $\Delta Ppl_{max} \geq 6$ mm Hg (25).

Finally, EPA affected horses respond well to removal from the inciting agents in pasture. Once stalled, these horses generally show marked respiratory improvement. Bronchodilators of the ß$_2$-adrenergic receptor agonist class of therapeutics (i.e. β_2-AR agonists: albuterol, levalbuterol) are typically used to initially reverse airway obstruction. Buscapam, a parsympatholytic agent, has also been demonstrated to effect bronchodilation in horses with SEA (28). The reversibility of airway obstruction (ie bronchodilation) in response to therapeutic intervention is a key criterion for asthma diagnosis. (29, 30). In addition to clinical improvement in dyspnea as evidence of bronchodilation, reversibility can also be documented using conventional pulmonary mechanics with decreases in ΔPpl_{max} and R_L accompanied by an increase in C_{dyn} being indicative of a bronchodilatory response to therapy. Oxygen insufflation may be needed in cases of prolonged hypoxemia (7). In the barn dust form of disease, soaking hay helps to reduce the effect of mold exacerbating disease. Many times, a decrease of clinical

signs following removal from pasture or from hay and bedding, is a good diagnostic indicator of the reversible airway obstruction component of disease (31).

Airway hyper-responsiveness in SEA

Airway hyper-responsiveness (AHR) is a state in which airways react to an airway spasmogen to a higher degree than they normally should. AHR was first associated with asthma by Dr. Szentivanyi in 1968, who suspected an association with an abnormality in the β-adrenergic system (32, 33). In asthma, AHR is characterized by persistence at a baseline level. While the etiology of this persistence is incompletely characterized, airway smooth muscle (ASM) from asthmatics has been demonstrated to be hyper-contractile, even in the absence of inflammation in primary cell culture (34–36). Lessons from *in vitro* models, which have been questioned because they do not successfully recapitulate the persistence of AHR that is characteristic of the asthma phenotype, have suggested roles for airway remodeling from chronic inflammation a well as acute, variable AHR from episodes of inflammatory increases at times of antigen exposure (37).

AHR is a key characteristic in the common respiratory conditions SEA and mild-moderate equine asthma (38). While ASM is considered to be the primary cell type responsible for AHR (39), differences in ASM contractile function, cell migration, proliferation, and secretion of pro-inflammatory cytokines from ASM cells have all been suggested to play a role in AHR (40). Smooth muscle contraction from asthmatics is greater than that of non-asthmatics (36). In human asthma, increase in contraction has been suggested to be due to increased ASM mass (41), with the increased thickness allowing increased tension forces. However, peripheral airway smooth muscle has also

been shown to have increased shortening velocity in horses with BDA relative to non-diseased controls, indicating that the phenomenon of increased contractility does not require increased ASM mass (42). Earlier studies had questioned if this is a universal truth, however (43). Still, greater increases in ASM have been associated with severity of asthma (44). Increases in ASM have also been found in horses with BDA (45) and EPA (6, 46).

The autonomic nervous system innervates airway smooth muscle. Through parasympathetic input, postganglionic nerves release acetylcholine that stimulates muscarinic receptors, leading to bronchoconstriction and mucus secretion. Cholinergic M3 receptor signaling is considered the principal mode of neuronally mediated bronchoconstriction in the human (47) and equine lung (48). Contraction of airway smooth muscle (ASM) surrounding conducting airways in the asthmatic lung contributes to shortness of breath that can be fatal. This contraction can also be induced by release of histamine and cysteinal leukotrienes of mast cells and basophils (49), to which asthmatics have been demonstrated to be hyper-responsive. In this investigation, responses to inhaled allergens were compared between asthmatics and people with a similar severe cutaneous immune response. Though both groups released similar amounts of histamines and leukotrienes (bronchoconstrictors) in airways, only asthmatics demonstrated exaggerated airway narrowing (49).

Inhibition of parasympathetic nervous system muscarinic receptor mediated bronchoconstriction is provided by parasympathetic M2 receptor signaling and by post-junctional non-adrenergic non-cholinergic (NANC) nerves (50). The M2 receptor is the most abundant in equine peripheral lung tissue (51). M2 muscarinic receptors on

parasympathetic nerves inhibit parasympathetic signaling by limiting further acetylcholine release and thus provide a negative feedback that limits neurotransmission and bronchoconstriction in diverse species (47, 52, 53). In addition, the M2 receptor can also inhibit β_2-AR relaxation of ASM by inhibiting adenylate cyclase (54). Simultaneous inhibition of both M2 and M3 receptors does not alter cholinergic activity in BDA airways (55). Both M2 receptor dysfunction (56–58) and lack of normal inhibition provided by non-adrenergic non-cholinergic (NANC) nerves at distal airways (31,32) have been implicated in the pathogenesis of AHR in horses with BDA.

Sutcliffe et al. (34) found that oxidative stress of ASM in asthmatics contributes to the degree of AHR. An overexpression of NOX4 in ASM of asthmatics has been suggested to cause oxidative stress and consequently lead to airway hyper-contractility. Another study found an increase in smooth muscle myosin light chain kinase (smMLCK) in the bronchial smooth muscle of asthmatics (35), but this has not been a consistent finding (36). Increased smMLCK would cause more bridging of myosin and actin chains (36). This would cause a greater shortening as demonstrated in dogs (59) and humans (60). The increased shortening would result in more luminal narrowing.

The heritability of AHR has been reported to be approximately 30% (61). In mice, hyper-reactivity to acetylcholine and serotonin have been demonstrated to be inherited in an autosomal recessive manner, with linkage analysis demonstrating that the associated genes were not closely linked and indicating that AHR in mice is a polygenic trait (62, 63). Congruent with this complexity, another study of house dust mite sensitized mice demonstrated epigenetically modulated transforming growth factor beta as associated

with AHR (62). RNA from bronchial biopsy identified 4 genes not only associated with asthma, but also with AHR: RPTOR, VANGL1, FAM129A, and LEPREL1 (64).

In summary AHR persists at a baseline level in human asthma. While the etiology of this persistence is incompletely characterized, ASM from asthmatics has been demonstrated to be hyper-contractile, even in the absence of inflammation in primary cell culture (34–36). Lessons from in vitro models (which have been questioned because they do not successfully recapitulate the persistence of AHR) have suggested roles for airway remodeling. These include chronic inflammation as well as acute, variable AHR from episodic increases in airway inflammation at times of antigen exposure (37). Seasonal increases of AHR have been found in allergic asthmatics (65). However, caution must be used when faced with a negative bronchial challenge (66). A negative challenge does not exclude asthma but must be considered with the presence or absence of clinical signs. Chapman and Irvin (66) suggest that in patients with a history of asthma-like signs, the term "currently negative AHR" may be more appropriate. While clearly AHR is complex, and the understanding of its pathogenesis is poorly refined, demonstrated relationships between magnitude of AHR and asthma severity warrant focused research efforts to decipher the mechanistic basis of AHR in order to moderate asthma.

Pulmonary Function Testing in Horses

Methods for pulmonary function testing that have been most employed in horses include conventional pulmonary mechanics, open plethysmography, and oscillometry (forced and impulse). Clinically, pulmonary function testing is indicated in equine patients with non-responsive cough, abnormal chest auscultation, exercise intolerance, increased mucus, and tachypnea that is not referrable to pneumonia (67). While these

tests reflect the magnitude of airway obstruction at the time of testing, a limit of such testing is that isolated measurements of airway obstruction do not necessarily correlate to longitudinal measures of asthma severity- including asthma frequency and the magnitude of asthma attacks over protracted periods. In human asthma, strong correlations between increasing sensitivity to bronchoprovocation with methacholine and longitudinal measures of asthma frequency and severity of attacks have been identified, leading to the common employment of methacholine bronchoprovocation in human asthma subjects in order to gauge longitudinal measures of asthma severity (27).

Conventional pulmonary mechanics is the gold standard for diagnosing airway obstruction in horses (9, 37, 41). Increases in intrapleural pressure were first identified in horses with respiratory illness in the 1960s using conventional pulmonary mechanics (68). Measurements of airway obstruction derived from conventional pulmonary mechanics in horses typically focus on three parameters: maximum change in intrapleural pressure (ΔPpl_{max}), total lung resistance (R_L), and dynamic compliance (C_{dyn}) (35). These measurements are calculated from input signals that quantify changes in intrapleural pressure, air flow, and air volume, dynamically, throughout the breathing cycle. Intrapleural pressure is derived from an esophageal balloon catheter that is placed via the nose, into the thoracic esophagus. Air flow is measured using a pneumotachograph which in horses is attached to an airtight mask that covers the horse's muzzle. Volume is derived from the integral of the flow signal.

The maximal change in intrapleural pressure (ΔPpl_{max}) refers to the difference in intrapleural pressue between maximal inspiration and maximal expiration. Total lung resistance (R_L) reflects friction of air against airway walls (67) and is calculated by

dividing the change in transpulmonary pressure by the associated change in airflow between isovolumetric points (69). Because the transpulmonary pressure (i.e. atmospheric pressure-alveolar pressure) cannot be directly measured, the measured intrapleural pressure difference serves as its surrogate. C_{dyn} is a measure of lung elastance defined as the ratio of the tidal volume to the difference in intrapleural pressure between zero airflow relating to beginning of inspiration and expiration (70). As airways constrict, R_L increases and C_{dyn} decreases. C_{dyn} can be affected by both obstruction and elasticity.

A drawback of conventional mechanics is its dependence on spontaneous respiratory frequency which slows acquisition with low respiratory rates (35). Respiratory frequency has been shown to impact R_L in the dog model (42). However, conventional mechanics provides the unparalleled advantage of directly measuring parameters of airway obstruction.

Forced oscillometry (FOM) is a technique where the horse is exposed to sinusoidal, external air flow, generated by compressed air, via a facemask (71). The external source is intended to produce oscillations of pressure that are measured at the airway opening (67). FOM has been proven to be a realiable test of lung function in the horse and has the advantage of differentiating small airway contraction from that of larger airways (72, 73). As a non-invasive technique, it is more appropriate for less compliant animals.

Another method of pulmonary function testing called open plethysmography has been done in horses (74, 75). This method uses external sensors placed on the animal to take measurements of dynamic changes in the thoracic circumference that are then correlated to measurements of air flow (74, 76). In the horse, these measurements are made using thoracic inductance bands that are attached to each horse along with a fitted mask and

pneumotachograph that measures changes in airflow at the nostril. Therefore changes in flow at the airway opening and changes in body surface area are used to analyze respiratory function (67). Changes in flow (Δ_{flow}) are used to find the PC (change in flow signal found from the thoracic volume change at the peak of expiration) (74, 76). This method has been shown to produce reproducible results for up to a year with caution if ambient temperature is low (i.e. less than 5°C) (76). Plethysmography is also able to give information on the breathing pattern through the thoracic bands (67). Plethysmography may be closed (within a closed environment) or open. The open method is more suitable for large animals since restraining a horse in a small, closed environment has safety concerns. This method has been successfully combined with methacholine bronchoprovocation to identify airway hyper-responsiveness in horses with mild-moderate asthma (ie IAD) (77).

Bronchoprovocation

The pulmonary function methods previously addressed can be combined with bronchoprovocation to diagnose airway disease and to understand the mechanics of AHR. The American Thoracic Society has established methacholine challenge guidelines for diagnosing asthma that rely on giving set concentrations of an airway spasmogen to measure reactivity (78). The two-minute tidal breathing protocol is one of the most common bronchoprovocation methods. It involves administering doubling doses of methacholine after baseline pulmonary parameters have been acquired. The guidelines for testing adults and children differ, since children are considered "non-compliant" (79).

For example, in children the dose of methacholine causing a 40% increase in R_L from baseline defines the study end-point (79) and asthma severity (Table 1) (27).

Typically either provocative dose (PD) or provocative concentration (PC) are used to report study end-points. The PC or PD is the inhaled concentration or dose, respectively, that is required to reach a set change from a baseline measurement (79). PC is typically used for 2 minute tidal breathing methods, and PD is used for breath dosimeter methods (78). The American Thoracic Society recommends both and has specified the best doses of methacholine for each (27). However, in a study comparing accuracy of PC_{20} to PD_{20} using tidal breathing with nebulizers, PD_{20} was more consistent and recommended for use in methacholine challenges (80). Tidal breathing incorporates measuring the tidal expiratory flow and/or thoracoabdominal motion (79). The dosimeter method involves using about half the delivered dose with deep inhalations (81). A caveat of using PD is that the dose delivered to the airways can only be roughly estimated (78). While both tests are used frequently, the type of test and nebulizer used has been shown to impact the number of AHR positives (82). Using the dosimeter method has also been found to have 25% more false negatives in mild AHR compared to the tidal breathing method (81). However, when using the 2-minute tidal breathing method as done in Dell et al. (80), the use of PD is still preferred to PC.

Studies of Lung Function and Bronchoprovocation in Horses

Pulmonary function testing has been performed predominantly in horses with SEA. A few studies will be reviewed in this section and their contributions discussed. The effect of the β-adrenergic system on asthma, as first proposed by Szentivanyi (32) has been evaluated in BDA horses (83). Horses were given β-adrenergic antagonists and

agonists that were nonspecific, as well as those specific for β_1-adrenoceptors or β_2-adrenoceptors during clinical remission and clinical exacerbation. The antagonists decreased C_{dyn} and increased R_L during exacerbation of BDA affected horses, but there was no effect during clinical remission. Normal horses were not affected by the antagonists. Agonists were able to attenuate airway constriction while bronchodilation was specifically attributed to β_2-adrenergic receptor signaling.

In the late 1980s, Armstrong et al. performed bronchoprovocation in ponies with BDA using methacholine and citric acid aerosolization (26). Lung function was measured during clinical remission (2 months on pasture), after barn dust exposure, then 1 and 2 weeks after returning to pasture. The group found that BDA affected horses had the greatest difference in pulmonary function parameters (C_{dyn} and R_L) when tested after barn dust exposure. The dose of methacholine causing a 65% decrease in C_{dyn} was lower for BDA affected ponies than control ponies. Also, the change in R_L and percent change in C_{dyn} caused by 0.1mg/ml methacholine was higher in affected ponies compared to controls. BDA affected horses did not maintain these parameter differences to the same degree when in the period of clinical remission. However, C_{dyn} was decreased and R_L was increased in BDA affected ponies versus controls during remission. Once horses were on pasture 1 and 2 weeks post barn dust exposure, C_{dyn} and R_L returned to similar levels as in clinical remission, indicating improvement in airway obstruction when the inciting environmental triggers were removed.

Some things to note about this study are: 1) that the horses were sedated, and the effect of sedation on lung parameters has been debated (84–86), 2) measurements were obtained via an esophageal balloon passed through the nares, 3) horses were force

ventillated before study, and 4) study end-point was when C_{dyn} reached 50% below initial saline challenge. Using this endpoint 0.1mg/ml was the maximum dose of methacholine administered.

In another study by Scott et al., a similar lack of AHR during clinical remission was observed for BDA affected horses (87). Unlike the previously mentioned study, these horses were not sedated during testing. Again, an esophageal balloon was passed through the nares and attached to a pressure transducer. Histamine nebulization was done until C_{dyn} decreased to 50% below baseline. The Scott et al. study also used a change in R_L from baseline to the 0.1mg/ml histamine dose as a measure of AHR (87). Before these experiments in 1973, measurement of changes in lung function of BDA horses was performed via a catheter inserted through a skin incision in the 16th intercostal space (70). The catheter was inserted through the parietal pleura into the pleural cavity. A manometer was attached to record intrapleural pressure measurements. Again, a decrease in C_{dyn} was observed in horses with BDA compared to normal horses along with greater work of breathing.

By testing affected horses with absences of clinical signs, two studies of pulmonary function testing showed the horses with BDA were still hyper-reactive (69, 88). In one of these studies measurements were made by using an esophageal catheter placed into the nares to the 11th intercostal space (69). Using a face mask and pneumotachograph, a single dose of histamine (62.5mg) was nebulized for the experiment. In this study, one BDA affected horse was tested that lacked evidence of respiratory disterss and had normal pulmonary function at rest. This horse showed significant hyper-reactivity to histamine characterized by decreases in C_{dyn} , increases in (ΔPpl_{max}, and increases in R_L.

In the second study (88), BDA horses in remission were compared with healthy horses using both impulse oscillometry and the traditional esophageal balloon method. Impulse oscillometry (IOS) is a non-invasive system that generates a short, pulse signal at a high frequency to determine respiratory impedance in a manner akin to forced oscillometry which has been previously discussed (88). While this study first found that IOS measurements were more sensitive than the esophageal balloon method, it also showed that BDA horses could still be hyper-reactive while in clinical remission. A study by Fairburn et al. (89) found that BDA horses still have AHR up to 3 days post allergen challenge while in clinical remission. To this author's knowledge, no study has yet to evaluate the persistence of AHR in the EPA form of SEA, which is an objective of this dissertation research.

Other Features of Asthma

There are three key defining features of asthma: reversible airway obstruction, chronic airway inflammation, and persistent AHR. (2, 90, 91). Since AHR in SEA is the focus of this dissertation, it has been previously discussed at length. Other important features of asthma are discussed briefly here. Airway obstruction during asthma occurs from airway narrowing due to bronchoconstriction (92). Other changes including mucus accumulation and airway remodeling contribute to obstruction (92–94). The key in asthma is that this obstruction is reversible, meaning that bronchodilation reverses the asthmatic episode. Classically, reversibility is confirmed with the administration of an inhaled $ß_2$-agonist or a parasympatholytic agent.

Prevalence of Human Asthma

According to the CDCs Morbidity and Mortality Weekly reports, the prevalence of asthma has been increasing since 1960 (95–97). As of 2011, there were an estimated 39.5 million people affected with asthma in the United States (98). 1 in 12 adults are affected with asthma, with estimates of increasing asthma prevalence in adults between 7.3% and 15% over the years 2001-2009 (17, 99). Asthma prevalence increased significantly from 2001-2009 in 22 states, one of which was Mississippi (17). Other southeastern states with a similar increase in asthma prevalence include Alabama, Tennessee, South Carolina, and North Carolina (17), which is of particular interest to this work as they constitute a region of the country in which EPA cases are concentrated. Asthma costs for the United States in 2007 were estimated to be $56 billion due to medical expenses, loss of productivity, and premature death (100). Productivity losses such as those from morbidity that includes lost work and school days made up 8%-12% of the total costs from 2002-2007 (100).

Certain populations are more likely to suffer from asthma. African-Americans are more likely to be affected with asthma than Caucasians (95). In 2009 asthma was more prevalent among females, non-Hispanic African-Americans, Puerto Ricans, those with lower incomes, people living in the Northeast and Midwest, and children (101). Asthma has also been shown to be higher among inner city children than other US populations, with a large proportion of children reporting asthma-like symptoms with no actual diagnosis (102). Similar to asthma seen in urban environments, which to some extent are caused by house dust (103, 104), BDA is caused by exposure to dust particles in stables

(14). These geographic and allergen similarities show important reasons to use SEA as a translational model for human asthma.

Diagnosis of Human Asthma

A more in-depth review of diagnostic equipment and protocols was discussed previously. A few points of relevance to the diagnosis of human asthma will be discussed here. Pulmonologists commonly employ spirometry (2) or impulse oscillometry (IOS) (105) in the diagnosis of human asthma. Spirometry assesses lung function by measuring how much air is inhaled, exhaled, and the timing of exhalation (106). IOS is a forced oscillation technique (FOT) requiring the patient to passively breathe while small pressure oscillations are delivered through a mouthpiece (107). The National Asthma Education and Prevention Program suggests diagnosis by a thorough patient history to confirm recurrent episodes of reversible airway obstruction, a physical exam focusing on abnormal sounds of the lungs, and/or spirometry (2). As previously indicated, the magnitude of AHR is also quantified using methacholine bronchoprovocation in human asthmatics as a means to characterize asthma severity (27). The American Thoracic Society has indicated that severe human asthma is characterized by a 20% decrease in forced expiratory volume in one minute by methacholine doses < 1 mg/ml. Methacholine bronchoprovocation thresholds, clinical signs, and symptoms, useful for the diagnosis of asthma are found in Tables 1 and 2.

Asthma is a separate disease from chronic obstructive pulmonary disease (COPD), though roughly 20% of the population are diagnosed with both diseases (108). COPD, as the name suggests, is characterized by chronic airway obstruction that is not reversible (109). Reversibility is a key characteristic differentiating the two and must be addressed

diagnostically in order to direct proper treatment of the patient for COPD versus asthma (110, 111).

Once a diagnosis of asthma has been made, monitoring is generally considered to be the responsibility of the patient. This may involve self-reporting such as keeping a diary of attacks and symptoms, worsening of severity, response to therapy, or perhaps even analysis of lung function via pulmonary mechanical testing (78). Recently, a number of home monitoring devices have been made available to facilitate self-monitoring and include SmartPhone applications such as the Asthma Health App (http://apps.icahn.mssm.edu/asthma/). The CDC's National Asthma Program recommends each person receiving an asthma diagnosis be given a written action plan, that includes daily instructions to reduce the chances of adverse events (1). Long term asthma therapy aims to prevent triggers that cause difficulty breathing, moving to less frequent use of short acting beta agonists (SABA) for quick relief, and allowing patients to have a normal activity level with near normal pulmonary function (2). The difficulty with meeting each of these expectations lies in the still evolving molecular pathogenesis of asthma.

Airway Remodeling

Lung biopsy can aid in the diagnosis and characterization of severe asthma. While both large and small airways are involved in asthma (112–114), much of the current understanding of asthma has been derived from investigations of endoscopic biopsies derived from large non-cartilaginous airways. A combination of changes including thickening of the airway walls, smooth muscle cell hyperplasia and hypertrophy, fibrosis, mucous metaplasia, and epithelial hypertrophy all are included in the over-arching term

"airway remodeling" (49). Airway remodeling is the collective process of structural alterations in cells and tissues in asthmatic airways (115). Epithelial injury can include a loss of epithelial integrity, damage to the barrier function of epithelium via disruption of tight junctions, and cell death all leading to worsening asthma severity (116–118). However, damaged epithelial integrity has also been found to be no different from that of non-asthmatics in bronchial biopsy, suggesting it may not be a unique feature of disease (119).

One investigation of large airway biopsies from patients with severe human asthma demonstrated thickening of the basement membrane and fewer inflammatory cells in patients taking IC than those taking only β ·agonists (113). Cartilaginous large airways from cases of fatal asthma have also been identified to have greater total wall, inner wall, outer wall, and smooth muscle area in fatal asthmatics relative to non-fatal asthmatic and control cases (114). Similarly, the inner wall area of small non-cartilaginous airways has been demonstrated to be greater in fatal versus non-fatal cases and controls (114). A histologic scoring system was similarly developed recently and applied for the evaluation of endobronchial biopsies from horses with BDA (120).

Mucus accumulation plays a role in obstruction to gas movement through the airways. Changes include mucus production, mucin cross-linking, the degree of mucus gel hydration, and mucus clearance (49). These changes in mucus are not limited to severe asthmatics. Abnormalities in mucus production, storage, and clearance have been seen in mild and moderate asthmatics as well (119). In addition to mucus accumulation, ASM constriction plays a critical role in obstruction. ASM mediates bronchoconstriction, hyper-responsiveness, and impacts lung function (121).

Though large cartilaginous airways do contribute to asthma, modeling along with inert gas washout and high-resolution CT have determined that the majority of airflow obstruction during asthma occurs in small non-cartilaginous airways (122, 123). Despite this, the relative inaccessibility of these airways has limited their histologic characterization. By employing lung samples collected at necropsy, the histologic features of airway remodeling noted in human asthma were evaluated in peripheral non-cartilaginous airways from lung sections of EPA-affected and clinically normal horses (6). Diseased airways had increases in ASM, goblet cell hyperplasia/metaplasia, peribronchiolar fibrosis, airway obstruction by mucus/inflammation, elastosis, and airway adventitial inflammation, all of which, are also found in human asthma (125–128).

Video-assisted thorascopic surgery (VATS) has become the gold standard to evaluate the small non-cartilaginous airways of the distal lung in human patients (124). VATS is not necessarily commonly performed in people with severe asthma, but has been performed with a low incidence of post-surgical complications (124). In addition to providing information about the remodeling status of the distal lung, VATS samples have the potential to provide a wealth of research information as they are suitable not only for histologic examination, but also for immunohistochemistry and diverse "-omics" technologies including proteomics, transcriptomics, and metabolomics. In part reflecting the increased risk and difficulty in attaining VATS samples, they present a particular value in a biorepository form that could be beneficial in further aiding asthmatic research. VATS was also employed in this dissertation research for biopsy of the peripheral lung in order to evaluate the transcriptome of EPA horses and non-diseased controls during seasons of asthma exacerbations and remission.

Asthma Classification: Extrinsic versus Intrinsic

In 1949 Williams and Williams described the "asthma diathesis" that included a familial history of disease, a history of allergic manifestations in the individual, and abnormalities of the upper respiratory tract and bronchial tree as necessary for the development of asthma (129). This asthma classification centers upon asthma arising from a heritable predisposition to allergic disease which Rackeman described in 1947 as reactivity to extrinsic allergens, giving rise to a classification system in which allergic asthma is also termed 'extrinsic asthma' (130). Rackemann reasoned that the onset of extrinsic allergic asthma presented initially in individuals under 30 years of age (130).

Among the allergic conditions that constitute asthma, the best characterized and most common is atopic asthma, which reflects an inherited propensity of atopic individuals to produce IgE in response to certain environmental antigens (131, 132). Reflecting their increased IgE responses, atopic asthmatics also respond positively to skin tests of common allergens. Allergens identified with atopic asthma include, but are not limited to house dust mite, cockroach, molds, fungi, and animal dander (133, 134).

Because the prevalence of asthma has increased so significantly over the last few decades, and genetic factors require much more protracted time frames to increase asthma prevalence, environmental factors are postulated to be the most likely cause for the reported increases in asthma prevalence (135). However, environmental factors leading to asthma development are most likely to have a greater effect on individuals who are genetically susceptible to the disease (135). In a review of population-based studies evaluating family history of asthma and atopy, Burke at al. found that a family history of atopic disease is a risk factor for asthma if that atopic disease is asthma (136).

Using a phenotypic algorithm, the loci 6p21.31, 9p21.2, and 10q21.3 have been found in association with asthma in European Americans while the PTGES gene has been found linked with asthma in African Americans (137). Meanwhile many genes have been shown to occur with higher frequency in asthma populations. Interleukin 4-receptor (IL4R) has been associated with severe asthma via genome-wide association studies (GWAS) (138). Specifically, the allele IL4R-589T promotor polymorphism has been associated with sudden-fatal onset asthma, and the allele IL4RA-576R amino acid substitution is associated with severe airway obstruction (139).

An example of extrinsic asthma is occupational, or work-related asthma (WRA). It is defined as a reversible respiratory disorder caused by exposure to substances including mold, dust, chemicals, fragrances, etc. (140). WRA is estimated to cause roughly 15% of adult asthma (141), and is typically caused by a sensitizer, most commonly a high molecular weight agent, causing an increased IgE response (142). Agents known to be of particular risk include animal allergens, plants and plant products, cereals and grains, fungi, and insects (142). Cleaning agents and moldy environments are particularly important factors in development of WRA (141). The type of exposure (i.e. chemical vs. aerosol) can largely determine the severity of asthmatic response. In one example from the 1970s, employees at an electronics factory exposed to soldering reported asthma-like exacerbations (140, 143). In another example, fragrances have been demonstrated to increase WRA (144).

In contrast to extrinsic asthma, Rackemann observed that patients whose asthma began over 40 years of age were less likely to have allergic components to their asthma and failed to respond to antigen desensitization (130). Postulating that factors inside the

body were relevant in such asthmatics the term 'intrinsic asthma' was coined to address non-allergic asthma phenotypes. Today intrinsic asthma is considered to be characterized by lack of allergic propensity (145), lack of clearly Th2-mediated disease (146, 147), and an absence of positive skin testing reactions to common allergens (130, 145). Intrinsic asthmatics generally have more severe clinical disease (130). However, the extrinsic and intrinsic terminology has several challenges which have resulted in the replacement of these terminologies with allergic and non-allergic asthma (148) or atopic and non-atopic asthma (147).

Classification of Asthma: Eosinophilic versus Non-eosinophilic

Of particular relevance to this work is the designation of asthma phenotypes as eosinophilic asthma and non-eosinophilic asthma (149, 150). As their names suggest, these terms respectively refer to airway inflammation in which eosinophils or another inflammatory cell type (most commonly neutrophils) predominate. (151). Eosinophils have been found at higher concentrations in mild to moderate asthmatics, while neutrophils were higher among severe asthmatics (152–154). People who have died from sudden asthma attacks have a higher neutrophil over eosinophil inflammatory cell type (155). Neutrophils have also been found to be the predominant cell type in asthma refractory to treatment with inhaled corticosteroids (IC) (156). Eosinophilic inflammation is more consistently associated in children with acute asthma (157), while neutrophils are more consistently associated with adult onset asthma (158). However, both eosinophilic and neutrophilic airway inflammation can occur in all age groups (159).

Woodruff et al. (160) also divided asthmatics into $Th2^{high}$ and $Th2^{low}$ phenotypes. $Th2^{high}$ asthmatics responded to IC and had higher IL-5 and IL-13 concentrations in

bronchial biopsies than the $Th2^{low}$ subgroup. Others have identified Th2 controlled inflammation due to upregulation of the inflammatory markers PGD_2, HPGDS, and CRTH2 (161). One investigation of severe asthma identified decreased Th2 and IL-17 responses and increased IFN-γ (162). Increased transcriptional expression of IL-17A has also been identified in severe asthmatics (163). The chemokine CXCL8, which codes for IL-8 and is induced by IL-17A, is also increased in severe asthmatics (163). CXCL8 is the strongest neutrophilic chemoattractant in the lung (164). Interestingly, the Th17 (inducing IL-17) immune response was found to be steroid resistant, while the Th2 immune response was not (165), suggesting the Th2 inflammatory response may not play a role in, or has a sparing effect on steroid resistance in severe asthma.

Neutrophilic influx leads to a cascade of airway changes by first stimulating goblet cells to secrete mucus, which plugs airways and causes obstruction (166). Neutrophil supernatant has been shown to increase responsiveness of bronchial tissue sensitized with 0.1% albumin (167). A decline in lung function, demonstrated by a decreased FEV1, has also been associated with increased neutrophils in sputum (168). These changes may be responsible for the finding that sudden-fatal onset of asthma was associated more commonly with neutrophilic versus eosinophilic inflammatory cell influx (155).

Systems Biology Investigations of Asthma

Asthma is a complex disease that results from the interaction of genetic and environmental factors. Extending from this realization is an increasing interest in research

methods that can capture and model this complexity. “Omics” technologies include research methodologies that are capable of identifying changes in the expression of biomolecules such as genes, proteins, metabolites, and lipids across an entire genome, and are particularly well suited to the goal of unraveling asthma’s complexity. These technologies include transcriptomics, proteomics, metabolomics, and lipidomics among others. Samples used for biomarker discovery with these technologies include whole lung tissue, cells isolated from lung, BALF, induced or spontaneous sputum, exhaled air, and blood (169). Application of systems biology approaches to study complex diseases is facilitated by the availability of large datasets that are generated by ‘omics’ technologies. Systems biology seeks “to understand biological systems, complex or otherwise, from the interactions of their components, using computational and mathematical tools” (170). Systems biology uses both clinical and experimental data in computational and mathematical modeling to gain biological insight (171). The U-BIOPRED experiment is a key example of an organized multicenter study that integrates data derived from “omics” technologies using systems biology to discover the underlying mechanistic basis of severe asthma and will be discussed below (172).

“Omics” technologies are rapidly evolving, and more platforms are being added. Genome-wide association studies (GWAS) identify markers across complete sets of DNA or genomes to find genetic variations associated with a disease (173). RNA-Sequencing uses deep sequencing technologies for transcriptome profiling and to catalogue all species of transcripts (mRNAs, non-coding RNAs, small RNAs, etc.) (174). Proteomics seeks to identify differences in protein expression, and protein-protein interactions, while also able to highlight post-translational modification, and identify temporal patterns of

expression (175). A newer "omics" field called breathomics uses exhaled breath to identify biomarkers (176, 177). This technique has been pivotal in the identification of the fraction exhaled nitric oxide (FeNO) as a marker of eosinophilic airway inflammation in human asthma (178–180). As a non-invasive procedure, breathomics could be particularly useful in a clinical setting for children, and perhaps one day for equine diagnostics.

The sequencing of the equine genome has allowed researchers the ability to use molecular genetics platforms such as proteomics, transcriptomics, and microarrays to study the molecular basis of disease processes (181). Ever improving structural and functional annotation of the equine genome has aided researchers when using modeling tools in systems biology (182). Previous work done in our lab has furthered functional characterization the equine genome (183). Both structural and functional annotation were improved by confirming protein expression through proteomics and adding to the Gene Ontology (GO). The GO consortium aims to create a vocabulary that can be applied across eukaryotes to describe biological information related to gene products (184). Through the three ontologies of biological process, cellular component, and molecular function, the goal is to keep the vocabulary malleable to changing biological knowledge and amendable to updated annotations (184).

There have been seven studies that utilize "omics" platforms to search for differences between SEA affected horses and clinically normal horses. Molecular approaches to the study of SEA have helped to solidify the importance of the disease as a translational model for human asthma. Studies done have found overexpression of cytokines and genes including IL-4R (185), NOD2 (185), CXCL13 (186), IL-8 (187), and TLR4 (187).

These genes have been found as overrepresented in human asthma studies as well (188–194).

Using genome-wide scanning of two half-sibling strings of horses with a severely BDA-affected stallion, two chromosome regions (ECA13 and ECA15) had genome wide association with BDA (195). These regions included the genes 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.

Racine et al. (185) then used the software Ingenuity Pathways Analysis (IPA®) to compare proteins from BAL fluid of BDA affected and control horses to investigate the 8 genes identified by Swinburne at al. (195). IL-4R had the greatest number of interactions in the pathway analysis, highlighting it as an important protein in the BDA development signaling cascade. They also suggest the proteins SOCS5, NOD2, RPS6KA5, and FOXP3 have importance as regulators.

Peripheral lung biopsies from BDA affected and antigen challenged horses as well as clinically normal horses were taken for comparative differential expression analysis with suppression subtractive hybridization (196). Genes found showing expression differences that are associated with asthmatic airway inflammation included PPP3CB/NFAT, RhoA, and LTB4/GPR44.

One investigation employed proteomic analysis to characterize the proteome of BAL fluid from BDA horses. These investigators identified 100 differentially expressed peaks (197). The highlighted proteins from this study were secretoglobin and transferrin

for use as biomarkers of disease. Both were negatively correlated with BDA but overexpressed in control horses.

In a more recent study, microRNA (miRNAs) from SEA horses and normal horses were compared (198). Eleven significantly differentially expressed miRNAs were found, and pathways analysis highlighted regulation of epithelial to mesenchymal transition and PIP3 signaling pathway. These are key mediators of airway remodeling (199) and CD4+ T cell modulators (200) highlighting the relevance of the technique to examine future novel biomarkers of disease.

Using peripheral blood mononuclear cells (PBMCs), an RNA-seq study of BDA horses and controls challenged with hay dust found that the cytokine CXCL13 was strongly upregulated (186). The study also found that genes involved with immune cell trafficking, cell cycle regulation, and development were also differentially expressed in affected versus control horses.

Finally, RNA-seq was used to identify transcriptomic differences taken from endobronchial biopsies in response to inhaled allergen challenge (187). In this study, neutrophil migration genes were centralized in interaction networks. Highlighted genes from this study included MMP9, TLR4, MMP1, and IL-8. This study was able to find significantly expressed genes and networks that are similar to those of human asthma, highlighting utility of this technology for translational studies.

All of these studies were able to use a molecular approach to investigate the molecular mechanisms responsible for SEA. The goal of these approaches is to highlight genes, proteins, networks, or pathways that may later be considered as biomarkers of disease. Human asthma molecular studies have the advantage of having access to larger

population sizes available for study. Thousands of people may be studied at one time (201). As an example, genome wide association studies (GWAS) done over the past 10 years have shown that genes including TSLP, TNFSF4, ADORA1, CHIT1, and USF1 are associated with asthma disease (201). However, human studies present certain challenges including difficulties in unifying the type and stage of asthma progression as well as the nature and timing of therapy.

The concept of "personalized medicine" based on "omics" studies is a current topic of great interest in human medicine (202, 203). It focuses on the ability of "omics" technologies to identify unique aspects of an individual's metabolic makeup both in health and disease in order to direct medical diagnosis and management. Using "omics" studies to identify the mechanistic basis of disease in individuals and to identify therapeutic targets for individuals is now a reality that is increasingly being applied to the human population (204, 205). In fact, in a recent study analyzing priorities for asthma research, the use of "omics" technologies for diagnosis and prediction was one of the most important tools discussed (206).

Genomic approaches are being pursued to characterize differences between the various types of asthma in people (207). For instance, a population of Icelandic individuals in whom asthma is disproportionately low were identified to have a loss of function mutation in IL-33 which is a tissue-derived cytokine that induces and amplifies eosinophilic inflammation and is considered a promising target for asthma and allergic disease (208). More recently, a systems biology approach has shed light on the poor steroid responsiveness that characterizes severe asthma, by determining that a protein not previously understood to interact with corticosteroids, FAM129A, modulates steroid

responsiveness in lung epithelial cells (209). Similar studies could be useful in further differentiating BDA from EPA, though it may be that the two have similar genetic mechanisms as various forms of asthma do (207). Still, differences between types of asthma (i.e. adult onset vs. childhood) have been found at a transcriptomic level (210), and should be considered for BDA and EPA analysis.

U-BIOPRED, Unbiased Biomarkers for the Prediction of Respiratory Disease Outcomes, is a consortium of academic institutions, pharmaceutical companies, and patient organizations with the goal of understanding mechanisms of asthma using systems biology (211). Samples from this study have shown that patients with severe asthma have worse quality of life (including anxiety and depression) than those with mild/moderate asthma, higher incidences of nasal polyps and gastric reflux, lower lung function, and sputum eosinophil counts higher than mild/moderate asthmatics (211). Use of U-BIOPRED has also determined that in patients with severe asthma, pathways in chemotaxis, migration, and myeloid cell trafficking are increased while B-lymphocyte development, hematopoietic progenitor cells, and lymphoid organ hypoplasia is decreased (212). Another similar systems approach proposed by Auffray et al. (213) seeks to find functional and regulatory pathways that play central roles in severe asthma. This is another collaborative effort utilizing global genome, transcriptome, proteome, and metabolome data sets integrated with biological and clinical data to form predictive models. Through computational and experimental models, well-classified phenotyping of severe asthma will hopefully be achieved and used to aid the development novel diagnostic and therapeutic biomarker targets (213).

Additional studies using the systems approach have been done to elicit mechanisms of severe asthma in particular. Transcriptome analysis has demonstrated that severe asthma is associated with activated CD8(+) T cells but not CD4(+) T cells (214). Sputum and blood transcriptomes of children and adults with asthma have identified gene expression patterns that are able to differentiate near-fatal, severe, and mild/moderate asthma (215). Clusters correlating with clinical, physiologic, and inflammatory characteristics of disease could be grouped as: 1) history of intubation, lower pre-bronchodilator FEV1, higher bronchodilator response, and higher exhaled nitric oxide levels. 2) hospitalized for asthma, 3) normal lung function, lower exhaled nitric oxide levels, and lower inhaled steroid requirements (215). Thymic stromal lymphopoietin (TSLP) is increased in airway epithelium and lamina propria of asthmatics, but especially in patients with severe asthma (216). It also correlates with severity of airflow obstruction (216). Sputum transcriptome analysis has shown the chitinase-like protein (YKL-40) is more associated in asthmatics with earlier onset and longer duration of disease, severe airflow obstruction, and near-fatal asthma attacks (217). Using epithelial brushings from severe asthmatics and controls has shown IL-13 is upregulated in severe asthmatics and is more pronounced in the peripheral rather than central airways (218). Proteomic studies on severe asthma have highlighted significance of proteins like galectin-3 (219), TGF-β induced epithelial mesenchymal transition (EMT) (220), and mitogen kinase kinase 3 (MKK3) (221), and have found clusters differentiated by asthma severity (with the more severe having higher sputum eosinophils) (222).

The most severe population of asthmatics are corticosteroid resistant and have their own distinct genetic features (223). The role of IC treatment and the effect of severe

asthma has also been studied with systems approaches. For example, a microarray found that mRNA encoding cFLIP, a modulator of caspase-mediated extrinsic apoptosis pathway, is decreased in corticosteroid resistant asthma (224). Microarrays done on a murine model of asthma found that expression of FK506 binding protein 51 gene (FKBP51) is increased after dexamethasone treatment (225). When FKBP51 expression was then induced in peripheral blood mononuclear cells, FKBP51 expression was significantly higher in severe asthmatics (225). In comparing sputum from severe asthmatics with that of non-severe asthmatics treated with inhaled corticosteroids (IC), brain-derived neutropenic factor (BDNF), B-lymphocyte chemoattractant (BLC/CXCL13), and epidermal growth factor (EGF) were significantly increased in severe asthmatics (226). Sputum with neutrophil counts greater than 70% were used to look for differential expression between patients with severe, uncontrolled asthma and controlled asthma (227). S1009A was highlighted as a potential biomarker of neutrophilic inflammation in severe, uncontrolled asthma. Vitamin D has been proposed to enhance the effects of IC in severe asthma patients where IC alone are ineffective (228). Bitter taste transduction receptors (TAS2Rs) are increased in transcriptome analysis of children with severe, IC resistant asthma compared to normal patients and have been confirmed using qPCR to be upregulated in leukocytes from adult severe asthmatics (229). Himes et al. (230) compared ASM transcriptomes of severe, fatal asthmatics and non-asthmatics when treated with vitamin D. 711 differentially expressed genes were found in the ASM transcriptome of fatal asthmatics after vitamin D treatment and included genes in the cytokine and chemokine categories. Follow-up

experiments then showed that vitamin D could inhibit TNFα induced IL-8 protein secretion to similar levels in both the fatal asthma and control groups (230).

Together these studies have demonstrated that systems biology approaches can be used to not only find biomarkers of severe asthma but can also be used to assess the effectiveness of potential therapeutics targeting these biomarkers. As shown above, asthma is a multifactorial disease, and it has many potential regulators. The increasing advancement of these technologies will be important to further characterize specific asthmatic phenotypes, most notably severe asthma that is refractory to IC.

Asthma is a dynamic disease for which "omics" platforms provide a unique advantage in characterizing how asthma changes in its underlying biology as the disease progresses in severity over time. Systems biology approaches allow for observations at selected points in time to be compared with other time points on a global 'genome scale' level, which provides a unique ability to decipher the biology behind these dynamic changes. Overall, numerous mediators of disease have been highlighted using systems biology to investigate "omics" samples from asthmatics. Certainly, the numbers of studies may never be able to reach the same level in SEA. However, SEA possesses characteristics that make it a superior translational model for investigating severe and neutrophilic forms of human asthma. Beyond the demonstrated homologies between SEA and human asthma that have been previously described, investigations of SEA can control for factors such as environment, treatment, and treatment compliance which are not well addressed in human studies, while also characterizing the natural progression of spontaneous severe and neutrophilic asthma. By extension, despite any challenges, investigations of SEA that employ systems biology and "omics" technologies have the unique and insightful

potential to contribute to investigations of severe and neutrophilic human asthma in a unique and novel manner.

Animal Asthma Models

Animal models for the study of disease have historically been, and are still, an important part of biomedical research. This is because ethical considerations prevent many studies on human subjects. The use of laboratory animals also has its own ethical considerations and cadre of compliance mandates that must be addressed (231). The best way to utilize various animal models of asthma is still being debated (232, 233), and there is no one model that recapitulates all aspects of human asthma (234). Still, understanding the pathophysiology and treatment of asthma, like many diseases, has benefited from the use of animal models.

Animal asthma models can be divided into those that are induced and those that are naturally occurring, also termed 'spontaneous.' Three animal species are reported to naturally develop asthma-like disease: 1) Feline asthma, which is characterized by eosinophilic airway inflammation (235, 236); 2) Equine asthma which has both a mild and severe form that is characterized by mild increases in neutrophils and eosinophils and moderate to severe increases in neutrophils, respectively (8, 14), and 3) asthma in dogs which includes Basenji dogs as well as asthma in sled dogs and is analogous to exercise-induced asthma (237, 238). While having the advantage of being naturally occurring, the disadvantage of using spontaneous models is that they occur in outbred species whose inherent biologic variation presents the potential to require large sample sizes to achieve sufficient statistical power to identify true differences in experimental outcomes (239).

Rodents make up the largest percentage of induced animal models (239). Coupled with current statistics that 85% of drugs that originate from mouse models do not effectively translate to their human disease counterpart (240, 241), the utility of commonly employed induced animal asthma models has been questioned (241, 242). In induced asthma models, the allergens responsible for sensitization can be delivered by inhalation, subcutaneous injection, intraperitoneal injection, or through intranasal drops (243). The most frequently used induction antigens and important findings from investigations that employ them are summarized in Table 3.

The most commonly employed induced animal model for asthma is the mouse, particularly the BALB/c strain (243). Protocols sensitizing mice with ovalbumin (OVA) (244–246) , house dust mite (247, 248), cockroach antigens (249, 250), *Aspergillus fumigatus* (251, 252) , and ragweed extracts (253) have all elicited an asthma-like phenotype. These models have aided in the identification of important cytokine pathways in the pathogenesis of asthma (254–258). Mice are popular because they have many advantages. Mice are easy to breed and maintain and, consequently, can be studied in large numbers (243). Their inbred nature is particularly advantageous because it limits variability in testing, facilitating the recognition of differences in experimental protocols with fewer animals than are needed for outbred individuals. There are also a wide variety of transgenic mouse strains available to study (259, 260), which has accounted for their massive popularity recently. For example, the most commonly used mouse strain (BALB/c) is employed in asthma research due to their ability to develop good Th2 responses (261, 262).

The most important disadvantage to murine models is the inability to maintain the disease state over time. Mice eventually develop tolerance to the sensitizing antigen (247, 263). Some groups have tried to develop chronic models with mice in order to develop a more clinically representative phenotype (261). These models involve exposing mice to an allergen for longer periods of time. A drawback with these models was that long-term exposure could lead to a decreased inflammation and AHR (264). After 55 days of being challenged 3 times a week with OVA, mice have been shown to maintain a predominantly eosinophilic infiltrate and airway remodeling, characterized by increased collage matrix deposition and ASM cell hyperplasia, up to 4 weeks after challenge ceases (265). This study also identified AHR to be maintained on day 35 of allergen challenge, but AHR was not identified one month after exposure stopped in response to doses of methacholine from 3-100mg/ml methacholine (265).

The mouse phenotype is predominantly characterized by eosinophilic airway inflammation (244), not the neutrophilic airway inflammation that has been associated with severe human asthma. These eosinophils rarely degranulate in the mouse models of asthma (266), whereas human eosinophils do (267). It should be noted that there is a murine model that results in airway remodeling more typical of that seen in humans (268). There is also a mouse asthma model elicited by sensitization to house dust mite antigen that resulted in longer termed airway remodeling consisting of goblet cell hyperplasia, collagen deposition, peribronchial accumulation of contractile tissue and what the authors call severe, only partially resolved airway hypersensitivity to methacholine up to 9 weeks after exposure (248). Despite these advances, many

therapeutics developed from murine protocols have proven ineffective in clinical trials (269, 270).

Another commonly used rodent for asthma studies is the rat. The Brown Norway is the most commonly used strain. Like mice, rats have been sensitized with ovalbumin (271, 272), HDM extracts (273), and additionally 2,4-dinitrophenylated (DNP)-*Ascaris/Bordetella pertussis* vaccine antigen (274). Rats have a larger body size providing an advantage for lung mechanics studies to non-specific bronchoconstricting agents and allergen inhalation. Brown Norway, Wistar, Sprague Dawley, Lewis, and Fischer 344 strains of rats have been shown to vary in response to sensitization, the most common being the Brown Norway (274, 275). Strain responses can be found in Table 3.

Another rodent, the guinea pig, is one of the oldest animal models of allergic airway responses (276, 277). Guinea pigs have been primarily sensitized to OVA and respond with a predominance of airway eosinophilic inflammation and increased airway responsiveness (278, 279). Guinea pigs are believed to be more eosinophilic in their allergic responses than people, constitutively producing more eosinophils in their normal 'non-sensitized' lung then do humans. This is thought to be influenced by constitutive production of eotaxin and RANTES in guinea pig lung (278). Guinea pigs have parasympathetic cholinergic input to ASM that mediates airway contraction through acetylcholine release at post junctional M3 receptors (258). Guinea pigs also have nonadrenergic noncholinergic (parasympathetic noncholinergic) innervation, like humans, that mediates airway dilation and is absent in rats and mice (279). However, the sympathetic innervation of guinea pigs differs from humans in that guinea pigs have extensive sympathetic innervation of their tracheal ASM with sparse to non-existent

innervation of ASM in the intrapulmonary airways (278). Humans, on the other hand, lack direct sympathetic innervation of ASM (280, 281). The majority of autonomic relaxant tone is mediated by $ß_2$-adrenergic sympathetic receptors on ASM in response to noradrenaline release, with parasympathetic non-cholinergic nerves (nonadrenergic noncholinergic nerves) also contributing to direct relaxant innervation of ASM in both guinea pigs and humans (282).

Guinea pigs have extensive advantages when investigating allergic asthma, which have been reviewed by Canning (281). Guinea pigs have well-characterized early and late phase responses (283) and experiments with precision cut lung slices have shown more agreement between pharmacological responses of guinea pig and human airways than other rodents (284). However, guinea pigs have some receptors not present in human airway smooth muscle such as neurokinin$_1$ receptor (285) and contractile prostanoid EP_1 receptors (286–288). In addition, few transgenic guinea pig models are available, and guinea pigs are prone to develop tolerance after repeated challenges just like mice and rats (289).

Cats are one of the few animals to spontaneously develop an asthma-like disease with respiratory signs of coughing, sneezing, and dyspnea (290). A model that sensitized cats to *Ascaris suum* has also been developed, with the animals developing AHR to acetylcholine and an increase in eosinophils in BAL fluid (291). Characteristics of AHR and remodeling in this model are detailed in Table 3. In the naturally occurring disease, bronchial washes have had a mixed population of cells including eosinophils, neutrophils, and macrophages, though positive bacteria cultures were also present in some of these cases (290).

Dogs have been challenged with *Ascaris suum* (292) and ragweed (293) to develop an asthma-like disease. Beagles sensitized with ragweed had increased IgE. They also develop prolonged AHR lasting up to five months after the final challenge (294). Similarly, *Ascaris suum* sensitized dogs develop airway hyper-responsiveness (290). Though ragweed sensitized dogs develop eosinophilic airway inflammation in BAL fluid, they lack a relationship between persistence of AHR and the magnitude of airway eosinophilic inflammation (295). Basenji greyhound crosses, that have a wide range of inherited bronchial sensitivity, have been proposed as a spontaneous model for asthma since they have shown bronchial responsiveness to hypotonic and isotonic aerosols (296). When challenged with *Ascaris* suum, citric acid, and methacholine, Basenji greyhounds responded to methacholine at concentrations 10-30 times lower than mongrel dogs, confirming non-specific airway hyper-responsiveness (297). In the same study, citric acid caused increases in R_L at 5-10 fold lower concentrations than mongrels (297). Interestingly, in the study, Basenji greyhounds responded to methacholine and citric acid regardless of their *Ascaris* sensitization status (297), suggesting a baseline level of bronchial hyper-reactivity in the breed. AHR also seems to be a characteristic of only the Basenji greyhound cross, and not the Basenji or greyhound ancestral lines (298).

Larger animal models have also been used to study asthma. Since animals like sheep, pigs, and calves have similar body weights as humans, they have been proposed as better models to evaluate pulmonary function (299). Sheep have been sensitized to *Ascaris suum* (300). Immediately after challenge R_L increases, and C_{dyn} and PaO_2 decrease (300). Sheep that respond both early and late to an allergen challenge, termed "dual responders", have increased airway eosinophil percentages following challenge (301). In

contrast, both early and late phase responders will also have an increase in airway neutrophilic inflammation post challenge (302). Only dual responders have AHR (in this study defined at 150% increase in R_L) 24 hours after challenge (303). However, therapeutics developed from sheep models have yet to prove effective in human clinical trials (304, 305). Pigs have also rarely been used and were sensitized with *Ascaris suum*, producing a late phase response with blood eosinophilia (306).

Finally, non-human primates have been used in asthma research, as well. Squirrel monkeys (*Saimiri sciureus*) (307), rhesus macaques (*Macaca mulatta*) (308), and cynomolgus macaques(*Macaca fascicularis*) (309) have been sensitized to *Ascaris suum* and house dust mites (310, 311). After multiple challenges, cynomologus macaques show AHR and eosinophilic inflammation to *Ascaris suum* (312). Inhalation challenge with *Ascaris* resulted in increased R_L in both types of single responders (acute or late phase), while only dual responders maintained an increased R_L for 6-8 hours after challenge (312). Neutrophilic inflammation has been observed in primates after antigen challenge with *Ascaris suum,* though to a lesser extent than eosinophilic inflammation (309). In this study, the percent of neutrophils in dual responder primates was greater than that of single responders. A direct relationship between the late phase response and neutrophilic inflammation was, therefore, proposed. In fact, another study by this same group found a late phase dependence on endothelial leukocyte adhesion molecule 1 (ELAM-1) for neutrophilic inflammation (313).

The inflammatory response of monkeys sensitized with house dust mite antigen includes increased eosinophils in BALF and elevated CD25 expression on circulating CD4(+) lymphocytes in peripheral blood (310). Sensitized monkeys exhibited clinical

signs of obstruction such as coughing and rapid shallow breathing. An increase in R_L that could be reversed by nebulized albuterol was also present. This model also has remodeling changes including mucus cell hyperplasia and epithelial cell hypertrophy in rhesus monkeys sensitized to house dust mites (310).

It should be mentioned that the fruit fly, *Drosophila melanogaster*, has been proposed as an animal asthma model due to potential homology of known asthma-susceptibility genes between humans and the fly (314). The fruit fly has also been proposed, particularly for GWAS comparison studies due to the similarity of the airway system's reaction to pathogens with mammals like the mouse (315). Due to their lack of lungs (315), they are not an appropriate model for induction studies, however.

Animal models remain integral to our understanding of asthma pathogenesis. There is no one perfect model that is able to address the multitude of disease characteristics that exist across the spectrum of asthma. As evidenced in Table 3, it is difficult to find a model that is able to answer all inquiries covered by various studies over time. Accordingly, animal models that capture specific facets of diverse asthma phenotypes constitute the best currently available research tools for investigating asthma. Understanding the limitations imposed by each model system is essential for selecting the appropriate model for a given hypothesis and for interpreting the resulting data within the context of human asthma.

Summary

Asthma is a multi-factorial disease, which is not yet completely understood. With increasing prevalence and costs of severe asthma, there is a growing need for animal models to investigate pathophysiologic mechanisms of disease. Various studies of lung

function, genetic mechanisms, and airway remodeling have been performed to this end. Many of these studies have utilized animal models, but most rely on induction of disease with antigens. Each model has advantages and disadvantages, but the largest drawback of induced models is the lack of chronic persistence and ability to consistently model the severe asthma phenotype. The horse, however, is unique since equine asthma develops spontaneously and is characterized by neutrophilic inflammation, an inflammatory characteristic that is reported to be associated with both severe and fatal human asthma. This creates a special opportunity for investigations targeting the neutrophilic, severe asthma phenotype. In consideration of the limited availability of spontaneous translational asthma models and knowledge that β -AR signaling contributes to the development of asthma in a murine model (ref), this dissertation first focuses on confirming that EPA recapitulates both the magnitude and persistence of airway hyper- that characterize severe human asthma. Next, using a systems biology approach, associations between β_2-AR signaling and genes that were identified as differentially expressed in the lung of EPA horses were explored to identify a novel mechanism of. ASM responsiveness. The overarching goal of this work was to validate Equine Pasture Asthma (EPA) as a translational asthma model in order to identify novel therapeutic targets for both horses affected with EPA and people suffering from severe asthma in concordance with One Health Initiatives. This has been accomplished by: 1) using methacholine bronchoprovocation to confirm AHR of EPA horses evident at methacholine concentrations equal to that of severe human asthmatics, 2) RNA-sequencing of lung samples of EPA and clinically normal horses to identify unique gene

targets for EPA disease, and 3) immunohistochemical and in vitro investigations of novel gene targets found to be unique to EPA horses during disease exacerbation.

Table 1 Categories of airway hyper-responsiveness based on methacholine challenge testing

$PC_{40}R_L$ (mg/ml)	**Analysis**
>16	Normal
4.0-16	Borderline AHR
1.0-4.0	Mild AHR
<1.0	Moderate to severe AHR

Information found in Guidelines for Methacholine and Exercise Challenge Testing (27).

Table 2 Symptoms indicating consideration for equine asthma diagnosis

Symptom	***Description***
Wheezing	Whistling noise heard on exhalation
Cough	Especially at night or when waking
Chest tightness	Historical
Difficulty breathing	Historical

Information found in the National Asthma Education and Prevention Program Expert Panel Report for diagnosing asthma (2).

Table 3 Summary of some induced animal models of asthma with relevant findings

Animal	**Antigens**	**Animal Strains-Breeds**	**Predomin-ant Cell Types (Predomin-ant Listed First)**	**Magnitude of AHR**	**Remodel-ing**	**References**
Mice	OVA	C57BL/6, BALB/c, A/J	Eosinophils	Inhaled Mch Range: 3-100 mg/ml - PC_{50} Mch Elastance: 3.3 mg/ml -Mch for Significant R_L Change: 25, 50, and 100mg/ml -Mch for Significant Penh Change: 10,30, 100mg/ml	Collagen matrix deposition, ASM hyperplasia	(265, 316–318)
	House dust mites	C57BL/6	Eosinophils, persists for up to 7 weeks and decrease with long term (11 weeks) exposure	Dose dependent increase in R_L from 1.0-100mg/ml Mch; Significant difference in AHR between sensitized and control mice occurs after 5 weeks of exposure	Goblet cell hyperplasia, peribronchial accumulation of contractile tissue, collagen deposition	(247, 319)

Table 3 (continued)

	Cockroach	C57BL/6	4-week-old mice have greater inflammation in airways and vasculature (specific cell type not defined)	4-week-old mice have higher R_L than control mice at 40 and 50mg/ml Mch	Peribronchial and perivascular inflammation	(250)
	Aspergillus fumigatus	BALB/c	Eosinophils	-	-	(251, 252)
	Ragweed extract	-	Eosinophils, neutrophils, lymphocytes, and macrophages	-	-	(253)

Table 3 (continued)

Rat	OVA	Brown Norway, Sprague Dawley	Eosinophils, neutrophils, mast cells	-	-	(271, 272)
	House Dust Mites	Brown Norway	Eosinophils, neutrophils	-	-	(273)
	DNP-*Ascaris/Bordetella pertussis* vaccine	Wistar, Lewis, Fischer 344	-	-	-	(274)

Table 3 (continued)

Guinea Pigs	OVA	Dunkin-Hartley	Eosinophils, macro-phages	AHR noted 12 hours following exposure but abates by 24 hours. –AHR noted with 0.5mg/ml histamine	-	(278, 320, 321)
Cats	*Ascaris suum*	Domestic Short Hair	Eosinophils	Ach challenge with resulted in a 1.0 log decrease eliciting a 200% increase in AHR 24 hours after challenge; 1.5 log decrease eliciting a 200% increase in AHR present after 4 to 6 weeks of challenge three times a week (reactivity assessed 72 hrs after last challenge)	Bronchocon striction, luminal narrowing, increased smooth muscle thickness, goblet cell and sub-mucosal gland hypertrophy and hyperplasia	(291)

Table 3 (continued)

Dog	*Ascaris suum*	Basenji-Greyhound	Neutrophils	R_L increases 4-6 hours after challenge	-	(292, 297)
	Ragweed	Beagle	Eosinophils	AHR persisted after 5 months after exposure to ragweed twice at 13-day intervals and again 45 days later.	-	(294)
Sheep	*Ascaris suum*	-	Eosinophils, neutrophils	-R_L increased and C_{dyn} decreased for 6.5-8 hrs post-challenge with carbachol -Carbachol (26.9 breath units) caused 400% increase in R_L - Dual responders (early and late phase) to carbachol exhibit AHR greater than baseline values up to 24 hours post challenge	-	(300, 302, 303, 322)

Table 3 (continued)

	House Dust Mites	Merino cross	Neutrophils, eosinophils (Initial recruitment of neutrophils, followed by eosinophils. Eosinophils continue to make up inflamma-tory cell type until 48 hours after challenge)	-	-	(323)
Non-human Primates	*Ascaris suum*	Cyno-molgus, Rhesus macaque, Squirrel monkey	Eosinophils	Single responders had 298% increase in R_L, and dual responders had 268% increase (determined by forced oscillations on tidal breathing)	-	(307–309)
	House Dust Mites	Rhesus macaque	Eosinophils	Increased AHR to metha-choline	Basement membrane thickening	(311, 324)
Pig	*Ascaris suum*	-	Late blood eosinophils	-	-	(306)

Notes: (-) indicates information not specified in study, OVA=ovalbumin, ASM=airway smooth muscle, Mch=methacholine, RL=resistance, AHR-airway hyper-responsiveness, Cdyn= dynamic compliance, Penh=enhanced pause

References-see Appendix

CHAPTER II

HORSES WITH PASTURE-INDUCED ASTHMA DEMONSTRATE SEVERE PERSISTENT AIRWAY HYPER-RESPONSIVENESS

Abstract

Rationale: Persistent airway hyper-responsiveness (AHR) is an asthma feature not effectively recapitulated by induced asthma models. An animal model with severe, non-specific and persistent AHR is advantageous for asthma investigation.

Objectives: To document magnitude and persistence of AHR in a naturally occurring horse asthma model, equine pasture asthma (EPA), elicited by exposure to pasture grasses during conditions of high heat and humidity and concentrated in the southeastern United States.

Methods: AHR was evaluated using the two-minute tidal breathing protocol to identify the methacholine concentration causing 40% increase in baseline lung resistance ($PC_{40}R_L$) in 10 horses with EPA during the season of asthma exacerbation. To confirm persistent AHR, $PC_{40}R_L$ was re-evaluated in 5 of the 10 diseased horses during seasonal asthma remission (approximately 3-34 months later). 1 additional horse was also tested during asthma remission. 10 non-diseased horses also underwent bronchoprovocation. Reversibility of airway obstruction was evaluated in 8 EPA horses during asthma exacerbation by evaluating changes in dynamic compliance (C_{dyn}), maximum change in

pleural pressure (ΔPpl_{max}), and lung resistance (R_L) in response to levalbuterol administration.

Measurements and Main Results: $PC_{40}R_L$ of diseased horses was ≤1 mg/ml methacholine during the seasons of asthma exacerbation (0.0625-1.0mg/ml) and remission (0.25-1.0mg/ml). $PC_{40}R_L$ of individual EPA horses did not differ significantly between exacerbation and remission (p=0.2176), confirming persistent AHR. Control horses were less reactive then diseased (p=0.0014), failing to react up to endpoint methacholine dilutions (8-16 mg/ml). Airway obstruction was reversible ($P < 0.05$), characterized by increased C_{dyn}, and decreased ΔPpl_{max} and R_L.

Conclusions: EPA is characterized by reversible airway obstruction with persistent non-specific AHR at methacholine concentrations considered diagnostic of severe asthma (≤1 mg/ml), confirming EPA's high fidelity with defining criteria of human asthma.

Introduction

More than 39.5 million people suffer from asthma in the United States (98). CDC estimates indicate that asthma affects 1 in 12 adults with a 15% increase in prevalence from 2001-2009 (99). Asthma is estimated to cost the United States $56 billion/year from medical expenses, loss of productivity, and premature death (100). While prevalence of adult asthma varies between states, Mississippi saw a significant increase from 2001-2009 (99). In 2016, the Asthma and Allergy Foundation of America listed Jackson, Mississippi as the most challenging capital in which to live with fall allergies (18).

A severe asthma-like disease, termed equine pasture asthma (EPA), also affects adult horses living on pastures during the summer months in the southeastern United States (13). A parallel clinical phenotype previously referred to as 'heaves' or recurrent airway obstruction (RAO) affects stabled horses in the winter in temperate regions (325–327) and has been increasingly employed as a naturally occurring animal asthma model based on similarities between the equine and human lung (328), ability to induce reversible airway obstruction (329), and the ability to acquire thoracoscopic lung biopsies for experimental analysis (330, 331). Whereas the stable 'heaves' phenotype is triggered by indoor aero-allergens that accompany feeding hay (325), the phenotype affecting horses on pasture in the southeastern US has been termed Equine Pasture Asthma (7) to address the unique geographic, seasonal, and aeroallergen characteristics of the pasture associated disease. Horses with EPA demonstrate progressive exacerbation of their clinical signs, which include cough, wheezing, and respiratory distress during successive warm seasons with periods of remission during cool seasons despite their continued housing on inciting pastures (5).

Horses with EPA and stable heaves experience key facets of asthma including variable airflow obstruction and chronic airway inflammation in which neutrophilic inflammation predominates (22, 332, 333). While non-eosinophilic airway inflammation is identified in at least 50% of adult asthmatic patients (149), neutrophilic airway inflammation correlates to severe and treatment refractory asthma phenotypes (152, 155). Despite the clinical relevance of non-eosinophilic inflammation in asthma, eosinophilic inflammation predominates in animal asthma models (334). Both EPA and stable heaves provide the only naturally occurring animal asthma model with neutrophilic airway inflammation.

Non-specific airway hyper-responsiveness (AHR), a key facet of asthma diagnosis, has been documented in horses with stable heaves (326) following challenge with moldy hay but with no persistence of non-specific AHR 1 to 2 weeks following moldy hay challenge. However, presence, severity and persistence of non-specific AHR has not been documented in EPA.

Considering the strengths of EPA as a severe and naturally occurring asthma disease with neutrophilic inflammation which correlates to disease severity in human asthma, the objective of this investigation was to characterize key facets of asthma diagnosis, namely the presence, severity and persistence of non-specific AHR and the reversibility of airway obstruction in order to refine the phenotype of horses with EPA. This information will improve the utility of this spontaneous animal model for investigating non-eosinophilic, and adult asthma phenotypes, as well as environmental factors that impact asthma prevalence in the southeastern United States.

We hypothesize that non-specific AHR in horses with EPA persists for protracted periods beyond seasonal exposure to inciting aeroallergens. To test this hypothesis, we characterize the magnitude of methacholine responsiveness in EPA affected horses during periods of clinical exacerbation and remission.

Materials and Methods

Study Subjects-

Twenty-two horses (12 EPA affected and 10 clinically normal controls) were enrolled in the study. The mean age of diseased horses was 19.9 years (SD=6.5 yrs). Control horses were 20.6 years old (SD=4.2 yrs). Diseased horses included 7males and 5 females. Control horses included 6 males and 4 females. EPA horses had 2 or more years of recurring episodes of warm season asthma-like disease that remitted during cool seasons, and a predominance of neutrophils on cytologic examination of BAL (12-97%, mean=58.6%). Only 2 horses had 1 % eosinophils identified. Control horses were selected from a closed herd also maintained in close proximity to principals at Mississippi State University and were identified based upon historical and clinical absence of seasonal respiratory distress in their lifetime. The study was approved by the IACUC committee at Mississippi State University (protocols 11-, 14-016, 16-035).

Study Design-

Reversible Airway Obstruction

Reversible airway obstruction was documented in 8 horses experiencing asthma during the season of clinical EPA exacerbation which is reported to span the months of June through November (10). Horses were tested when their clinical score of respiratory effort (CSRE) exceeded 5/8. CSRE is calculated based upon nostril flare and expiratory effort as previously described (335). The effect of nebulized levalbuterol (1.25 mg) on maximal change in pleural pressure (ΔPpl_{max}), lung resistance (R_L), and dynamic

compliance (C_{dyn}) were determined using conventional pulmonary mechanics (67). Pleural pressure was measured indirectly using a 10 cm latex balloon secured to a catheter that was placed at the 11-13th intercostal space. The catheter was connected to a pressure transducer (Validyne Model DP45-28, Northridge, CA). For each horse, balloon placement was initially determined by passing the balloon and catheter through a nasogastric tube that was introduced via the nares into the rostral esophagus. The balloon and catheter were advanced into the stomach, inflated with 15 cc of air, and withdrawn slowly to identify site of maximal negative deflection during inspiration between the diaphragm and heart.(22) Flow was measured using a Fleisch #6 pneumotachograph fitted into a mask (Air-o-mask, Trudell, Inc., London, Ontario) that covered the horse's mouth and nose(23). $\Delta Ppl_{max,}$ $R_{L,}$ and C_{dyn} were measured prior to and at 7 and 15 minutes after levalbuterol administration. All signals were recorded via Buxco Biosystems XA software (v. 2.5). Pressure and flow signals were interfaced via a preamplifier (Buxco Max II, Wilmington, NC) to Buxco Biosystems XA software (v. 2.5, Buxco, Wilmington, NC).

Methacholine Bronchoprovocation

Following levalbuterol administration, horses were housed for 48-72 hours in a climate-controlled stall until signs of respiratory distress abated. Each horse underwent methacholine bronchoprovocation using the 2-minute tidal breathing protocol (27), employing doubling concentrations of methacholine from 0.0625 to 16 mg/ml (337). Based upon guidelines for noncompliant human subjects (79), the methacholine concentration causing a 40% increase in baseline resistance ($PC_{40}R_L$) was determined.

Control horses were similarly challenged except for 5 control horses that were poorly tolerant of the instrumentation and were challenged with a single methacholine concentration (16 mg/ml) via 2-minute tidal breathing. Measurements of R_L for each methacholine dose were derived from 10-20 representative breaths. To determine the persistence of non-specific airway hyper-responsiveness, methacholine bronchoprovocation was repeated in 5 EPA-affected horses during the season of EPA remission (December-April) (10) when clinical scores of respiratory effort were consistently <4/8. An additional horse also underwent methacholine bronchoprovocation during seasonal remission which had not been previously tested the season of disease exacerbation. At the end of each test, bronchodilation was induced using 1.25mg of levalbuterol by nebulization.

Statistical Methods

Nonparametric models were employed because the data did not meet assumptions of normality and homoscedasticity. For measures of reversible airway obstruction, the effect of sample time on R_L, C_{dyn}, and ΔPpl_{max} was assessed by a method similar to the non-parametric Friedman's test. The data was first ranked within each horse. Analysis of variance for each of the outcomes using PROC GLM in SAS for Windows v9.4 (SAS Institute, Inc., Cary, NC) was then conducted on the ranked data with sample time and horse as fixed effects. Where the effect of time was significant, pairwise comparisons were made between the Time 0 (baseline), Time 7, and Time 14 values, using Tukey's adjustment for multiple comparisons. Differences in R_L values for horses in exacerbation compared to control horses and for horses in persistence compared to control horses were assessed with separate Wilcoxon Rank Sum tests using PROC NPAR1WAY in SAS for

Windows v9.4. Differences in $PC_{40}R_L$ values while horses were in exacerbation compared to persistence were assessed using a paired t-test. An alpha level of 0.05 was used to determine statistical significance.

Results

Reversible Airway Obstruction

Significant decreases in R_L between T=0 and T=7 (p=0.0003), and between T=0 and T=14 (p<0.0001) were identified. A slight decrease in R_L between T=7 and T=14 was not statistically significant (p=0.1134) (Figure 1A). ΔPpl_{max} decreased significantly between T=0 and T=7 (p=0.0003) and between T=0 and T=14 (P<0.0001). There was no difference in ΔP_{max} between T=7 and T=14 (P=0.6092) (Figure 1B). Significant increases in C_{dyn} between T=0 and T=7 (P=0.0146) and between T=0 to T=14 (P=0.0003) were also identified. Reflecting large variations in C_{dyn} between T=7 and T=14, a slight increase C_{dyn} during this interval was not significant (P=0.1389) (Figure 1C).

Methacholine Bronchoprovocation

When tested during the season of clinical EPA exacerbations, 10 EPA-affected horses achieved their $PC_{40}R_L$ at methacholine concentrations between 0.0625 and 1.0mg/ml (Figure 2). During this season, control horses failed to develop significant increases in R_L at all tested methacholine concentrations (Figure 2). Accordingly, the maximal R_L achieved by EPA-affected horses in response to the $PC_{40}R_L$ was significantly greater than

the R_L achieved by control horses in response to maximal methacholine concentrations (p= 0.0014).

6 EPA-affected horses received methacholine bronchoprovocation during seasonal EPA remission, achieving their maximal R_L concentrations between 0.25-1.0mg/ml (Figure 3). 5 of these horses had been tested between 3 and 34 months prior, during the season of EPA exacerbations. Non-specific AHR persisted in all five horses tested. There was no significant difference in the $PC_{40}R_L$ for each horse between seasonal exacerbation and remission (p=0.2176) (Figure 4). Three of the 5 horses achieved their $PC_{40}R_L$ at methacholine concentrations less than 0.1mg/ml difference between both time periods, while two horses reacted to concentrations less than 0.4mg/ml during remission relative to the preceding exacerbation test. (Table 1). The maximal R_L achieved by EPA-affected horses in response to the $PC_{40}R_L$ during seasonal remission was significantly greater than the R_L achieved by control horses in response to maximal methacholine concentrations (p= 0.0074).

Discussion

This investigation identified reversible airway obstruction, and non-specific AHR that persists despite the absence of inciting aeroallergens for periods up to three years in horses with documented equine pasture asthma. Neutrophilic airway inflammation with minimal eosinophilic inflammation has been previously documented in horses with EPA, and is a diagnostic feature of the disease (7, 22, 338). While the triumvirate of reversible airway obstruction, inflammation, and persistent non-specific AHR are considered key diagnostic features of human asthma (2, 109, 149), induced animal models are challenged by the inability to adequately recapitulate these diagnostic features (235, 262).

EPA-affected horses exhibit asthma while grazing pastures during summer months in the southeastern United States, and demonstrate improvement, consistent with reversible obstruction, when removed from the inciting pasture environment into a climate-controlled stall (15). In this investigation, reversible airway obstruction was confirmed in 8 horses with EPA during a naturally occurring asthma attack, as evidenced by significant decreases in $R_{L,}$ $\Delta Ppl_{max,}$ and a significant increase in C_{dyn} in response to the β2-adrenoceptor agonist bronchodilator levalbuterol.

To quantify the magnitude of non-specific AHR in EPA horses, we determined the concentration of methacholine that elicits a 40% increase in baseline R_L ($PC_{40}R_L$) in 10 EPA affected horses. This threshold increase in R_L was selected based upon methods for methacholine challenge in non-compliant humans (26), because of the inability of horses to voluntarily mount a forced expiratory maneuver (339). EPA horses achieved their $PC_{40}R_L$ at methacholine concentrations $\leq$1.0mg/ml, which have been previously considered to be diagnostic of moderate to severe human asthma (24). Ten non-diseased horses failed to react to methacholine doses of 8-16 mg/ml, congruent with methacholine reactivity described for non-asthmatic human subjects (24). These results are of interest because they substantiate that the methacholine reactivity of both normal and EPA horses is physiologically relevant to non-asthmatic and asthmatic humans, respectively. This is in contrast to the commonly employed mouse asthma models, whose magnitude of AHR at baseline and following antigen sensitization, as measured by methacholine responsiveness, is not well aligned to that of non-diseased and asthmatic humans (27, 79, 316, 317). In addition, during methacholine bronchoprovocation clinical signs associated with asthma including increased abdominal effort, higher respiratory

frequency and coughing were noted in EPA horses with increasing methacholine concentrations (data not shown). Congruent with previous findings for human asthma (26), baseline R_L and the magnitude of AHR, as documented by the $PC_{40}R_L$ during methacholine challenge, differed between horses in this investigation.

Like human asthmatics, EPA horses were demonstrated in this investigation to have non-specific AHR that is persistent. Persistence of non-specific AHR to methacholine was confirmed in 5 EPA affected horses with the durations between re-evaluation being 3 to 34 months. Though the sample size of 5 horses tested for persistence could influence the lack of statistically significant differences in $PC_{40}R_L$ between the 2 time points, two points are noteworthy. First, 3 of 5 horses achieved their $PC_{40}R_L$ at near identical methacholine concentrations at each of the two separate evaluations. Second, the $PC_{40}R_L$s of the remaining two horses during the two separate evaluations were within a single doubling dilution. In its guidelines for methacholine challenge, the American Thoracic Society has previously reported that methacholine reactivity within 1.5 doubling doses over short time periods is not considered significantly different (27). Accordingly, there is strong evidence for persistent and non-specific AHR in EPA.

Mouse models sensitized to ovalbumin and house dust mite, as well as rat and guinea pig asthma models, which are commonly employed (247, 271, 273, 316, 340), present several shortcomings as asthma models that were not identified in EPA horses. First, rodents do not spontaneously develop asthma (248, 252, 271, 340–343). Second, rodents do not develop AHR that persists for protracted periods beyond the duration of antigen sensitization (247, 334, 344). Further, as previously indicated, in most rodent asthma models the magnitude of AHR, as measured by methacholine reactivity, is not within

physiologic ranges that are typical of human asthma (247, 316). Finally, rodent models develop tolerance to their sensitizing antigen, which is also not a characteristic of human asthma (263, 334, 344). By contrast, EPA is a lifelong disease that worsens in successive years in affected horses that remain in the southeastern US (7), indicating that specific AHR persists in EPA horses for life. Similarly, in the BDA form of SEA commonly termed heaves, which is a spontaneous asthma model, tolerance to the sensitizing antigen most commonly employed (moldy hay) has not been reported. However, persistence of non-specific AHR to methacholine after hay and straw challenge has been reported to persist for as few as 3 days following antigen sensitization (89). Though rodent asthma models are cost effective, with an unprecedented array of immunologic reagents and ability to generate diverse strains necessary to adequately investigate the molecular underpinnings of asthma, the inability of rodent models to recapitulate aspects of AHR that are well aligned between EPA and human asthma highlights the strengths and relevance of EPA as a translational asthma model.

While addressing important aspects of AHR, the neutrophilic airway inflammation that characterizes EPA BALF also supports the relevance of EPA to a population of asthmatics that are not well modeled by other animal models. Murine models are characterized by eosinophilic inflammation rather than neutrophilic (27). This makes them ineffective in modeling the population of asthmatics that are demonstrated to be most likely to have fatal attacks (155). Further, while some investigations have identified eosinophilic airway inflammation in more severe asthmatics (345), Wenzel et al. (149) identified a subset of people suffering from severe asthma characterized by predominately neutrophilic inflammation. Though the neutrophilic phenotype has been

reported to be present in approximately 50% of patients with symptomatic asthma (346), it remains poorly modeled in translational studies where eosinophilic airway inflammation predominates (247, 265, 317, 343). Neutrophils also predominate in people with corticosteroid resistant asthma, which is now considered a defining criteria for severe asthma (347). Accordingly, EPA presents a translational asthma model that encompasses aspects of AHR that have been previously associated with severe human asthma, while also demonstrating neutrophilic BALF infiltrates that segregate with severe asthma, indicating that EPA provides an appropriate animal model for the study of non-eosinophilic, severe asthma.

While working with a large animal species has associated increased costs, EPA affected horses used in this investigation had been previously diagnosed with documented histories of seasonally recurring asthma signs and exhibited naturally occurring asthma exacerbation of sufficient severity and consistency to document reversible airway obstruction using conventional pulmonary mechanics. The seasonal recurrence of EPA exacerbations provides high fidelity as a disease for the study of severe asthma based upon its natural occurrence, chronicity, and pathologic and physiological similarities to severe, non-eosinophilic asthma in humans. The prevalence of EPA in a region of the United States seeing a significant increase in the number of adults reporting to be severely asthmatic increases the relevance of further study of EPA. Here we have demonstrated that an equine asthma model with spontaneous reversible airway obstruction and neutrophilic BALF inflammation recapitulates persistent and non-specific AHR that characterize severe human asthma, in a manner that other translational models lack.

Acknowledgements

We gratefully acknowledge the skilled assistance of many student workers and summer interns including Melissa Steichen, whose efforts in training horses to the pulmonary equipment were integral to this study.

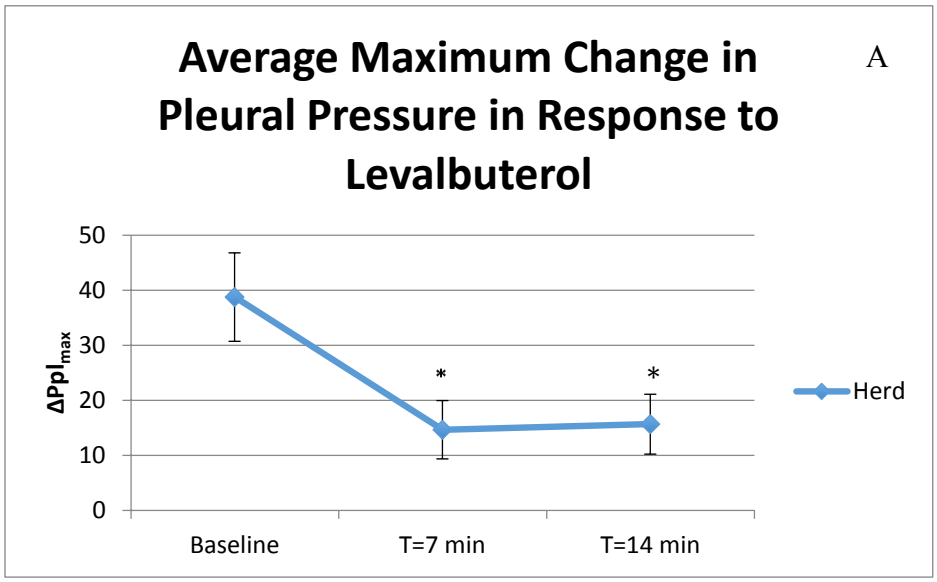

Figure 1 Horse with EPA have reversible airway obstruction.

The average maximum change in pleural pressure (A), resistance (B), and dynamic compliance (C) of 8 horses experiencing EPA exacerbations (baseline) and changes in these parameters at 7 and 14 minutes following the nebulization of 1.25mg of levalbuterol are graphed. Error bars represent standard error of the mean. * significantly different from T=o ($p<0.05$)

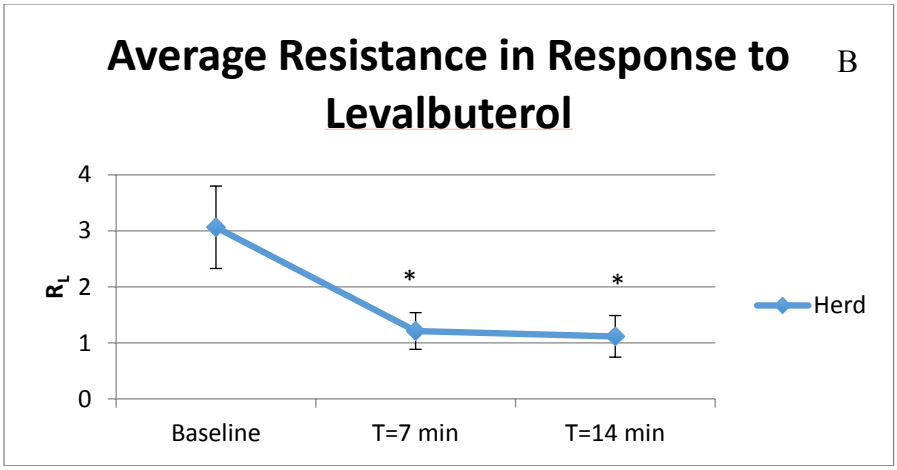

Figure 1 (continued)

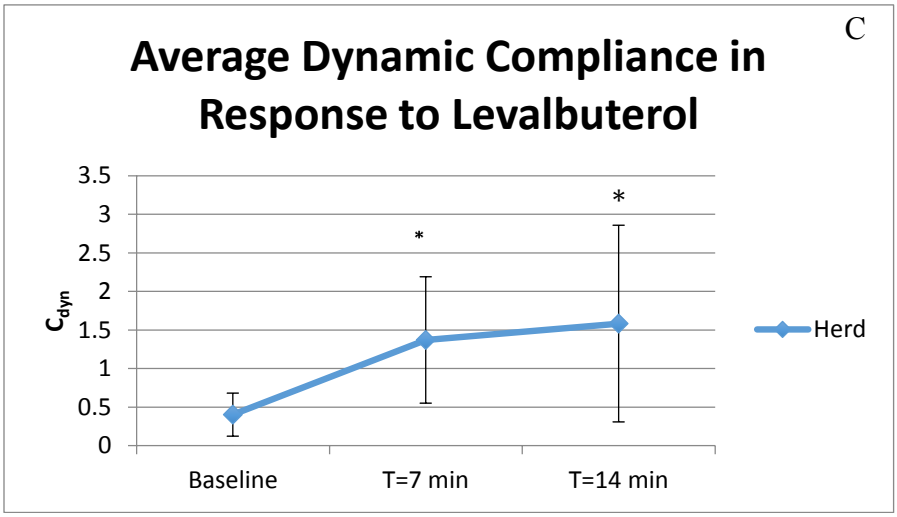

Figure 1 (continued)

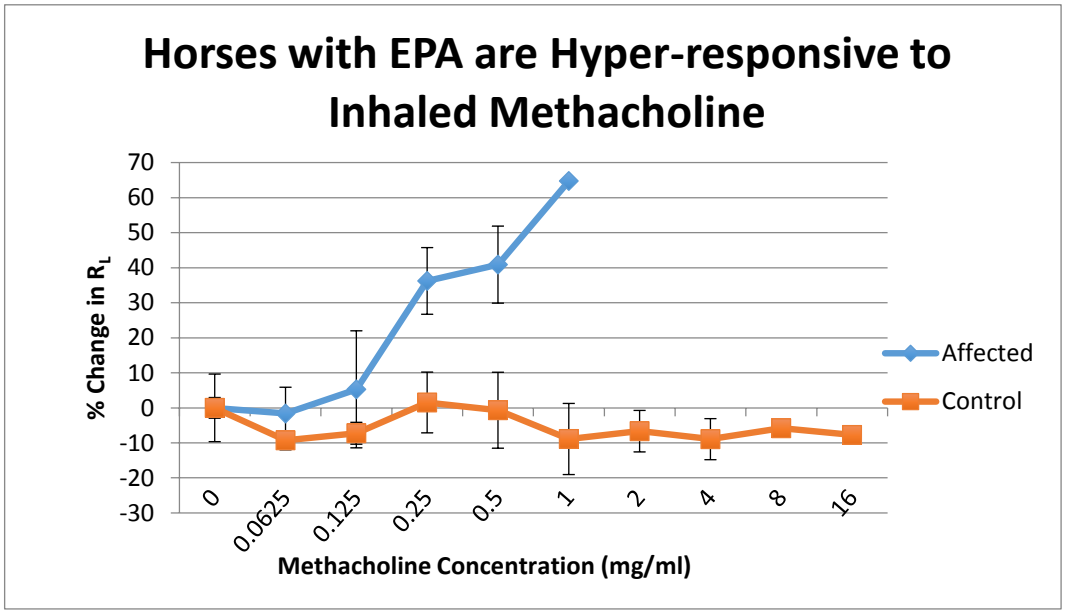

Figure 2 Horses with EPA are hyper-responsive to inhaled methacholine.

The average % change of lung resistance (RL) from baseline was determined for horses with EPA and clinically normal horses in response to serially increasing concentrations of inhaled methacholine. Concentrations of methacholine <1mg/ml induced a 40% increase in baseline resistance ($PC40R_L$), while control horses failed to demonstrate significant increases in RL to methacholine concentrations up to 8-16 mg/ml ($p<0.0014$). Error bars represent the standard error of the mean.

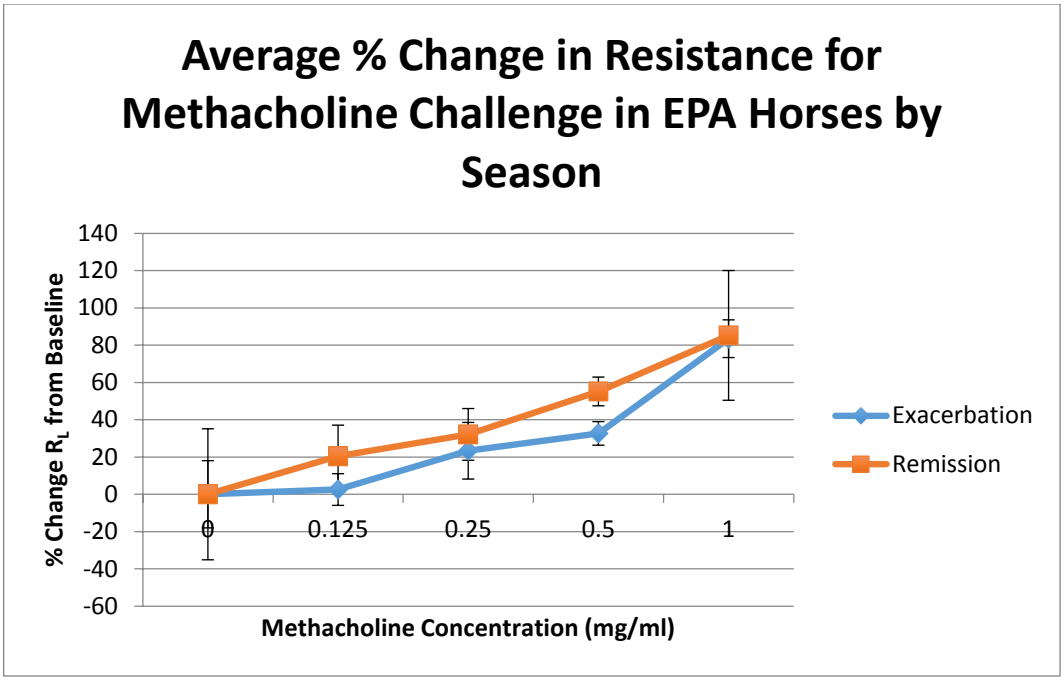

Figure 3 Average % change in resistance for methacholine challenge in EPA horses by season.

Average % change from baseline in resistance for EPA affected horses in exacerbation and remission seasons. Graphs included bars for standard error the mean.

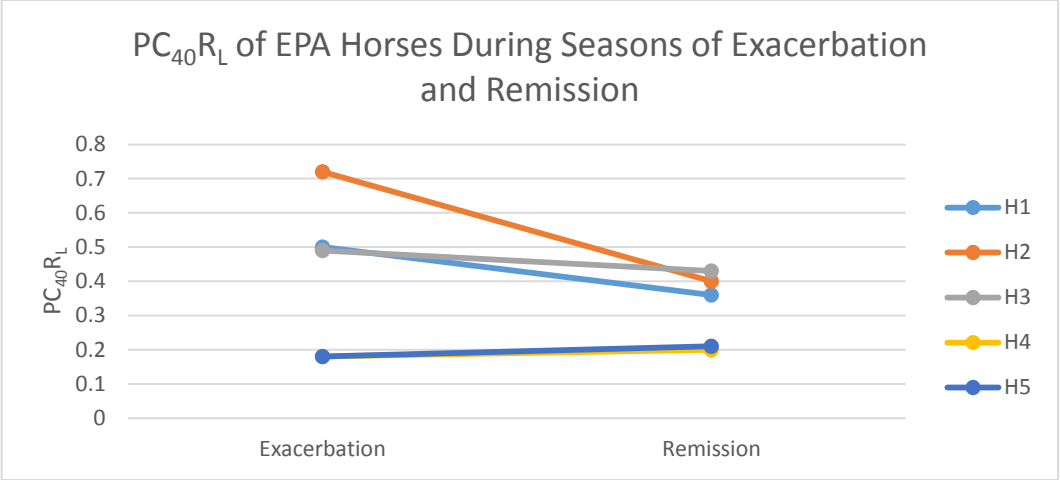

Figure 4 $PC_{40}R_L$ of EPA horses during seasons of exacerbation and remission.

PC40RL of five EPA horses receiving methacholine bronchoprovocation in seasons of exacerbation and remission. No significant difference was found between seasons for any of the horses tested (p=0.2176).

Table 4 Comparison of $PC_{40}R_L$ between horses in exacerbation and remission seasons

Horse	$PC_{40}R_L$ in Exacerbation (mg/ml)	$PC_{40}R_L$ in Remission (mg/ml)	Months Between Exacerbation and Remission Testing
1	0.50	0.36	34
2	0.72	0.40	30
3	0.49	0.43	3
4	0.18	0.20	19
5	0.18	0.21	9

References-see Appendix

CHAPTER III

INCREASED HCN4 EXPRESSION ASSOCIATED WITH AIRWAY SMOOTH MUSCLE DURING ASTHMA-LIKE DISEASE IN A SPONTANEOUS ANIMAL ASTHMA MODEL

Main

Airway hyper-responsiveness (AHR) is a defining criterion of asthma that is impacted by airway smooth muscle excitability (348). Decreasing AHR in asthmatics heralds decreased asthma severity (27), making it a pharmacologic goal in the development of asthma therapeutics (349). Hyperpolarization Activated Cyclic Nucleotide-Gated Potassium (HCN) channels generate hyperpolarizing ('funny') current (I_h or I_f) that regulates membrane excitability in cardiac muscle and nerves (350), in association with smooth muscle in certain organs systems (351–354). HCN channels have recently been demonstrated to modulate autonomic control of ASM in guinea pigs (355), but have not been identified in ASM. Herein, we demonstrate a significant increase in HCN4 message in lung tissue during exacerbation of a spontaneous asthma-like disease in horses. Validation of this finding in a separate cohort of horses with asthma identified increased

expression of the HCN4 protein to be localized to ASM, and to a lesser extent airway epithelium during asthma exacerbations. Less HCN4 protein was identified in ASM collected during asthma remission relative to exacerbation, but HCN4 protein was not identified in lung tissue from control horses. The HCN4 channel blocker ivabradine caused dose-dependent inhibition of carbachol-induced contraction in isolated bronchi from horses with asthma, as well as from control horses. Collectively, these findings indicate that HCN4 mediated current in ASM contributes to airway contractile responses.

HCN4 function has been deciphered in the SA node, where I_f current determines heart rate by influencing the rate of diastolic depolarization (356). Accordingly, the presence of HCN4 channels in ASM identified in this work indicates that I_f current has a role in ASM excitability. Increased HCN4 expression in ASM during asthma-like disease then advances a paradigm in which changes in the magnitude of I_f current contribute to ASM excitability, presenting the potential to direct novel asthma therapeutics.

Associations between long-acting β_2-adrenoceptor (β_2-AR) agonist use and severe asthma exacerbations (357) and asthma related deaths (358) highlight a critical need for novel asthma therapeutics. Translational discovery is challenged by commonly employed animal asthma models which are induced and do not adequately mirror the human condition (299, 334). To improve the translational relevance of discovery, we investigate the spontaneous equine asthma model (14, 299, 334), specifically the endotype that affects horses grazing pasture during conditions of heat and humidity termed Summer Pasture Associated Recurrent Airway Obstruction (SPARAO) (10), and more recently termed Equine Pasture Asthma (EPA) (7). EPA affected horses demonstrate clinical

features of asthma including reversible airway obstruction, airway hyper-responsiveness (359), chronic inflammation and asthmatic airway remodeling (6). EPA horses exhibit seasonal exacerbation remission cycles and are clustered in the southeastern United States (10). Neutrophils rather than eosinophils predominate in the BAL fluid of affected horses (22), mimicking a population of human asthmatics that are not well modeled by eosinophilic airway inflammation that predominates in rodent asthma models (299, 334).

Genes segregating with asthma-like disease were identified using differential expression analysis (RNA Sequencing) by comparing transcriptomes from serially sampled lung biopsies. Biopsies were collected during disease remission and exacerbation in both EPA-affected and non-diseased control horses. 1376 genes had seasonal differential expression conserved in diseased horses but were not differentially expressed by season in control horses (Online supplement table 1). Among these 1376 DEGs were genes with demonstrated relevance to asthma (Online supplement table 2), including potassium calcium-activated channel subfamily M regulatory beta subunit 1 (KCNMB1) (360), tumor necrosis factor (TNF) (361), and synaptotagmin (362), substantiating the translational relevance of our approach to the human condition. Based on evidence that β_2-AR signaling is necessary for the asthma phenotype in a murine model (363), and that long-acting β_2-AR agonist use increases severe asthma exacerbations (357) and asthma related deaths (358), we sought genes with functional relevance to β_2-AR signaling that were not previously investigated in asthma to identify novel facets of asthma pathophysiology. Among these, (HCN4), belonging to the gene family that regulates membrane excitability (350), was targeted based upon evidence of

strong selective differential regulation directly linked to asthma-like disease (e.g. raw read counts approaching zero during disease remission in both affected horses and in control horses with significant expression during seasonal exacerbation only in diseased horses).

Though there is ample evidence of HCN4 transcription in human lung expression databases (363, 364), evidence of HCN4 translation in the lung and its cellular location are absent. To confirm increased HCN4 expression at the protein level in our asthma model, and to localize its cellular expression, HCN4 protein was stained in archival lung tissues collected from an unrelated cohort of EPA-affected and age matched control horses. Immunohistochemical staining confirmed strong HCN4 protein expression in ASM, and to a lesser extent in airway epithelium during disease exacerbation (Fig 1A-C), contrasting non-diseased age matched control lung harvested during seasonal disease exacerbation in which HCN4 staining was not identified (Fig 1D-F). In lung harvested from diseased horses during seasonal disease remission, HCN4 staining remained evident but reduced in ASM and was rare in bronchial epithelium (Fig 1 G-I), in comparison to HCN4 staining during clinical disease (Fig 1 A-C). HCN4 staining in ASM of diseased horses during remission (Fig 1 G-I) was clearly greater than the nadir of expression demonstrated by control horses (Fig 1 D-F).

To identify the effect of HCN4 inhibition on bronchial ring contractility, we tested the effect of various concentrations of ivabradine, a known specific HCN4 blocker, on tensions generated by isolated equine bronchi in response to the constriction agent carbochol. Evaluations were performed on the day of lung harvest and repeated 24 hours

later. Disease status had no significant effect on the degree of contraction generated. However, this finding may reflect the fact that only 3 of 12 EPA horses employed in this investigation were euthanized during disease exacerbation when HCN4 expression in ASM is significantly increased. Significant interactions between concentrations of ivabradine and carbachol were identified, highlighting the need for further investigation into the mechanistic basis of ivabradine inhibition of ASM contractile responses.

Materials and Methods

Animal Tissues

A total of 27 horses, 12 with a diagnosis of EPA and 15 control horses without EPA were used in this investigation. Diagnosis of EPA was based on seasonally recurring and reversible obstructive respiratory disease when exposed to pasture during summers in Louisiana or Mississippi. Signs of disease included overt respiratory distress, pronounced expiratory wheezes throughout the lung fields, fluid in the trachea upon auscultation, intermittent cough, and airway neutrophilic inflammation (>10% non-degenerate neutrophils in BAL fluid and/or histologic evidence of neutrophilic airway inflammation in lung sections). Control horses lacked evidence of respiratory disease and had <3% neutrophils in BAL fluid and/or an absence of neutrophilic airway inflammation in lung sections. In 26 of the 27 horses, the diagnosis as EPA or control was confirmed at necropsy from gross and histologic pathology as we have described previously (6,22) During disease remission, EPA-affected horses had normal bronchovesicular sounds during auscultation of the lungs with the aid of a rebreathing bag. Limited neutrophilic airway inflammation was confirmed in EPA-affect horses during remission by <10%

non-degenerate neutrophils in BAL fluid and/or direct histologic evaluation of the airways as described (6,22). Airway hyper-responsiveness was confirmed and quantified using methacholine bronchoprovocation in 6 of the 12 EPA horses, as described in the previous chapter, identifying a 40% increase in lung resistance in response to methacholine doses $\leq$1 mg/ml. Tissues were harvested from animal procedures approved by the Animal Care and Use Committee of Mississippi State University except for archival tissue used for immunohistochemical staining which was harvested from animal procedures approved by the Animal Care and Use Committee of Louisiana State University.

Serial lung biopsies for transcriptome analysis were acquired from two EPA affected horses during asthma exacerbation and remission via standing thorascopic surgery. Diseased horses were paired to non-diseased control horses sharing the same environment that were similarly biopsied. EPA affected horses were Tennessee Walking Horses: an 18-year-old castrated male and a 17-year-old female. Control horses were castrated male American Quarter Horses aged 18 and 13. Lung biopsies were stored at -80°C in RNALater preservative (Ambion, Austin, TX).

Immunohistochemical staining was performed on archived formalin-fixed, paraffin embedded lung. Tissues had been collected immediately following humane euthanasia from 3 EPA-affected horses (7, 16, and 19 years old) and 3 age matched non-diseased control horses that were euthanized during seasonal disease exacerbation. This diseased cohort included 1 Appaloosa, 1 Quarter Horse, and 1 Welsh Pony; 2 castrated males and 1 female; controls included 2 Quarter Horses and one American Saddlebred, 1

castrated male and 2 females. 2 female EPA-affected horses, both Quarter Horses, 12 and 17 years of age, were euthanized during disease remission. The magnitude of neutrophilic inflammation and gross postmortem findings in this EPA cohort has been previously correlated to disease severity and peribronchial inflammation (22).

Bronchial rings were harvested from the right lung of seven EPA-affected and ten clinically normal horses for use in tissue bath analysis. EPA affected horses consisted of 6 castrated males and 1 female, mean age=17.0 years (sd=6.68), 4 Quarter Horses, 1 Paint, 1 Appaloosa, and 1 Mustang. Control horses consisted of 6 castrated males, 4 females, mean age= 18.1 years (sd=7.89), 9 Quarter Horses, and 1 Dutch Warmblood. Horses were sedated with xylazine (0.33-0.66 mg/kg) followed by humane euthanasia with sodium pentobarbital (100mg/kg). The lung was quickly removed from the thorax and placed in chilled modified Krebs-Henslett solution (KHS), which was also poured into the right mainstem bronchus. Bronchial rings (approximately 4-5mm in diameter and 3 mm thick) were dissected from the right caudodorsal lung lobe and incubated in KHS in 24 well plates at 4^{o} C until assayed.

RNA Sequencing and Differential Expression Analysis

Total RNA was isolated from equine lung biopsies using Qiagen RNeasy Maxi protocol (Qiagen Inc., Valencia, CA, USA) per the manufacturer's instructions. Paired end sequencing of isolated RNA (RIN= 7.1-9.3) was performed using Illumina TrueSeq v2. with Hi-Seq 2000 v3. chemistry (Illumina, San Diego, CA). Differential expression analysis was limited to the forward reads. Filtration and trimming were performed using FAST QC (Brabham bioinformatics,

http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc). Reads were aligned to the equine reference genome (GenBank Assembly ID: GCF_000002305.1) with Bowtie 2[1s] (http://bowtie-bio.sourceforge.net) and differential expression determined using ERANGE[2s].

Gene products with significant differential expression (DEGs) between seasonal exacerbation and remission at a 5% false discovery rate (FDR) were identified for each horse, sorted, and matched to identify a set of DEGs common to both affected horses and a second set of DEGs common to both control horses. From these two sets, differentially expressed gene products present in affected horses, but absent in control horses, were identified.

Immunohistochemistry

Translation of HCN4 mRNA, its differential expression in association with disease, and cellular localization were confirmed using immunohistochemistry. Lung sections were incubated at 75°C to remove excess paraffin. Slides were placed in serial alcohol dilutions for a total of three minutes to remove excess paraffin: 100% xylene, 100% xylene, 1:1 xylene to 100% EtOH, 100% EtOH, 100% EtOH, 95% EtOH, 70% EtOH, 50% EtOH, and deionized water to rinse. Antigen retrieval was performed for 30 minutes using a vegetable steamer. Dako's target retrieval solution (Dako North America Inc., Carpinteria, CA, USA) was used for antigen unmasking. Slides were treated with 0.2% Triton-X100 (Sigma-Aldrich, St. Louis, MO, USA) in TBS for 20 minutes to induce membrane permeability and blocked with Dako's serum free universal protein block (Dako Inc). To minimize background, an avidin/biotin blocking step was performed

according to manufacturer's instructions (Vector Laboratories Inc., Burlingame, CA, USA).

Slides were incubated with rat monoclonal anti-HCN4 (Abcam ab32675, Cambridge, MA, USA), 1:1500 for 16 hours and then treated with H_2O_2 for 10 minutes. A biotinylated link and streptavidin conjugated to HRP were applied for 45 minutes (Dako LSAB+ Detection System), followed by DAB chromogen for 10 minutes (Dako). Slides were then counterstained with hematoxylin for 2 minutes, dipped in 0.3% ammonia, mounted and cover slipped. Differential staining for HCN4 in lung samples was evaluated using light microscopy by a board certified veterinary pathologist.

Effect of Ivabradine on Carbachol Induced Bronchial Contraction

Each tissue bath assay consisted of four bronchial rings that were suspended to force transducers in the EMKA Bath 4 system (Emka Technologies, Falls Church, VA). During harvest of the bronchial rings, efforts were made to isolate at least 4 rings from a single bronchus to populate each assay. Prior to assay, bronchial rings were gently blotted with filter paper, and trimmed to identical mass within 0.01 grams. Bronchi were suspended to force transducers within isolated baths and equilibrated with 2g tension for 120 minutes in KHS with carbogen (95% O_2, 5% CO_2) aeration. Two bronchi were pretreated with ivabradine for 15 minutes, beginning at 1uM. Carbachol reactivity was characterized for all four bronchi using serially increasing half log concentrations of carbochol (0.1nM-33uM) every 15 minutes. Force (g) was measured continuously and recorded in 5 minutes intervals. The process was repeated with 4 new bronchial rings for each additional ivabradine concentration: 3, 5, and 10uM. In addition, bronchial rings were

incubated overnight at 4 °C in KHS and the experiment was repeated the following day. The contractile response of each bronchial ring was then calculated as a percentage of the maximal contraction achieved.

Statistical Methods

Effects of disease status, ivabradine dose, and carbachol dose on the contractile response were assessed in separate mixed multivariable linear regression models developed for Day 1 and Day 2 measurements using PROC MIXED in SAS for Windows 9.4 (SAS Institute, Inc., Cary, NC). Disease status, ivabradine dose, carbachol dose, all two-way interactions, and the three-way interaction were initially included as fixed effects. The dose group within horse and horse identity were included as random effects in the models. The interaction terms, starting with the three-way interaction term, were sequentially removed from the models if not statistically significant. Disease status, ivabradine dose, and carbachol dose remained in all models even when they were deemed to not have a statistically significant effect. A LSMESTIMATE statement with SIMULATE adjustment for multiple comparisons was used to make comparisons between ivabradine dose at each carbachol dose for the ivabradine dose x carbachol dose interaction in both models. In addition, for the Day 2 model, comparisons were made between affected and control groups at each carbachol and at each ivabradine for the Disease Status x carbachol dose and Disease Status x ivabradine interactions, respectively. While Disease status was initially significant, significance did not hold when broken down by ivabradine and carbachol dose. The distribution of the conditional residuals was evaluated for each model to assess if the assumptions of

normality and homoscedasticity had been met. An alpha level of 0.05 was used to determine statistical significance.

Discussion

Employing a self-controlled RNA sequencing protocol in which serial lung biopsies were collected during asthma exacerbation and remission in horses with naturally occurring severe asthma and paired controls, HCN4 message was identified to be increased during asthma exacerbation. Using immunohistochemistry, increased HCN4 protein expression was confirmed in the lung of a second cohort of asthmatic horses that were sacrificed during exacerbations of severe asthma. Histochemical staining localized the increase in HCN4 protein expression during asthma exacerbations to ASM and epithelium. Further, lung from age matched asthmatic horses that were sacrificed during asthma remission also demonstrated HCN4 expression in ASM that was decreased relative to HCN4 expression in ASM of asthmatic horses sacrificed during asthma exacerbation. By contrast, HCN4 protein was not identified in the lung of age matched control horses using immunohistochemistry. Collectively, these findings indicate that HCN4 expression is constitutively increased in epithelium and ASM of horses with asthma during remission with a significant increase occurring in ASM in association with asthma exacerbations. To our knowledge, this is the first report of differential expression of HCN4 and its localization to ASM and epithelium in association with asthma.

HCN channels, as reviewed by Postea (350) are six transmembrane domain, single pore-loop cation channels capable of activation by membrane hyperpolarization or cyclic nucleotide binding. Four HCN isoforms (HCN1-4) are characterized with differing tissue

distributions, activation kinetics, and cAMP sensitivity. The resulting cation current is termed funny current (I_f) because channel opening is activated by membrane hyperpolarization at the negative cellular voltage extremes that follow action potentials, a reverse of the voltage dependence that characterizes most ion channels. In this manner, HCN channels provide excitable tissues the unique ability to depolarize without external influences through sustained positive increases in membrane potential that are initiated by hyperpolarization and raise the membrane potential to the threshold for subsequent depolarization. This characteristic contributes to membrane responsiveness and makes funny current a pacemaker of rhythmic nerve and muscle firing.

Based upon the inherent ability of increased HCN4 expression in both epithelium and ASM to alter muscle membrane potentials, we contrasted the contractile responses of isolated bronchial rings from asthmatic horses and controls in the presence and absence ivabradine, an HCN4 antagonist. Ivabradine caused a dose dependent right shift in the percent maximal contraction versus carbachol concentration curve in airways from both asthmatic and control horses. These results differ from the absence of altered contraction during HCN inhibition described by McGovern et al. (365) using isolated tracheal strips from guinea pigs. Several differences in the experimental protocols are noteworthy. First, there is substantial evidence that contractile responses of trachealis muscle and ASM are inherently different in both guinea pig and equine asthmatics to account for the differing behavior observed between the two experiments (42, 366). Next, in the guinea pig experiments, HCN channel blockers were added 60 minutes before contraction agents. In

our investigation, the initial ivabradine equilibration was 15 minutes with serial carbachol concentrations increasing in 15-minute increments. The time of effectiveness with HCN channel inhibitor treatment should be further investigated. Also, the guinea pig trachealis strips were pre-incubated with 2 μM propranolol and 3 μM indomethacin which differs from our protocol in which the only agents within the tissue bath protocol were ivabradine and carbachol.

For an in vivo analysis, guinea pigs were anesthetized and mechanically ventilated for pulmonary inflation pressure (PIP) measurements in response to intravenous histamine. The HCN4 channel inhibitor ZD7288 caused significant bronchoconstriction. However, the authors note that their study was done on large airways and could not rule out a different response in the small airways to HCN channel inhibition. The proposed mechanism of this finding centers on an unmasked NK receptor activation of C-fibers in the airways. However, there are interspecies differences in the ability of tachykinins to effect trachea and bronchial smooth muscle tone (367, 368). For example, the electrical field stimulation bronchoconstriction of isolated guinea pig bronchi mediated by tachykinins is completely reversed in mice and rats (367). In these models, EFS causes a tachykinin dependent bronchodilation (368). In BDA affected horses, NK-2 receptors are significantly increased in bronchial epithelium and ASM compared to non-diseased horses (369). Neurokinin A and B also caused bronchoconstriction in BDA affected bronchial rings (369). The role of tachykinins has yet to be studied in the EPA variant of SEA, however. This, coupled with our findings, highlight a future need to study the effect of ivabradine at the small airways.

Finally, the guinea pig is an induced model for asthma with a predominance TH2, IgE mediated eosinophilic inflammation which differs from equine asthma in which neutrophilic inflammation predominates (22, 340). While these differences could alter the relevance of HCN4 current to airway contraction, it is noteworthy that HCN4 inhibition was efficacious in decreasing the contractile responses of bronchi from normal controls, suggesting that species-specific pharmacodynamic interactions with ivabradine or inherent species differences in HCN4 current may also be relevant.

The finding that ivabradine decreased contractile responses of both asthmatic and control bronchi was unanticipated. We hypothesize that this may reflect the fact that only 3 of 7 asthmatic horses were euthanized during the season of disease remission when, based upon our findings at both the transcript and protein level, HCN4 expression would be predicted to be decreased in the lung and ASM. Though HCN4 expression in ASM of horses with pasture asthma that were euthanized during remission was increased relative to that of non-diseased control horses, we did not identify differences in the ivabradine mediated decrease in carbachol contractile responses between diseased and control horses. This suggests that this difference is not physiologically significant in the in vitro model system that was employed. A challenge to this experimental protocol is the fact that horses with equine pasture asthma became available for the experimental protocols reported here through spontaneous donations, the timing of which was not under the control of the investigators. Similar experimental protocols that contrast ivabradine's effect on airway contractility in association with a determination of HCN4 expression in the airways from larger cohorts during exacerbation and remission are warranted.

Nonetheless, the primary finding that ivabradine moderated carbachol induced airway contraction at concentrations that are attained using standard human ivabradine dosing regimens remains relevant.

We also observed a significant difference in the ivabradine interaction with carbachol between day 1 and day 2 in our study. We hypothesize that the abundance of day 1 manipulations, which included the actual tissue harvest and bronchial ring dissection, prolong the tissue's ability to reach a true equilibrium. Following 24 hours in Krebs-Henslett, bronchial rings were more stable in their responsiveness, allowing more significant interactions to be observed. It is also feasible that following 24 hours of incubation, medications known to alter muscle contractility that were used for euthanasia (sodium pentobarbital and xylazine) (370, 371) had decreased activity through a combination of diffusion and metabolism during incubation. Interestingly, we have observed a similar phenomenon in day 1 versus day 2 precision cut lung slices from horses.

In the heart, the magnitude of funny current resulting from HCN4 channel activation regulates the slope (duration) of phase 4 diastolic depolarization; determining the frequency of spontaneous action potential firing of SA myocytes and, therefore heart rate. The specific HCN4 channel blocker ivabradine, binds to an intracellular site in the HCN4 channel pore, inhibiting If current, which blunts the diastolic depolarization rate and decreases heart rate (372). Cyclic nucleotide gating makes HCN channels also responsive to the autonomic nervous system (350). HCN4 channels, which co-localize with B_2-AR in the heart (373), are slowly gating and strongly sensitive to cAMP.

Ivabradine has been demonstrated to increase cAMP which would account for the moderation of airway contraction observed in this investigation. cAMP binds directly to f-channels and shifts the I_f activation range to more positive voltages, increasing I_f channel open probability. In the heart, this increases the steepness of diastolic depolarization, shortening its duration and causing heart rate acceleration. Autonomic regulation of heart rate is accordingly achieved by increases or depletion of cAMP that result from β-adrenergic or muscarinic M2 receptor stimulation, respectively. Recently, cyclic dinucleotides have been demonstrated to inhibit HCN4 opening, mimicking the effects of muscarinic I_f modulation and providing a mechanism for crosstalk with the immune system where cNDP signaling is well recognized (374).

Beyond the effects of increasing cAMP, another scenario for the observed moderation of airway contractile responses in this investigation is that HCN4 mediated I_f current raises ASM membrane potential to thresholds that activate other voltage gated ion channels including voltage gated calcium channels, providing an alternate route of excitation to the GPCR-IP_3-mediated Ca^{+2} release from SR that predominates in ASM contraction. Myometrium, which shares this primary contractile pathway with ASM and has a parallel dependence on intracellular Ca2+ stores for contraction, exhibits changes in the calcium machinery of the gravid rat uterus to support this hypothesis. Hyperpolarization-activated inward currents are identified in advancing pregnancy in rat myometrium (375) and both L-type and T-type voltage gated Ca2+ channels (376) as well as gap junctions (377) increase in gravid myometrium with advancing gestation. The resulting current contributes to maintenance of the resting membrane potential and

spontaneous activity in smooth muscle cells of late pregnant rats (375). I_f current in ASM also indicates a mechanistic explanation for the paradoxical ability of β_2-AR agonists to mediate bronchodilation in most asthmatic patients, while simultaneously effecting bronchoconstriction in more severe asthmatics. Specifically, while β_2-AR agonist mediated increases in cAMP drive ASM relaxation through PKC signaling and downstream effects on MLC-20, cAMP gating simultaneously increases HCN4 channel opening, facilitating ASM contraction via a depolarizing shift in the ASM membrane potential. The outcome of β_2-AR signaling would thus reflect the balance between PKC driven ASM relaxation and HCN4 driven ASM contraction. From this line of evidence emerges the hypothesis that I_f modulation may be of value in changing ASM responsiveness in certain populations of asthmatics.

Overall this work is the first to report the preferential transcription and translation of HCN4 in association with ASM in a spontaneous asthma model. It is also the first to demonstrate moderation of ASM contraction via inhibition of HCN4 in an asthma model. This work highlights the need for further investigations that characterize the effect of ivabradine on ASM contraction in horses with EPA during disease exacerbation and remission, as well as the degree of effect at large compared to small airways. Interpreted with the documented role of HCN4 in regulating membrane excitability, our findings indicate a role for I_f current in ASM in asthma and highlight the need to decipher the roles of HCN4 in bronchial hyper-responsiveness and bronchoconstriction to determine the therapeutic potential of I_f modulation in asthma management.

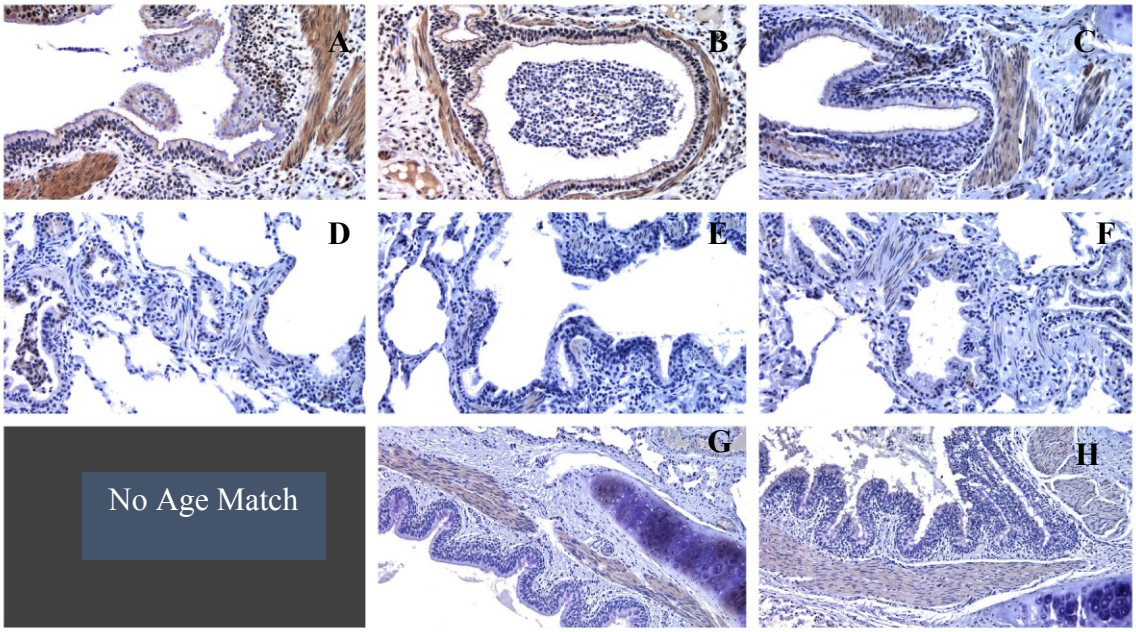

Figure 5 HCN4 protein expression increases in ASM and airway epithelium during disease in the equine asthma model, and decreases during remission, maintaining constitutive expression in diseased ASM exceeding that of non-diseased horses.

HCN4 stained lung sections collected during disease exacerbation from three SPARAO affected horses (panels A-C, ages 7, 16, and 19; respectively), and their respective age and season matched lung sections from non-diseased control horses (panels D-F), demonstrate strong HCN4 staining in ASM and to a lesser extent in the apical regions of epithelial cells during disease. In panels G-H HCN4 stained lung sections collected during disease remission (ages 12 and 17 respectively) identify constitutive HCN4 staining in ASM that is less than observed during disease exacerbation (Panels A-C) but exceeds non-diseased controls. HCN4 staining is no longer evident in airway epithelium during disease remission. No comparable remission age match was available for the first column.

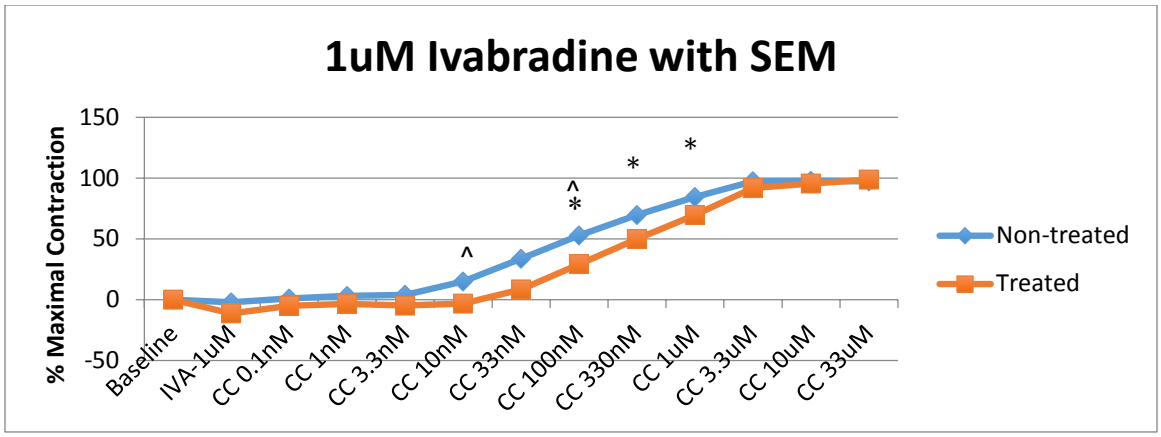

Figure 6 Ivabradine decreases bronchial hyper-responsiveness to carbachol mediated contraction.

The ability of ivabradine (1μM), ivabradine (3μM), ivabradine (5μM), ivabradine (10μM), to moderate carbachol induced contractile responses was evaluated using two ivabradine treated and two nontreated control bronchi isolated from 17 horses. Results are graphed as the average percentage of maximal contraction of 17 horses. Error bars represent SEM. * are significantly different on day 1, and ^ are significant on day 2 ($p \leq 0.05$).

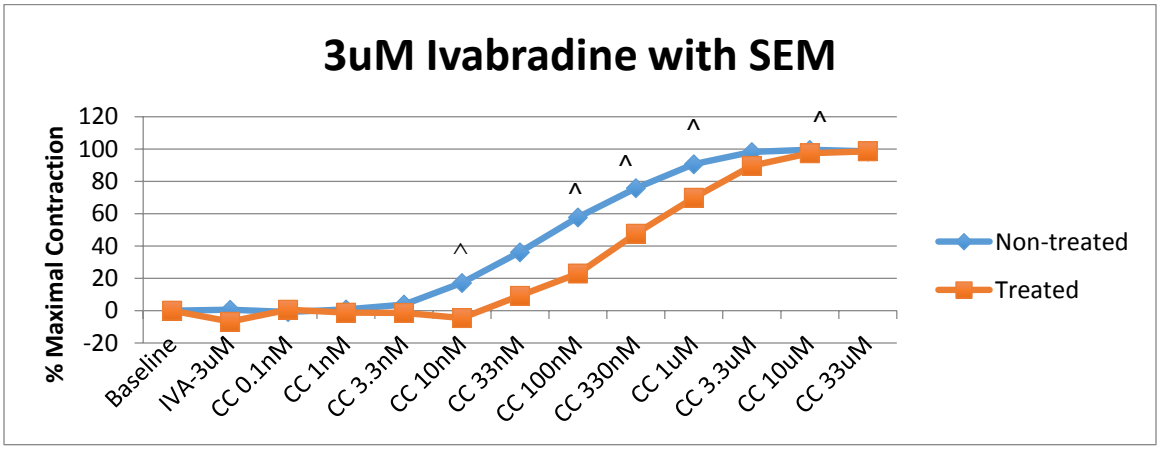

Figure 6 (continued)

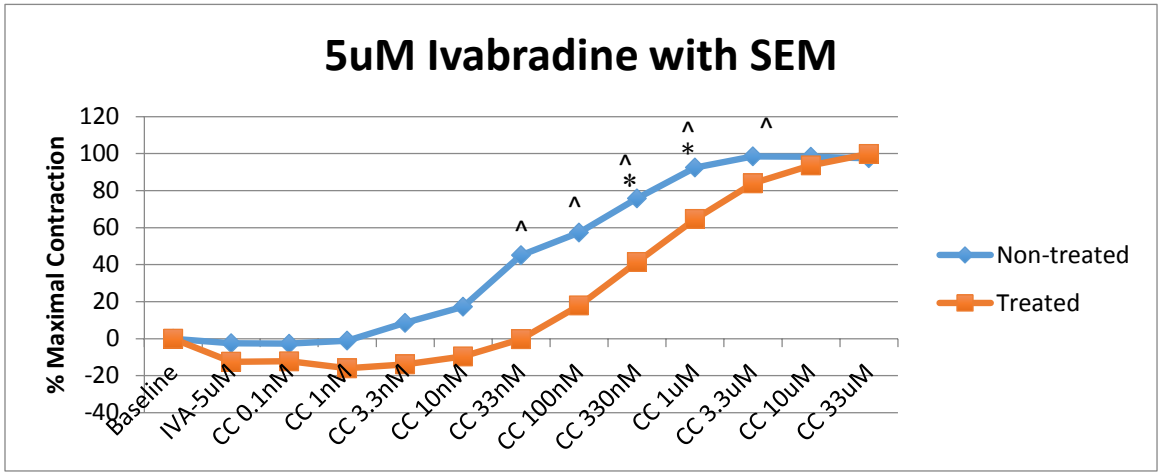

Figure 6 (continued)

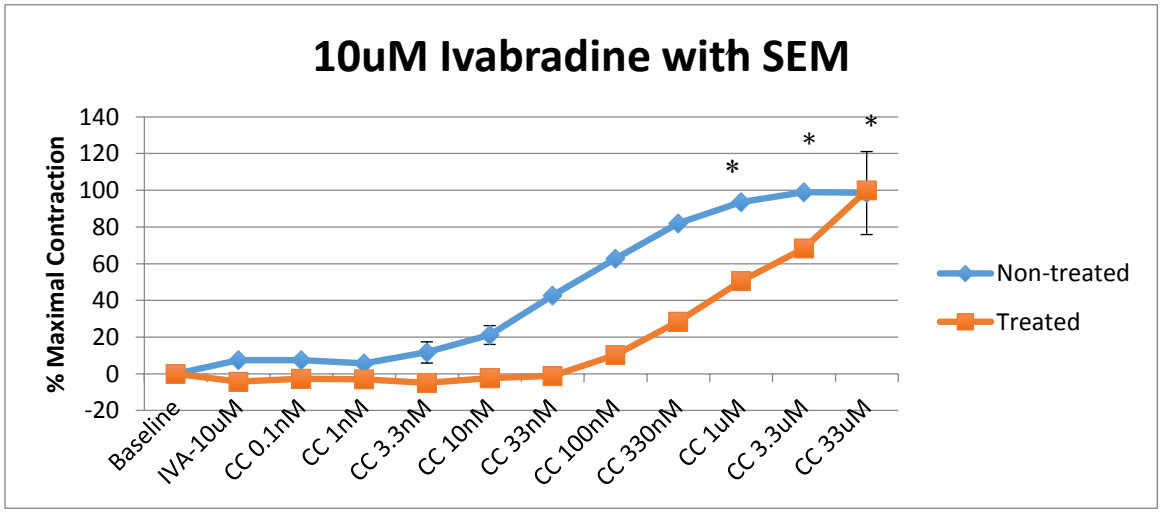

Figure 6 (continued)

Table 5 P-values for bronchial rings treated vs. untreated with ivabradine

	Day 1				Day 2			
Ivabradine dose	1uM	3uM	5uM	10 uM	1uM	3uM	5uM	10 uM
0.1 nM CC	0.9997	0.9980	1.0000	0.9999	1.0000	0.9982	0.9998	0.9991
1 nM CC	0.9998	0.9887	1.0000	1.0000	1.0000	0.9712	0.9885	0.9914
3.3nM CC	0.9976	0.9159	1.0000	1.0000	0.7379	0.8994	1.0000	1.0000
10nM	0.5314	0.7560	1.0000	1.0000	0.0328*	0.0200*	0.1741	0.9992
33nM	0.2425	0.9571	0.9962	1.0000	0.3209	0.1004	0.0148*	1.0000
100nM	0.0058*	0.4161	0.0640	0.9695	0.0126*	<.0001*	<.0001*	0.8539
330nM	0.0102*	0.8612	0.0040*	0.1307	0.1234	<.0001*	<.0001*	0.0982
1uM	0.0287*	0.9962	0.0045*	0.0044*	0.1961	0.0008*	<.0001*	0.0117*
3.3uM	0.8545	1.0000	0.0102*	0.0006*	0.9999	0.0530	0.0024*	0.0854
10uM	0.8808	1.0000	0.9999	0.9967	0.4873	0.0055*	0.6234	n/a
33uM	1.0000	1.0000	0.8608	0.0159*	1.0000	0.6411	0.4217	0.7158

Notes: * indicates significant p-value (<0.05). n/a=comparison unable to be made

CHAPTER IV

SUMMARY

The overarching goal of this research is to substantiate the clinical relevance of equine pasture asthma (EPA) to severe adult human asthma as a foundation for employing EPA as an asthma model capable of identifying novel mediators of airway hyper-responsiveness. From a position of improved relevance as an asthma model, it is reasoned that mediators identified in EPA horses would have an increased likelihood of relevance to severe human asthma. This is necessary because discoveries made in the most commonly employed animal asthma model, the mouse, demonstrate less than an 85% success of translation (240, 241). In addition, asthma models that employ mice, rats, guinea pigs as well as larger animal models including sheep and non-human primates require sensitization with an antigen to develop the asthma phenotype (247,250,251, 265, 271,273, 278, 300, 302, 307, 308). The disease that develops in these models differs substantially from human asthma in that these models eventually develop tolerance after repeated challenge with the sensitizing antigen (334), require higher doses of airway spasmogens than human asthmatics to achieve clinically significant airway hyper-responsiveness (247,250,265), and develop inflammatory cell types (eosinophils) that only model half of the population of human asthmatics (265,300, 334). Therefore, EPA

was chosen for scrutiny in this dissertation as a model for severe human asthma because it fills the aforementioned voids of other animal asthma models, being not only a naturally occurring asthma disease of horses that therefore does not require initial sensitization (8,9,10), but also an asthma condition which persists for the life of the horse (7,8) effectively substantiating that tolerance to the inciting triggers does not occur. Finally, EPA was pursued for further investigation because of its documented neutrophilic airway inflammation (8,22) which is a characteristic of not only more than 50% of the adult asthmatic population (149), but also of severe and corticosteroid resistant human asthmatics which constitute the most difficult and costly asthmatics to treat (155,347).

My first hypothesis predicted that EPA affected horses would possess a critical facet of severe asthma that is not well addressed in other animal asthma models, specifically the presence of airway hyper-responsiveness (AHR) that is persistent and of a magnitude that mirrors AHR in severe human asthmatics. Induced asthma models are limited as translational models because the magnitude of their AHR is much lower than human asthmatics and is not persistent, meaning that the AHR abates following cessation of exposure to the sensitizing antigens used to induce an asthma phenotype (247, 263, 334). To this end methacholine bronchoprovocation was performed using a threshold established for noncompliant humans who are unable to mount forced expiratory maneuvers that are typically used in bronchoprovocation protocols that quantify AHR in human asthma. Bronchoprovocation of EPA horses employing this threshold (the concentration of methacholine that caused a 40% increase in lung resistance) identified

methacholine concentrations to achieve the threshold that are employed by the American Thoracic Society to diagnose moderate to severe human asthma (79). Further, the magnitude of the AHR identified in 5 EPA horses was identical or within one doubling dose of methacholine when re-evaluated between 3 and 34 months following the initial methacholine bronchoprovocation, confirming persistent AHR. In addition, non-diseased control horses failed to react to methacholine thresholds that similarly rule out asthma in humans.

Finally, to further substantiate the model, reversibility of airway obstruction, also a key diagnostic feature of human asthma, was documented in 8 horses that were experiencing clinical disease and were treated with the β_2-adrenergic agonist levalbuterol. Together with the previously mentioned chronic neutrophilic airway inflammation, these findings substantiate the critical components of clinical asthma diagnosis namely, reversible airway obstruction and in particular the persistent AHR (2,109) that is not well modeled in other animal asthma models (334). This latter feature has similarly not been demonstrated in the Severe Equine Asthma variant of Barn Dust Asthma for longer than 3 days (89).

From the vantage of proven clinical relevance of EPA to the severe human asthma phenotype, and particularly given the model's unique ability to model persistent AHR of a magnitude that is diagnostic of severe human asthma, we hypothesized that mediators of AHR identified in EPA horses would have improved relevance to the human condition. Employing RNA sequencing of serial lung biopsies from EPA affected horses and clinically normal controls, HCN4 was identified as differentially expressed at the

transcript level in EPA affected horses during seasonal exacerbation. HCN4 was prioritized for further investigation because of its known role in increasing resting muscle membrane potentials to thresholds that trigger voltage gated calcium channels (350, 356). It was reasoned that such activity in airway smooth muscle could lead to irritability and alter contractile functions. HCN4 expression was not identified in the transcriptomes of non-diseased control horses, nor in the transcriptomes of EPA horses during disease remission. These findings were confirmed at the protein level using immunohistochemical staining which revealed increased HCN4 protein in the airway smooth muscle of EPA affected horses that were euthanized during disease exacerbation, relative to EPA affected horses that were euthanized during disease remission in which a low level of constitutive HCN4 expression was identified in airway smooth muscle. Congruent with the transcriptome findings, HCN4 protein was not identified in the airway smooth muscle of control horses.

Based upon the hypothesis that HCN4 mediated current contributes to airway contraction, the ability of ivabradine (a specific HCN4 inhibitor (356)) to moderate bronchial contraction in the horse was evaluated. In these experiments, bronchial rings bathed in ivabradine concentrations known to be achieved with labeled human dosing (1-10uM) demonstrated a dose dependent right shift of the % maximal contraction versus carbachol concentration curve. Accordingly, these results substantiate that HCN4 mediated current contributes to the generation of airway contractile forces. At the time of this discovery, HCN4 had yet to be characterized in the lung. Though the Gene Expression Omnibus contains entries relevant to the expression of HCN4 in human lung

transcriptomes, these entries do not identify differences in association with asthma. However, it is critical to recognize that each of the GEO entries fail to examine the changes in HCN4 in individual asthmatics during disease exacerbation and remission. This timing in our approach was critical to our discovery because HCN4 increases in association with disease exacerbation and decreases in association with disease remission. In addition, the human asthmatic populations represented within the GEO database are much more heterogenous with respect to disease severity and treatment effects then were horses in this investigation. It is noteworthy however that ivabradine decreased airway responsiveness in normal bronchi, indicating efficacy that is likely to not be dependent on the magnitude of HCN4 expression. Beyond GEO, only one other study has investigated the effect of HCN4 mediated current on airway contractility in another animal asthma model.

Despite the evidence presented herein that HCN4 current contributes to airway contraction, further characterization of the role of HCN4 in AHR is needed. A major challenge to this work is the inability to predict the ultimate relevance of HCN4 to airway contraction in humans with asthma. One next step would be to examine HCN4 expression in bronchial biopsy samples from a well-controlled cohort of severe asthmatics that possess neutrophilic airway inflammation. However, a series of investigations remain indicated in the horse including determining the role of HCN4 in asthma exacerbation versus remission, the role of HCN4 at small airways in the equine lung, as well as *in vivo* investigations of HCN4 blockade in lessening AHR.

To investigate the expression of HCN4 in affected horses while in clinical season, my lab will continue to analyze ivabradine effects on carbachol induced bronchial contractions *in vitro* from horses euthanized in EPA exacerbation. HCN4 expression will also be quantified using qPCR from these bronchial rings in order to associate the contractile responses of bronchial rings treated with ivabradine to the magnitude of HCN4 expression. From the differential protein expression already identified in the airway smooth muscle of EPA affected horses in exacerbation compared to controls and EPA affected horses in remission, I hypothesize that HCN4 expression will be increased in reactions run from bronchial rings of EPA horses euthanized in exacerbation. However, given that ivabradine reduced bronchial contractions in both EPA affected and normal control horses, I expect there to be some level of expression in control horses.

Our lab has recently begun investigating the potential of ivabradine to reduce airway contraction at the small airways. A similar tissue bath experiment as that used in chapter three will be used for this investigation. We hypothesize that a similar reduction in bronchial contraction in ivabradine treated rings will be observed in the smaller airways, expanding the differences observed in our equine model of asthma relative to the guinea pig experiment in which HCN4 blockade increased the contractile responses of trachealis muscle discussed in chapter 3.

Finally, our lab aspires to investigate the *in vivo* effect of ivabradine on EPA affected horses. To accomplish this, a pharmacokinetic study will need to be done to determine the dose of ivabradine needed to reach the therapeutic levels achievable in humans and used in chapter 3 of this study (1-10uM ivabradine). Once doses are established, we

hypothesize that ivabradine can be used as a drug to reduce AHR. Therefore, we propose to perform methacholine challenges similar to those performed in chapter two prior to and following treatment with ivabradine to determine the effect of HCN4 blockade on moderating airway hyper-responsiveness. We hypothesize that horses treated with ivabradine will reach study $PC_{40}R_L$ at higher methacholine concentrations than those untreated, proving reduced airway hyper-reactivity.

In conclusion, this dissertation research has proven equine pasture asthma to be diagnostically similar to severe human asthma and has demonstrated the utility of the model in its elucidation of novel gene targets that mediate airway contraction. It is this author's hope that the role of HCN4 antagonism in asthma will one day be pharmacologically relevant in treatment of both equine and human asthma.

REFERENCES

1. Centers for Disease Control and Prevention. *Asthma Facts—CDC's National Asthma Control Program Grantees. Atlanta, GA US Dep Heal Hum Serv Centers Dis Control Prev* 2013.

2. National Heart, Lung and BI. National Asthma Education and Prevention Program Expert Panel Report 3 Guidelines for the Diagnosis and Management of Asthma S U M M A R Y R E P O R T 2 0 0 7. 2007;at <https://www.nhlbi.nih.gov/files/docs/guidelines/asthsumm.pdf>.

3. Holgate ST. A brief history of asthma and its mechanisms to modern concepts of disease pathogenesis. *Allergy Asthma Immunol Res* 2010;2:165–71.

4. Marketos S, Ballas C. Bronchial asthma in medical literature of Greek antiquity. *Hist Sci Med* 1982;17:35–9.

5. Sakula A. Henry Hyde Salter (1823-71): a biographical sketch. *Thorax* 1985;40:887–8.

6. Ferrari CR, Cooley J, Mujahid N, Costa LR, Wills RW, Johnson ME, Swiderski CE. Horses With Pasture Asthma Have Airway Remodeling That Is Characteristic of Human Asthma. *Vet Pathol* 2018;55:144–158.

7. Swiderski CE, Hunter CL, Bowser JE, Costa LR, Claude A, Eddy AL, Bright LA. Deciphering the Role of Bronchial Hyper-Responsiveness in Equine Pasture Asthma. *J Equine Vet Sci* 2017;52:29–35.

8. Couëtil LL, Cardwell JM, Gerber V, Lavoie J-P, Léguillette R, Richard EA. Inflammatory Airway Disease of Horses--Revised Consensus Statement. *J Vet Intern Med* 2016;30:503–15.

9. Seahorn TL, Beadle RE. Summer pasture-associated obstructive pulmonary disease in horses: 21 cases (1983-1991). *J Am Vet Med Assoc* 1993;202:779–82.

10. Costa LRR, Johnson JR, Baur ME, Beadle RE. Temporal clinical exacerbation of summer pasture-associated recurrent airway obstruction and relationship with climate and aeroallergens in horses. *Am J Vet Res* 2006;67:1635–42.

11. HOTCHKISS JW, REID SWJ, CHRISTLEY RM. A survey of horse owners in Great Britain regarding horses in their care. Part 2: Risk factors for recurrent airway obstruction. *Equine Vet J* 2007;39:301–308.

12. Robinson NE. International Workshop on Equine Chronic Airway Disease Michigan State University 16–18 June 2000. *Equine Vet J* 2001;33:5–19.

13. Seahorn TL, Groves MG, Harrington KS, Beadle RE. Chronic obstructive pulmonary disease in horses in Louisiana. *J Am Vet Med Assoc* 1996;208:248–51.

14. LECLERE M, LAVOIE-LAMOUREUX A, LAVOIE J-P. Heaves, an asthma-like disease of horses. *Respirology* 2011;16:1027–1046.

15. Clarke AF, Madelin T. Technique for assessing respiratory health hazards from hay and other source materials. *Equine Vet J* 1987;19:442–7.

16. Hallas TE, Gudmundsson B. Storage mites in hay in Iceland. *Eur J Respir Dis Suppl* 1987;154:60–4.

17. Centers for Disease Control and Prevention (CDC). Vital signs: asthma prevalence, disease characteristics, and self-management education: United States, 2001--2009. *MMWR Morb Mortal Wkly Rep* 2011;60:547–52.

18. Foundation of America A. THE MOST CHALLENGING PLACES TO LIVE WITH. at <http://www.aafa.org/media/Fall-Allergy-Capitals-Report-Dec-2016.pdf>.

19. Couëtil LL, Cardwell JM, Gerber V, Lavoie J-P, Léguillette R, Richard EA. Inflammatory Airway Disease of Horses-Revised Consensus Statement. *J Vet Intern Med* 2016;30:503–515.

20. McPherson EA, Lawson GH, Murphy JR, Nicholson JM, Breeze RG, Pirie HM. Chronic obstructive pulmonary disease (COPD): factors influencing the occurrence. *Equine Vet J* 1979;11:167–71.

21. Ramseyer A, Gaillard C, Burger D, Straub R, Jost U, Boog C, Marti E, Gerber V. Effects of genetic and environmental factors on chronic lower airway disease in horses. *J Vet Intern Med* 21:149–56.

22. Costa LR, Seahorn TL, Moore RM, Taylor HW, Gaunt SD, Beadle RE. Correlation of clinical score, intrapleural pressure, cytologic findings of bronchoalveolar fluid, and histopathologic lesions of pulmonary tissue in horses with summer pasture-associated obstructive pulmonary disease. *Am J Vet Res* 2000;61:167–73.

23. Horohov DW, Beadle RE, Mouch S, Pourciau SS. Temporal regulation of cytokine mRNA expression in equine recurrent airway obstruction. *Vet Immunol Immunopathol* 2005;108:237–45.

24. Couëtil LL, Hoffman AM, Hodgson J, Buechner-Maxwell V, Viel L, Wood JLN, Lavoie J-P. Inflammatory airway disease of horses. *J Vet Intern Med* 21:356–61.

25. Willoughby RA, McDonell WN. Pulmonary function testing in horses. *Vet Clin North Am Large Anim Pract* 1979;1:171–96.

26. Armstrong PJ, Derksen FJ, Slocombe RF, Robinson NE. Airway responses to aerosolized methacholine and citric acid in ponies with recurrent airway obstruction (heaves). *Am Rev Respir Dis* 1986;133:357–61.

27. Crapo RO, Casaburi R, Coates AL, Enright PL, Hankinson JL, Irvin CG, MacIntyre NR, McKay RT, Wanger JS, Anderson SD, Cockcroft DW, Fish JE, Sterk PJ. Guidelines for methacholine and exercise challenge testing-1999. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, July 1999. *Am J Respir Crit Care Med* 2000;161:309–29.

28. de Lagarde M, Rodrigues N, Chevigny M, Beauchamp G, Albrecht B, Lavoie JP. N-butylscopolammonium bromide causes fewer side effects than atropine when assessing bronchoconstriction reversibility in horses with heaves. *Equine Vet J* 2014;46:474–8.

29. Robinson NE, Derksen FJ, Berney C, Goossens L. The airway response of horses with recurrent airway obstruction (heaves) to aerosol administration of ipratropium bromide. *Equine Vet J* 1993;25:299–303.

30. Derksen FJ, Robinson NE, Berney CE. Aerosol pirbuterol: bronchodilator activity and side effects in ponies with recurrent airway obstruction (heaves). *Equine Vet J* 1992;24:107–12.

31. McPherson E, Lawson G, Murphy J, Nicholson J, Fraser J, Breeze R, Pirie H. Chronic obstructive pulmonary disease (COPD): indentification of affected horses. *Equine Vet J* 1978;10:47–53.

32. Szentivanyi A. The beta adrenergic theory of the atopic abnormality in bronchial asthma. *J Allergy* 1968;42:203–232.

33. Townley RG. Interleukin 13 and the beta-adrenergic blockade theory of asthma revisited 40 years later. *Ann Allergy Asthma Immunol* 2007;99:215–24.

34. Sutcliffe A, Hollins F, Gomez E, Saunders R, Doe C, Cooke M, Challiss RAJ, Brightling CE. Increased nicotinamide adenine dinucleotide phosphate oxidase 4 expression mediates intrinsic airway smooth muscle hypercontractility in asthma. *Am J Respir Crit Care Med* 2012;185:267–74.

35. Ma X, Cheng Z, Kong H, Wang Y, Unruh H, Stephens NL, Laviolette M. Changes in biophysical and biochemical properties of single bronchial smooth muscle cells from asthmatic subjects. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L1181-9.

36. Matsumoto H, Moir LM, Oliver BGG, Burgess JK, Roth M, Black JL, McParland BE. Comparison of gel contraction mediated by airway smooth muscle cells from patients with and without asthma. *Thorax* 2007;62:848–54.

37. Meurs H, Gosens R, Zaagsma J. Airway hyperresponsiveness in asthma: lessons from in vitro model systems and animal models. doi:10.1183/09031936.00023608.

38. Robinson NE, Derksen FJ, Olszewski MA, Buechner-Maxwell VA. The pathogenesis of chronic obstructive pulmonary disease of horses. *Br Vet J* 1996;152:283–306.

39. Prakash YS. Airway smooth muscle in airway reactivity and remodeling: what have we learned? *Am J Physiol Lung Cell Mol Physiol* 2013;305:L912-33.

40. Mahn K, Ojo OO, Chadwick G, Aaronson PI, Ward JPT, Lee TH. Ca(2+) homeostasis and structural and functional remodelling of airway smooth muscle in asthma. *Thorax* 2010;65:547–52.

41. Lambert RK, Wiggs BR, Kuwano K, Hogg JC, Pare PD. Functional significance of increased airway smooth muscle in asthma and COPD. *J Appl Physiol* 1993;74:2771–2781.

42. Matusovsky OS, Kachmar L, Ijpma G, Bates G, Zitouni N, Benedetti A, Lavoie J-P, Lauzon A-M. Peripheral Airway Smooth Muscle, but Not the Trachealis, Is Hypercontractile in an Equine Model of Asthma. *Am J Respir Cell Mol Biol* 2016;54:718–727.

43. SEOW CY, SCHELLENBERG RR, PARÉ PD. Structural and Functional Changes in the Airway Smooth Muscle of Asthmatic Subjects. *Am J Respir Crit Care Med* 1998;158:S179–S186.

44. Benayoun L, Druilhe A, Dombret M-C, Aubier M, Pretolani M. Airway structural alterations selectively associated with severe asthma. *Am J Respir Crit Care Med* 2003;167:1360–8.

45. Herszberg B, Ramos-Barbon D, Tamaoka M, Martin JG, Lavoie JP. Heaves, an asthma-like equine disease, involves airway smooth muscle remodeling. *J Allergy Clin Immunol* 2006;118:382–388.

46. Mujahid N, Bowers C, Costa LR, Ferrari C, Cooley J, Burgess SC, Mays S, Swiderski CE. Smooth Muscle Proliferation is a Histologic Feature of Airway Remodeling in Summer Pasture Associated Recurrent Airway Obstruction. *J Equine Vet Sci* 2011;31:246.

47. Moulton BC, Fryer AD. Muscarinic receptor antagonists, from folklore to pharmacology; finding drugs that actually work in asthma and COPD. *Br J Pharmacol* 2011;163:44–52.

48. Menozzi A, Pozzoli C, Poli E, Delvescovo B, Serventi P, Bertini S. Pharmacological characterization of muscarinic receptors in the contractions of isolated bronchi in the horse. *J Vet Pharmacol Ther* 2014;37:325–331.

49. Erle DJ, Sheppard D. The cell biology of asthma. *J Cell Biol* 2014;205:621–31.

50. Scott GD, Fryer AD. Role of parasympathetic nerves and muscarinic receptors in allergy and asthma. *Chem Immunol Allergy* 2012;98:48–69.

51. Abraham G, Kottke C, Ammer H, Dhein S, Ungemach FR. Segment-dependent expression of muscarinic acetylcholine receptors and G-protein coupling in the equine respiratory tract. *Vet Res Commun* 2007;31:207–26.

52. White M V. Muscarinic receptors in human airways. *J Allergy Clin Immunol* 1995;95:1065–8.

53. Fryer AD, Jacoby DB. Muscarinic receptors and control of airway smooth muscle. *Am J Respir Crit Care Med* 1998;158:S154-60.

54. Fernandes LB, Fryer AD, Hirshman CA. M2 muscarinic receptors inhibit isoproterenol-induced relaxation of canine airway smooth muscle. *J Pharmacol Exp Ther* 1992;262:119–26.

55. Abraham G, Kottke C, Ungemach FR. Equine recurrent airway obstruction does not alter airway muscarinic acetylcholine receptor expression and subtype distribution. *J Vet Pharmacol Ther* 2007;30:401–9.

56. Yu MF, Wang ZW, Robinson NE, Derksen FJ. Modulation of bronchial smooth muscle function in horses with heaves. *J Appl Physiol* 1994;77:2149–2154.

57. Broadstone R V, LeBlanc PH, Derksen FJ, Robinson NE. In vitro responses of airway smooth muscle from horses with recurrent airway obstruction. *Pulm Pharmacol* 1991;4:191–202.

58. Matera MG, Calzetta L, Sanduzzi A, Page CP, Cazzola M. Effects of neuraminidase on equine isolated bronchi. *Pulm Pharmacol Ther* 2008;21:624–9.

59. Jiang H, Rao K, Halayko AJ, Kepron W, Stephens NL. Bronchial smooth muscle mechanics of a canine model of allergic airway hyperresponsiveness. *J Appl Physiol* 1992;72:39–45.

60. Mitchell RW, Ruhlmann E, Magnussen H, Leff AR, Rabe KF. Passive sensitization of human bronchi augments smooth muscle shortening velocity and capacity. *Am J Physiol Cell Mol Physiol* 1994;267:L218–L222.

61. Palmer LJ, Burton PR, James AL, Musk AW, Cookson WO. Familial aggregation and heritability of asthma-associated quantitative traits in a population-based sample of nuclear families. *Eur J Hum Genet* 2000;8:853–60.

62. Cheng RY, Shang Y, Limjunyawong N, Dao T, Das S, Rabold R, Sham JS, Mitzner W, Tang W-Y. Alterations of the lung methylome in allergic airway hyper-responsiveness. *Environ Mol Mutagen* 2014;55:244–55.

63. Levitt RC, Mitzner W. Autosomal recessive inheritance of airway hyperreactivity to 5-hydroxytryptamine. *J Appl Physiol* 1989;67:1125–32.

64. Yick CY, Zwinderman AH, Kunst PW, Grünberg K, Mauad T, Chowdhury S, Bel EH, Baas F, Lutter R, Sterk PJ. Gene expression profiling of laser microdissected airway smooth muscle tissue in asthma and atopy. *Allergy* 2014;69:1233–40.

65. Boulet LP, Cartier A, Thomson NC, Roberts RS, Dolovich J, Hargreave FE. Asthma and increases in nonallergic bronchial responsiveness from seasonal pollen exposure. *J Allergy Clin Immunol* 1983;71:399–406.

66. Chapman DG, Irvin CG. Mechanisms of airway hyper-responsiveness in asthma: the past, present and yet to come. *Clin Exp Allergy* 2015;45:706–719.

67. Hoffman AM. Clinical Application of Pulmonary Function Testing in Horses (1-Aug-2002). at <https://pdfs.semanticscholar.org/0edd/055695f9eccbebc02522a107e5d4659d2b36.pdf>.

68. Gillespie JR, Tyler WS, Eberly VE. Pulmonary ventilation and resistance in emphysematous and control horses. *J Appl Physiol* 1966;21:416–22.

69. Doucet MY, Vrins AA, Ford-Hutchinson AW. Histamine inhalation challenge in normal horses and in horses with small airway disease. *Can J Vet Res* 1991;55:285–93.

70. Muylle E, Oyaert W. Lung function tests in obstructive pulmonary disease in horses. *Equine Vet J* 1973;5:37–44.

71. Mazan MR, Hoffman AM, Kuehn H, Deveney EF. Effect of aerosolized albuterol sulfate on resting energy expenditure determined by use of open-flow indirect calorimetry in horses with recurrent airway obstruction. *Am J Vet Res* 2003;64:235–42.

72. Young SS, Hall LW. A rapid, non-invasive method for measuring total respiratory impedance in the horse. *Equine Vet J* 1989;21:99–105.

73. Young SS, Tesarowski D. Respiratory mechanics of horses measured by conventional and forced oscillation techniques. *J Appl Physiol* 1994;76:2467–2472.

74. Hoffman A, Kuehn H, Riedelberger K, Kupcinskas R, Miskovic MB. Flowmetric comparison of respiratory inductance plethysmography and pneumotachography in horses. *J Appl Physiol* 2001;91:2767–75.

75. Amory H, Lomba F, Lekeux PM, Solal AN, Jauniaux TP, Desmecht DJ. Bilateral diaphragmatic paralysis in a pony. *J Am Vet Med Assoc* 1994;205:587–91.

76. Nolen-Walston RD, Kuehn H, Boston RC, Mazan MR, Wilkins PA, Bruns S, Hoffman AM. Reproducibility of airway responsiveness in horses using flowmetric plethysmography and histamine bronchoprovocation. *J Vet Intern Med* 2009;23:631–5.

77. Wichtel M, Gomez D, Burton S, Wichtel J, Hoffman A. Relationships between equine airway reactivity measured by flowmetric plethysmography and specific indicators of airway inflammation in horses with suspected inflammatory airway disease. *Equine Vet J* 2016;48:466–71.

78. Reddel HK, Taylor DR, Bateman ED, Boulet L-P, Boushey HA, Busse WW, Casale TB, Chanez P, Enright PL, Gibson PG, de Jongste JC, Kerstjens HAM, Lazarus SC, Levy ML, O'Byrne PM, Partridge MR, Pavord ID, Sears MR, Sterk PJ, Stoloff SW, Sullivan SD, Szefler SJ, Thomas MD, Wenzel SE, American Thoracic Society/European Respiratory Society Task Force on Asthma Control and Exacerbations. An Official American Thoracic Society/European Respiratory Society Statement: Asthma Control and Exacerbations. *Am J Respir Crit Care Med* 2009;180:59–99.

79. Beydon N, Davis SD, Lombardi E, Allen JL, Arets HGM, Aurora P, Bisgaard H, Davis GM, Ducharme FM, Eigen H, Gappa M, Gaultier C, Gustafsson PM, Hall GL, Hantos Z, Healy MJR, Jones MH, Klug B, Lødrup Carlsen KC, McKenzie SA, Marchal F, Mayer OH, Merkus PJFM, Morris MG, Oostveen E, Pillow JJ, Seddon PC, Silverman M, Sly PD, *et al.* An Official American Thoracic Society/European Respiratory Society Statement: Pulmonary Function Testing in Preschool Children. *Am J Respir Crit Care Med* 2007;175:1304–1345.

80. Dell SD, Bola SS, Foty RG, Marshall LC, Nelligan KA, Coates AL. Provocative dose of methacholine causing a 20% drop in FEV1 should be used to interpret methacholine challenge tests with modern nebulizers. *Ann Am Thorac Soc* 2015;12:357–63.

81. Allen ND, Davis BE, Hurst TS, Cockcroft DW. Difference Between Dosimeter and Tidal Breathing Methacholine Challenge. *Chest* 2005;128:4018–4023.

82. Mazi A, Lands LC, Zielinski D. Methacholine challenge test: Comparison of tidal breathing and dosimeter methods in children. *Pediatr Pulmonol* 2018;53:174–180.

83. Scott JS, Berney CE, Derksen FJ, Robinson NE. Beta-adrenergic receptor activity in ponies with recurrent obstructive pulmonary disease. *Am J Vet Res* 1991;52:1416–22.

84. Reitemeyer H, Klein HJ, Deegen E. The effect of sedatives on lung function in horses. *Acta Vet Scand Suppl* 1986;82:111–20.

85. Broadstone R V, Gray PR, Robinson NE, Derksen FJ. Effects of xylazine on airway function in ponies with recurrent airway obstruction. *Am J Vet Res* 1992;53:1813–7.

86. AC L, JE B, CJ W, RM W, AJ T, EK M, CE S. Sedated versus nonsedated methacholine challenge for the diagnosis of airway hyper-responsiveness in horses. Minneapolis, MN: Journal of Equine Veterinary Science; 2017. p. 52–53.

87. Scott JS, Broadstone R V, Derksen FJ, Robinson NE. Beta-adrenergic blockade in ponies with recurrent obstructive pulmonary disease. *J Appl Physiol* 1988;64:2324–8.

88. van Erck E, Votion DM, Kirschvink N, Art T, Lekeux P. Use of the impulse oscillometry system for testing pulmonary function during methacholine bronchoprovocation in horses. *Am J Vet Res* 2003;64:1414–20.

89. Fairbairn SM, Lees P, Page CP, Cunningham FM. Duration of antigen-induced hyperresponsiveness in horses with allergic respiratory disease and possible links with early airway obstruction. *J Vet Pharmacol Ther* 1993;16:469–76.

90. Hargreave FE, Ryan G, Thomson NC, O'Byrne PM, Latimer K, Juniper EF. Bronchial responsiveness to histamine or methacholine in asthma: measurement and clinical significance. *Eur J Respir Dis Suppl* 1982;121:79–88.

91. Howarth PH. The airway inflammatory response in allergic asthma and its relationship to clinical disease. *Allergy* 1995;50:13–21.

92. Schofield ML. Asthma Pharmacotherapy. *Otolaryngol Clin North Am* 2014;47:55–64.

93. Aikawa T, Shimura S, Sasaki H, Ebina M, Takishima T. Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack. *Chest* 1992;101:916–21.

94. Boulet L, Bélanger M, Carrier G. Airway responsiveness and bronchial-wall thickness in asthma with or without fixed airflow obstruction. *Am J Respir Crit Care Med* 1995;152:865–71.

95. Mannino DM, Homa DM, Akinbami LJ, Moorman JE, Gwynn C, Redd SC. Surveillance for asthma--United States, 1980-1999. *MMWR Surveill Summ* 2002;51:1–13.

96. Mannino DM, Homa DM, Pertowski CA, Ashizawa A, Nixon LL, Johnson CA, Ball LB, Jack E, Kang DS. Surveillance for asthma--United States, 1960-1995. *MMWR CDC Surveill Summ Morb Mortal Wkly report CDC Surveill Summ* 1998;47:1–27.

97. Moorman JE, Rudd RA, Johnson CA, King M, Minor P, Bailey C, Scalia MR, Akinbami LJ, Centers for Disease Control and Prevention (CDC). National surveillance for asthma--United States, 1980-2004. *MMWR Surveill Summ* 2007;56:1–54.

98. Centers for Disease Control and Prevention (CDC). *National Health Interview Survey (NHIS) data: 2011 lifetime and current asthma*. Atlanta, GA: 2012. at <https://www.cdc.gov/asthma/nhis/2011/data.htm>.

99. Center for Environmental Health N, of Environmental Hazards D, Effects H. *Asthma's Impact on the Nation Data from the CDC National Asthma Control Program*. at <https://www.cdc.gov/asthma/impacts_nation/asthmafactsheet.pdf>.

100. Barnett SBL, Nurmagambetov TA. Costs of asthma in the United States: 2002-2007. *J Allergy Clin Immunol* 2011;127:145–52.

101. Akinbami LJ, Moorman JE, Liu X. Asthma prevalence, health care use, and mortality: United States, 2005-2009. *Natl Health Stat Report* 2011;1–14.at <http://www.ncbi.nlm.nih.gov/pubmed/21355352>.

102. Grant EN, Daugherty SR, Moy JN, Nelson SG, Piorkowski JM, Weiss KB. Prevalence and burden of illness for asthma and related symptoms among kindergartners in Chicago public schools. *Ann Allergy Asthma Immunol* 1999;83:113–20.

103. Chandra S, Wingender G, Greenbaum JA, Khurana A, Gholami AM, Ganesan A-P, Rosenbach M, Jaffee K, Gern JE, Wood R, O'Connor G, Sandel M, Kattan M, Bacharier L, Togias A, Horner AA, Kronenberg M. Development of Asthma in Inner-City Children: Possible Roles of MAIT Cells and Variation in the Home Environment. *J Immunol* 2018;ji1701525.doi:10.4049/jimmunol.1701525.

104. Kakumanu S, Jaffee K, Visness CM, Dresen A, Burger M, Witter FR, O'Connor GT, Cruikshank WW, Shreffler WG, Bacharier LB, Gern JE. The influence of atopy and asthma on immune responses in inner-city adults. *Immunity, Inflamm Dis* 2016;4:80–90.

105. Saadeh C, Cross B, Saadeh C, Gaylor M. Retrospective Observations on the Ability to Diagnose and Manage Patients with Asthma through the Use of Impulse Oscillometry: Comparison with Spirometry and Overview of the Literature. *Pulm Med* 2014;2014:1–9.

106. Mayo Clinic Staff. Spirometry - Overview - Mayo Clinic. *Patient Care Heal Inf* 2017;at <http://www.mayoclinic.org/tests-procedures/spirometry/home/ovc-20346849>.

107. Komarow HD, Myles IA, Uzzaman A, Metcalfe DD. Impulse oscillometry in the evaluation of diseases of the airways in children. *Ann Allergy Asthma Immunol* 2011;106:191–9.

108. Bateman ED, Reddel HK, van Zyl-Smit RN, Agusti A. The asthma-COPD overlap syndrome: towards a revised taxonomy of chronic airways diseases? *Lancet Respir Med* 2015;3:719–728.

109. Vestbo J, Hurd SS, Agustí AG, Jones PW, Vogelmeier C, Anzueto A, Barnes PJ, Fabbri LM, Martinez FJ, Nishimura M, Stockley RA, Sin DD, Rodriguez-Roisin R. Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med* 2013;187:347–365.

110. Rabe KF, Hurd S, Anzueto A, Barnes PJ, Buist SA, Calverley P, Fukuchi Y, Jenkins C, Rodriguez-Roisin R, van Weel C, Zielinski J, Global Initiative for Chronic Obstructive Lung Disease. Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med* 2007;176:532–555.

111. Reddel HK, Bateman ED, Becker A, Boulet L-P, Cruz AA, Drazen JM, Haahtela T, Hurd SS, Inoue H, de Jongste JC, Lemanske RF, Levy ML, O'Byrne PM, Paggiaro P, Pedersen SE, Pizzichini E, Soto-Quiroz M, Szefler SJ, Wong GWK, FitzGerald JM, FitzGerald JM. A summary of the new GINA strategy: a roadmap to asthma control. *Eur Respir J* 2015;46:622–39.

112. Dolhnikoff M, da Silva LFF, de Araujo BB, Gomes HAP, Fernezlian S, Mulder A, Lindeman JH, Mauad T. The outer wall of small airways is a major site of remodeling in fatal asthma. *J Allergy Clin Immunol* 2009;123:1090–1097.e1.

113. Ollerenshaw SL, Woolcock AJ. Characteristics of the Inflammation in Biopsies from Large Airways of Subjects with Asthma and Subjects with Chronic Airflow Limitation. *Am Rev Respir Dis* 1992;145:922–927.

114. Carroll N, Elliot J, Morton A, James A. The Structure of Large and Small Airways in Nonfatal and Fatal Asthma. *Am Rev Respir Dis* 1993;147:405–410.

115. Elias JA, Elias JA, Zhu Z, Zhu Z, Chupp G, Chupp G, Homer RJ, Homer RJ. Airway remodeling in asthma. *Heart* 1999;104:1001–1006.

116. Jeffery PK, Wardlaw AJ, Nelson FC, Collins J V., Kay AB. Bronchial Biopsies in Asthma: An Ultrastructural, Quantitative Study and Correlation with Hyperreactivity. *Am Rev Respir Dis* 1989;140:1745–1753.

117. Barbato A, Turato G, Baraldo S, Bazzan E, Calabrese F, Panizzolo C, Zanin ME, Zuin R, Maestrelli P, Fabbri LM, Saetta M. Epithelial damage and angiogenesis in the airways of children with asthma. *Am J Respir Crit Care Med* 2006;174:975–81.

118. Holgate ST. Epithelium dysfunction in asthma. *J Allergy Clin Immunol* 2007;120:1233–1244.

119. ORDOÑEZ C, FERRANDO R, HYDE DM, WONG HH, FAHY JV. Epithelial Desquamation in Asthma. *Am J Respir Crit Care Med* 2000;162:2324–2329.

120. Bullone M, Hélie P, Joubert P, Lavoie J-P. Development of a Semiquantitative Histological Score for the Diagnosis of Heaves Using Endobronchial Biopsy Specimens in Horses. *J Vet Intern Med* 2016;30:1739–1746.

121. Tliba O, Amrani Y. Airway Smooth Muscle Cell as an Inflammatory Cell Lessons Learned from Interferon Signaling Pathways. doi:10.1513/pats.200705-060VS.

122. Hamid Q, Song Y, Kotsimbos TC, Minshall E, Bai TR, Hegele RG, Hogg JC. Inflammation of small airways in asthma. *J Allergy Clin Immunol* 1997;100:44–51.

123. Haley KJ, Sunday ME, Wiggs BR, Kozakewich HP, Reilly JJ, Mentzer SJ, Sugarbaker DJ, Doerschuk CM, Drazen JM. Inflammatory cell distribution within and along asthmatic airways. *Am J Respir Crit Care Med* 1998;158:565–72.

124. Doberer D, Trejo Bittar HE, Wenzel SE. Should lung biopsies be performed in patients with severe asthma? *Eur Respir Rev* 2015;24:525–39.

125. Al-Muhsen S, Johnson JR, Hamid Q. Remodeling in asthma. *J Allergy Clin Immunol* 2011;128:451–462.

126. Fahy J V. Goblet cell and mucin gene abnormalities in asthma. *Chest* 2002;122:320S–326S.

127. Faul JL, Tormey VJ, Leonard C, Burke CM, Farmer J, Horne SJ, Poulter LW. Lung immunopathology in cases of sudden asthma death. *Eur Respir J* 1997;10:301–7.

128. Shiang C, Mauad T, Senhorini A, de Araújo BB, Ferreira DS, da Silva LFF, Dolhnikoff M, Tsokos M, Rabe KF, Pabst R. Pulmonary periarterial inflammation in fatal asthma. *Clin Exp Allergy* 2009;39:1499–1507.

129. WILLIAMS EO, WILLIAMS GEO. The natural history of asthma; a review of 300 cases. *Br Med J* 1949;2:897–900.

130. Rackeman FM. A Working Classification of Asthma. *Am J Med* 1947;3:601–606.

131. Litzky LA, Green LK. 20 – Emphysema and Diseases of Large Airways. *Pulm Pathol* 2018;409–426.e1.doi:10.1016/B978-0-323-39308-9.00020-0.

132. Fontenot AP, Simonian PL. Adaptive Immunity. *Murray Nadel's Textb Respir Med* Elsevier; 2016. p. 206–224.e3.doi:10.1016/B978-1-4557-3383-5.00013-0.

133. Sokol K, Sur S, Ameredes BT. Inhaled environmental allergens and toxicants as determinants of the asthma phenotype. *Adv Exp Med Biol* 2014;795:43–73.

134. Jenerowicz D, Silny W, Dańczak-Pazdrowska A, Polańska A, Osmola-Mańkowska A, Olek-Hrab K. Environmental factors and allergic diseases. *Ann Agric Environ Med* 2012;19:475–81.

135. Patiño CM, Martinez FD. Interactions between genes and environment in the development of asthma. *Allergy* 2001;56:279–86.

136. Burke W, Fesinmeyer M, Reed K, Hampson L, Carlsten C. Family history as a predictor of asthma risk. *Am J Prev Med* 2003;24:160–9.

137. Almoguera B, Vazquez L, Mentch F, Connolly J, Pacheco JA, Sundaresan AS, Peissig PL, Linneman JG, McCarty CA, Crosslin D, Carrell DS, Lingren T, Namjou-Khales B, Harley JB, Larson E, Jarvik GP, Brilliant M, Williams MS, Kullo IJ, Hysinger EB, Sleiman PMA, Hakonarson H. Identification of four novel loci in Asthma in European American and African American Populations. *Am J Respir Crit Care Med* 2017;195:456–463.

138. Melén E, Pershagen G. Pathophysiology of asthma: Lessons from genetic research with particular focus on severe asthma. *J Intern Med* 2012;272:108–120.

139. Sandford AJ, Chagani T, Zhu S, Weir TD, Bai TR, Spinelli JJ, FitzGerald JM, Behbehani NA, Tan WC, Pare PD. Polymorphisms in the IL4 , IL4RA , and FCERIB genes and asthma severity. *J Allergy Clin Immunol* 2000;106:135–140.

140. Fish JE. Occupational asthma: a spectrum of acute respiratory disorders. *J Occup Med* 1982;24:379–86.

141. Malo JL, Tarlo SM, Sastre J, Martin J, Jeebhay MF, Le Moual N, Heederik D, Platts-Mills T, Blanc PD, Vandenplas O, Moscato G, De Blay F, Cartier A. An Official American Thoracic Society Workshop Report: Presentations and discussion of the fifth Jack Pepys Workshop on Asthma in the Workplace: Comparisons between asthma in the workplace and non-work-related asthma. *Ann Am Thorac Soc* 2015;12:S99–S110.

142. Tarlo SM, Lemiere C. Occupational Asthma. *N Engl J Med* 2014;370:640–649.

143. Burge PS, Perks W, O'Brien IM, Hawkins R, Green M. Occupational asthma in an electronics factory. *Thorax* 1979;34:13–8.

144. Weinberg JL, Flattery J, Harrison R. Fragrances and work-related asthma–California surveillance data, 1993–2012. *J Asthma* 2017;1–10.doi:10.1080/02770903.2017.1299755.

145. Barnes PJ. Intrinsic asthma: not so different from allergic asthma but driven by superantigens? *Clin Exp Allergy* 2009;39:1145–1151.

146. Humbert M, Menz G, Ying S, Corrigan CJ, Robinson DS, Durham SR, Kay AB. The immunopathology of extrinsic (atopic) and intrinsic (non-atopic) asthma: more similarities than differences. *Immunol Today* 1999;20:528–33.

147. Holt PG, Sly PD. Non-atopic intrinsic asthma and the "family tree" of chronic respiratory disease syndromes. *Clin Exp Allergy* 2009;39:807–811.

148. Romanet-Manent S, Charpin D, Magnan A, Lanteaume A, Vervloet D. Allergic *vs* nonallergic asthma: what makes the difference? *Allergy* 2002;57:607–613.

149. Wenzel SE, Schwartz LB, Langmack EL, Halliday JL, Trudeau JB, Gibbs RL, Chu HW. Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics. *Am J Respir Crit Care Med* 1999;160:1001–8.

150. Gibson PG, Simpson JL, Saltos N. Heterogeneity of airway inflammation in persistent asthma : evidence of neutrophilic inflammation and increased sputum interleukin-8. *Chest* 2001;119:1329–36.

151. Thomson NC. Novel approaches to the management of noneosinophilic asthma. *Ther Adv Respir Dis* 2016;10:211–34.

152. Wenzel SE, Szefler SJ, Leung DY, Sloan SI, Rex MD, Martin RJ. Bronchoscopic evaluation of severe asthma. Persistent inflammation associated with high dose glucocorticoids. *Am J Respir Crit Care Med* 1997;156:737–43.

153. Moore WC, Hastie AT, Li X, Li H, Busse WW, Jarjour NN, Wenzel SE, Peters SP, Meyers DA, Bleecker ER, National Heart, Lung, and Blood Institute's Severe Asthma Research Program. Sputum neutrophil counts are associated with more severe asthma phenotypes using cluster analysis. *J Allergy Clin Immunol* 2014;133:1557–63.e5.

154. The ENFUMOSA cross-sectional European multicentre study of the clinical phenotype of chronic severe asthma. European Network for Understanding Mechanisms of Severe Asthma. *Eur Respir J* 2003;22:470–7.

155. Sur S, Crotty TB, Kephart GM, Hyma BA, Colby T V, Reed CE, Hunt LW, Gleich GJ. Sudden-onset fatal asthma. A distinct entity with few eosinophils and relatively more neutrophils in the airway submucosa? *Am Rev Respir Dis* 1993;148:713–9.

156. Green RH, Brightling CE, Woltmann G, Parker D, Wardlaw AJ, Pavord ID. Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. *Thorax* 2002;57:875–9.

157. Norzila MZ, Fakes K, Henry RL, Simpson J, Gibson PG. Interleukin-8 secretion and neutrophil recruitment accompanies induced sputum eosinophil activation in children with acute asthma. *Am J Respir Crit Care Med* 2000;161:769–74.

158. Green RH, Brightling CE, Woltmann G, Parker D, Wardlaw AJ, Pavord ID. Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. *Thorax* 2002;57:875–9.

159. Miranda C, Busacker A, Balzar S, Trudeau J, Wenzel SE. Distinguishing severe asthma phenotypes: role of age at onset and eosinophilic inflammation. *J Allergy Clin Immunol* 2004;113:101–8.

160. Woodruff PG, Modrek B, Choy DF, Jia G, Abbas AR, Ellwanger A, Koth LL, Arron JR, Fahy J V. T-helper type 2-driven inflammation defines major subphenotypes of asthma. *Am J Respir Crit Care Med* 2009;180:388–95.

161. Fajt ML, Gelhaus SL, Freeman B, Uvalle CE, Trudeau JB, Holguin F, Wenzel SE. Prostaglandin D_2 pathway upregulation: relation to asthma severity, control, and TH2 inflammation. *J Allergy Clin Immunol* 2013;131:1504–12.

162. Raundhal M, Morse C, Khare A, Oriss TB, Milosevic J, Trudeau J, Huff R, Pilewski J, Holguin F, Kolls J, Wenzel S, Ray P, Ray A. High IFN-γ and low SLPI mark severe asthma in mice and humans. *J Clin Invest* 2015;125:3037–50.

163. Bullens DMA, Truyen E, Coteur L, Dilissen E, Hellings PW, Dupont LJ, Ceuppens JL. IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx? *Respir Res* 2006;7:135.

164. Kunkel SL, Standiford T, Kasahara K, Strieter RM. Interleukin-8 (IL-8): the major neutrophil chemotactic factor in the lung. *Exp Lung Res* 17:17–23.

165. McKinley L, Alcorn JF, Peterson A, Dupont RB, Kapadia S, Logar A, Henry A, Irvin CG, Piganelli JD, Ray A, Kolls JK. TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. *J Immunol* 2008;181:4089–97.

166. Nadel JA. Role of neutrophil elastase in hypersecretion during COPD exacerbations, and proposed therapies. *Chest* 2000;117:386S–9S.

167. Anticevich SZ, Hughes JM, Black JL, Armour CL. Induction of hyperresponsiveness in human airway tissue by neutrophils--mechanism of action. *Clin Exp Allergy* 1996;26:549–56.

168. Stănescu D, Sanna A, Veriter C, Kostianev S, Calcagni PG, Fabbri LM, Maestrelli P. Airways obstruction, chronic expectoration, and rapid decline of FEV1 in smokers are associated with increased levels of sputum neutrophils. *Thorax* 1996;51:267–71.

169. Wheelock CE, Goss VM, Balgoma D, Nicholas B, Brandsma J, Skipp PJ, Snowden S, Burg D, D'Amico A, Horvath I, Chaiboonchoe A, Ahmed H, Ballereau S, Rossios C, Chung KF, Montuschi P, Fowler SJ, Adcock IM, Postle AD, Dahlén S-E, Rowe A, Sterk PJ, Auffray C, Djukanovic R, U-BIOPRED Study Group the U-BS. Application of 'omics technologies to biomarker discovery in inflammatory lung diseases. *Eur Respir J* 2013;42:802–25.

170. Thamrin C, Frey U, Kaminsky DA, Reddel HK, Seely AJE, Suki B, Sterk PJ. Systems biology and clinical practice in respiratory medicine:the twain shall meet. *Am J Respir Crit Care Med* 2016;194:1053–1061.

171. Sittka A, Vera J, Lai X, Schmeck BT. Asthma phenotyping, therapy, and prevention: what can we learn from systems biology? *Pediatr Res* 2013;73:543–52.

172. Altman M, Busse W. EDITORIALS A Deep Dive into Asthma Transcriptomics. 2017;195:1279–1280.

173. Genome-Wide Association Studies Fact Sheet - National Human Genome Research Institute (NHGRI). at <https://www.genome.gov/20019523/genomewide-association-studies-fact-sheet/>.

174. Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet* 2009;10:57–63.

175. Verrills NM. Clinical proteomics: present and future prospects. *Clin Biochem Rev* 2006;27:99–116.

176. Bush A. Translating Asthma: Dissecting the Role of Metabolomics, Genomics and Personalized Medicine. *Indian J Pediatr* 2017;doi:10.1007/s12098-017-2520-0.

177. van der Schee MP, Paff T, Brinkman P, van Aalderen WMC, Haarman EG, Sterk PJ. Breathomics in Lung Disease. *Chest* 2015;147:224–231.

178. PAYNE DNR, ADCOCK IM, WILSON NM, OATES T, SCALLAN M, BUSH A. Relationship between Exhaled Nitric Oxide and Mucosal Eosinophilic Inflammation in Children with Difficult Asthma, after Treatment with Oral Prednisolone. *Am J Respir Crit Care Med* 2001;164:1376–1381.

179. Schleich FN, Seidel L, Sele J, Manise M, Quaedvlieg V, Michils A, Louis R. Exhaled nitric oxide thresholds associated with a sputum eosinophil count ≥3% in a cohort of unselected patients with asthma. *Thorax* 2010;65:1039–44.

180. Wadsworth S, Sin D, Dorscheid D. Clinical update on the use of biomarkers of airway inflammation in the management of asthma. *J Asthma Allergy* 2011;4:77–86.

181. 2007 Release: Horse Genome Assembled - National Human Genome Research Institute (NHGRI). at <https://www.genome.gov/20519480/>.

182. Bright LA, Burgess SC, Chowdhary B, Swiderski CE, McCarthy FM. Structural and functional-annotation of an equine whole genome oligoarray. *BMC Bioinformatics* 2009;10 Suppl 11:S8.

183. Bright LA, Mujahid N, Nanduri B, McCarthy FM, Costa LRR, Burgess SC, Swiderski CE. Functional modelling of an equine bronchoalveolar lavage fluid proteome provides experimental confirmation and functional annotation of equine genome sequences. *Anim Genet* 2011;42:395–405.

184. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G, Sherlock G. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* 2000;25:25–9.

185. Racine J, Gerber V, Feutz MM, Riley CP, Adamec J, Swinburne JE, Couetil LL. Comparison of genomic and proteomic data in recurrent airway obstruction affected horses using Ingenuity Pathway Analysis®. *BMC Vet Res* 2011;7:48.

186. Pacholewska A, Jagannathan V, Drögemüller M, Klukowska-Rötzler J, Lanz S, Hamza E, Dermitzakis ET, Marti E, Leeb T, Gerber V. Impaired Cell Cycle Regulation in a Natural Equine Model of Asthma. In: Wade C, editor. *PLoS One* 2015;10:e0136103.

187. Tessier L, Côté O, Clark ME, Viel L, Diaz-Méndez A, Anders S, Bienzle D. Impaired response of the bronchial epithelium to inflammation characterizes severe equine asthma. *BMC Genomics* 2017;18:708.

188. Massoud AH, Charbonnier L-M, Lopez D, Pellegrini M, Phipatanakul W, Chatila TA. An asthma-associated IL4R variant exacerbates airway inflammation by promoting conversion of regulatory T cells to TH17-like cells. *Nat Med* 2016;22:1013–1022.

189. Davoodi P, Mahesh P, Holla A, Ramachandra N. A preliminary study on the association of single nucleotide polymorphisms of interleukin 4 (IL4), IL13, IL4 receptor alpha (IL4Rα) & Toll-like receptor 4 (TLR4) genes with asthma in Indian adults. *Indian J Med Res* 2015;142:675.

190. Ni G, Chen Y, Wu F, Zhu P, Song L. NOD2 promotes cell proliferation and inflammatory response by mediating expression of TSLP in human airway smooth muscle cells. *Cell Immunol* 2017;312:35–41.

191. Al-Kufaidy R, Vazquez-Tello A, BaHammam AS, Al-Muhsen S, Hamid Q, Halwani R. IL-17 enhances the migration of B cells during asthma by inducing CXCL13 chemokine production in structural lung cells. *J Allergy Clin Immunol* 2017;139:696–699.e5.

192. Charrad R, Kaabachi W, Rafrafi A, Berraies A, Hamzaoui K, Hamzaoui A. IL-8 Gene Variants and Expression in Childhood Asthma. *Lung* 2017;195:749–757.

193. Roviezzo F, Sorrentino R, Terlizzi M, Riemma MA, Iacono VM, Rossi A, Spaziano G, Pinto A, D'Agostino B, Cirino G. Toll-Like Receptor 4 Is Essential for the Expression of Sphingosine-1-Phosphate-Dependent Asthma-Like Disease in Mice. *Front Immunol* 2017;8:1336.

194. Zhao J, Shang H, Cao X, Huang Y, Fang X, Zhang S, Xie M, Xie J, Liu X. Association of polymorphisms in TLR2 and TLR4 with asthma risk. *Medicine (Baltimore)* 2017;96:e7909.

195. Swinburne JE, Bogle H, Klukowska-Rötzler J, Drögemüller M, Leeb T, Temperton E, Dolf G, Gerber V. A whole-genome scan for recurrent airway obstruction in Warmblood sport horses indicates two positional candidate regions. *Mamm Genome* 2009;20:504–515.

196. Lavoie J-P, Lefebvre-Lavoie J, Leclere M, Lavoie-Lamoureux A, Chamberland A, Laprise C, Lussier J. Profiling of differentially expressed genes using suppression subtractive hybridization in an equine model of chronic asthma. In: Schönbach C, editor. *PLoS One* 2012;7:e29440.

197. Feutz MM. Proteomics technology used for discovery in recurrent airway obstruction in horses. 2010;

198. Pacholewska A, Kraft MF, Gerber V, Jagannathan V. Differential Expression of Serum MicroRNAs Supports $CD4^+$ T Cell Differentiation into Th2/Th17 Cells in Severe Equine Asthma. *Genes (Basel)* 2017;8:.

199. Fang L, Wu J, Huang T, Zhang P, Xin X, Shi Y. TGF-β1 stimulates epithelial- mesenchymal transition mediated by ADAM33. *Exp Ther Med* 2017;15:985–992.

200. Han JM, Patterson SJ, Levings MK. The Role of the PI3K Signaling Pathway in CD4(+) T Cell Differentiation and Function. *Front Immunol* 2012;3:245.

201. Vicente CT, Revez JA, Ferreira MAR. Lessons from ten years of genome-wide association studies of asthma. *Clin Transl Immunol* 2017;6:e165.

202. Canonica GW, Ferrando M, Baiardini I, Puggioni F, Racca F, Passalacqua G, Heffler E. Asthma: personalized and precision medicine. *Curr Opin Allergy Clin Immunol* 2017;18:1.

203. Saqi M, Pellet J, Roznovat I, Mazein A, Ballereau S, De Meulder B, Auffray C. Systems Medicine: The Future of Medical Genomics, Healthcare, and Wellness. *Methods Mol Biol* 2016. p. 43–60.

204. Bainbridge MN, Wiszniewski W, Murdock DR, Friedman J, Gonzaga-Jauregui C, Newsham I, Reid JG, Fink JK, Morgan MB, Gingras M-C, Muzny DM, Hoang LD, Yousaf S, Lupski JR, Gibbs RA. Whole-genome sequencing for optimized patient management. *Sci Transl Med* 2011;3:87re3.

205. Welch JS, Westervelt P, Ding L, Larson DE, Klco JM, Kulkarni S, Wallis J, Chen K, Payton JE, Fulton RS, Veizer J, Schmidt H, Vickery TL, Heath S, Watson MA, Tomasson MH, Link DC, Graubert TA, DiPersio JF, Mardis ER, Ley TJ, Wilson RK. Use of whole-genome sequencing to diagnose a cryptic fusion oncogene. *JAMA* 2011;305:1577–84.

206. Garcia-Marcos L, Edwards J, Kennington E, Aurora P, Baraldi E, Carraro S, Gappa M, Louis R, Moreno-Galdo A, Peroni DG, Pijnenburg M, Priftis KN, Sanchez-Solis M, Schuster A, Walker S, EARIP Collaboration. Priorities for future research into asthma diagnostic tools: A PAN-EU consensus exercise from the European asthma research innovation partnership (EARIP). *Clin Exp Allergy* 2018;48:104–120.

207. Cao C, Li W, Hua W, Yan F, Zhang H, Huang H, Ying Y, Li N, Lan F, Wang S, Chen X, Li J, Liu J, Lai T, Bao Z, Cao Y, Zhao Y, Huang G, Huang L, Huang Y, Wu P, Peng C, Chen Z, Chung KF, Zhong N, Ying S, Shen H. Proteomic analysis of sputum reveals novel biomarkers for various presentations of asthma. *J Transl Med* 2017;15:171.

208. Smith D, Helgason H, Sulem P, Bjornsdottir US, Lim AC, Sveinbjornsson G, Hasegawa H, Brown M, Ketchem RR, Gavala M, Garrett L, Jonasdottir A, Jonasdottir A, Sigurdsson A, Magnusson OT, Eyjolfsson GI, Olafsson I, Onundarson PT, Sigurdardottir O, Gislason D, Gislason T, Ludviksson BR, Ludviksdottir D, Boezen HM, Heinzmann A, Krueger M, Porsbjerg C, Ahluwalia TS, Waage J, *et al.* A rare IL33 loss-of-function mutation reduces blood eosinophil counts and protects from asthma. In: Lappalainen T, editor. *PLOS Genet* 2017;13:e1006659.

209. McGeachie MJ, Clemmer GL, Hayete B, Xing H, Runge K, Wu AC, Jiang X, Lu Q, Church B, Khalil I, Tantisira K, Weiss S. Systems biology and in vitro validation identifies FAM129A as asthma steroid response modulator. *J Allergy Clin Immunol* 2018;doi:10.1016/j.jaci.2017.11.059.

210. Hekking P-P, Loza MJ, Pavlidis S, de Meulder B, Lefaudeux D, Baribaud F, Auffray C, Wagener AH, Brinkman P, Lutter R, Bansal AT, Sousa AR, Bates SA, Pandis Y, Fleming LJ, Shaw DE, Fowler SJ, Guo Y, Meiser A, Sun K, Corfield J, Howarth PH, Bel EH, Adcock IM, Chung KF, Djukanovic R, Sterk PJ, Adcock IM, Ahmed H, *et al.* Pathway discovery using transcriptomic profiles in adult-onset severe asthma. *J Allergy Clin Immunol* 2017;doi:10.1016/j.jaci.2017.06.037.

211. Shaw DE, Sousa AR, Fowler SJ, Fleming LJ, Roberts G, Corfield J, Pandis I, Bansal AT, Bel EH, Auffray C, Compton CH, Bisgaard H, Bucchioni E, Caruso M, Chanez P, Dahlén B, Dahlen S-E, Dyson K, Frey U, Geiser T, Gerhardsson de Verdier M, Gibeon D, Guo Y, Hashimoto S, Hedlin G, Jeyasingham E, Hekking P-PW, Higenbottam T, Horváth I, *et al.* Clinical and inflammatory characteristics of the European U-BIOPRED adult severe asthma cohort. *Eur Respir J* 2015;46:1308–1321.

212. Bigler J, Boedigheimer M, Schofield JPR, Skipp PJ, Corfield J, Rowe A, Sousa AR, Timour M, Twehues L, Hu X, Roberts G, Welcher AA, Yu W, Lefaudeux D, Meulder B De, Auffray C, Chung KF, Adcock IM, Sterk PJ, Djukanović R, U-BIOPRED Study Group ‖ . A Severe Asthma Disease Signature from Gene Expression Profiling of Peripheral Blood from U-BIOPRED Cohorts. *Am J Respir Crit Care Med* 2017;195:1311–1320.

213. Auffray C, Adcock IM, Chung KF, Djukanovic R, Pison C, Sterk PJ. An Integrative Systems Biology Approach to Understanding Pulmonary Diseases. *Chest* 2010;137:1410–1416.

214. Tsitsiou E, Williams AE, Moschos SA, Patel K, Rossios C, Jiang X, Adams O-D, Macedo P, Booton R, Gibeon D, Chung KF, Lindsay MA. Transcriptome analysis shows activation of circulating CD8+ T cells in patients with severe asthma. *J Allergy Clin Immunol* 2012;129:95–103.

215. Yan X, Chu J-H, Gomez J, Koenigs M, Holm C, He X, Perez MF, Zhao H, Mane S, Martinez FD, Ober C, Nicolae DL, Barnes KC, London SJ, Gilliland F, Weiss ST, Raby BA, Cohn L, Chupp GL. Noninvasive Analysis of the Sputum Transcriptome Discriminates Clinical Phenotypes of Asthma. *Am J Respir Crit Care Med* 2015;191:1116–1125.

216. Shikotra A, Choy DF, Ohri CM, Doran E, Butler C, Hargadon B, Shelley M, Abbas AR, Austin CD, Jackman J, Wu LC, Heaney LG, Arron JR, Bradding P. Increased expression of immunoreactive thymic stromal lymphopoietin in patients with severe asthma. *J Allergy Clin Immunol* 2012;129:104–111.e9.

217. Gomez JL, Yan X, Holm CT, Grant N, Liu Q, Cohn L, Nezgovorova V, Meyers DA, Bleecker ER, Crisafi GM, Jarjour NN, Rogers L, Reibman J, Chupp GL, SARP Investigators. Characterisation of asthma subgroups associated with circulating YKL-40 levels. *Eur Respir J* 2017;50:1700800.

218. Singhania A, Rupani H, Jayasekera N, Lumb S, Hales P, Gozzard N, Davies DE, Woelk CH, Howarth PH. Altered Epithelial Gene Expression in Peripheral Airways of Severe Asthma. In: Mersha TB, editor. *PLoS One* 2017;12:e0168680.

219. Riccio AM, Mauri P, De Ferrari L, Rossi R, Di Silvestre D, Benazzi L, Chiappori A, Dal Negro RW, Micheletto C, Canonica GW. Galectin-3: an early predictive biomarker of modulation of airway remodeling in patients with severe asthma treated with omalizumab for 36 months. *Clin Transl Allergy* 2017;7:6.

220. Zhao Y, Tian B, Sadygov RG, Zhang Y, Brasier AR. Integrative proteomic analysis reveals reprograming tumor necrosis factor signaling in epithelial mesenchymal transition. *J Proteomics* 2016;148:126–138.

221. Holand T, Riffo-Vasquez Y, Spina D, O'Connor B, Woisin F, Sand C, Marber M, Bacon KB, Rohlff C, Page CP. A role for mitogen kinase kinase 3 in pulmonary inflammation validated from a proteomic approach. *Pulm Pharmacol Ther* 2014;27:156–163.

222. Lefaudeux D, De Meulder B, Loza MJ, Peffer N, Rowe A, Baribaud F, Bansal AT, Lutter R, Sousa AR, Corfield J, Pandis I, Bakke PS, Caruso M, Chanez P, Dahlén S-E, Fleming LJ, Fowler SJ, Horvath I, Krug N, Montuschi P, Sanak M, Sandstrom T, Shaw DE, Singer F, Sterk PJ, Roberts G, Adcock IM, Djukanovic R, Auffray C, *et al.* U-BIOPRED clinical adult asthma clusters linked to a subset of sputum omics. *J Allergy Clin Immunol* 2017;139:1797–1807.

223. Gibeon D, Chung KF. The investigation of severe asthma to define phenotypes. *Clin Exp Allergy* 2012;42:678–692.

224. Lazarev D, Miller RL, Dimango E, Fu X-D, Li H-R, Logan CJ, Manley JL. cFLIP expression is altered in severe corticosteroid-resistant asthma. *Genomics Data* 2014;2:99–104.

225. Chun E, Lee H-S, Bang B-R, Kim T-W, Lee S-H, Kim J-H, Cho S-H, Min K-U, Kim Y-Y, Park H-W. Dexamethasone-Induced FKBP51 Expression in Peripheral Blood Mononuclear Cells Could Play a Role in Predicting the Response of Asthmatics to Treatment with Corticosteroids. *J Clin Immunol* 2011;31:122–127.

226. Hastie AT, Moore WC, Meyers DA, Vestal PL, Li H, Peters SP, Bleecker ER, National Heart, Lung, and Blood Institute Severe Asthma Research Program the NSARP. Analyses of asthma severity phenotypes and inflammatory proteins in subjects stratified by sputum granulocytes. *J Allergy Clin Immunol* 2010;125:1028–1036.e13.

227. Lee T-H, Jang A-S, Park J-S, Kim T-H, Choi YS, Shin H-R, Park S-W, Uh S-T, Choi J-S, Kim YH, Kim Y, Kim S, Chung IY, Jeong SH, Park C-S. Elevation of S100 calcium binding protein A9 in sputum of neutrophilic inflammation in severe uncontrolled asthma. *Ann Allergy Asthma Immunol* 2013;111:268–275.e1.

228. Gupta A, Bush A, Hawrylowicz C, Saglani S. Vitamin D and Asthma in Children. *Paediatr Respir Rev* 2012;13:236–243.

229. Orsmark-Pietras C, James A, Konradsen JR, Nordlund B, Söderhäll C, Pulkkinen V, Pedroletti C, Daham K, Kupczyk M, Dahlén B, Kere J, Dahlén S-E, Hedlin G, Melén E. Transcriptome analysis reveals upregulation of bitter taste receptors in severe asthmatics. *Eur Respir J* 2013;42:65–78.

230. Himes BE, Koziol-White C, Johnson M, Nikolos C, Jester W, Klanderman B, Litonjua AA, Tantisira KG, Truskowski K, MacDonald K, Panettieri RA, Weiss ST. Vitamin D Modulates Expression of the Airway Smooth Muscle Transcriptome in Fatal Asthma. In: Morty RE, editor. *PLoS One* 2015;10:e0134057.

231. Animals NRC (US) C for the U of the G for the C and U of L. *Guide for the Care and Use of Laboratory Animals*. *Guid Care Use Lab Anim* National Academies Press (US); 2011. doi:10.17226/12910.

232. Chapman DG, Tully JE, Nolin JD, Janssen-Heininger YM, Irvin CG. Animal models of allergic airways disease: where are we and where to next? *J Cell Biochem* 2014;115:2055–64.

233. van der Worp HB, Howells DW, Sena ES, Porritt MJ, Rewell S, O'Collins V, Macleod MR. Can animal models of disease reliably inform human studies? *PLoS Med* 2010;7:e1000245.

234. Sagar S, Akbarshahi H, Uller L. Translational value of animal models of asthma: Challenges and promises. *Eur J Pharmacol* 2015;759:272–277.

235. Kirschvink N, Reinhold P. Use of alternative animals as asthma models. *Curr Drug Targets* 2008;9:470–84.

236. Trzil JE, Reinero CR. Update on Feline Asthma. *Vet Clin North Am Small Anim Pract* 2014;44:91–105.

237. Martin JG. [Animal models of bronchial hyperreactivity]. *Rev Mal Respir* 1994;11:93–9.

238. Darowski MJ, Hannon VM, Hirshman CA. Corticosteroids decrease airway hyperresponsiveness in the Basenji-Greyhound dog model of asthma. *J Appl Physiol* 1989;66:1120–1126.

239. Williams K, Roman J. Studying human respiratory disease in animals--role of induced and naturally occurring models. *J Pathol* 2016;238:220–32.

240. Mullane K. Asthma translational medicine: report card. *Biochem Pharmacol* 2011;82:567–85.

241. Perrin S. Preclinical research: Make mouse studies work. *Nature* 2014;507:423–425.

242. Jucker M. The benefits and limitations of animal models for translational research in neurodegenerative diseases. *Nat Med* 2010;16:1210–1214.

243. Aun M, Bonamichi-Santos R, Arantes-Costa FM, Kalil J, Giavina-Bianchi P. Animal models of asthma: utility and limitations. *J Asthma Allergy* 2017;Volume10:293–301.

244. Zosky GR, von Garnier C, Stumbles P a, Holt PG, Sly PD, Turner DJ. The pattern of methacholine responsiveness in mice is dependent on antigen challenge dose. *Respir Res* 2004;5:15.

245. Renz H, Smith HR, Henson JE, Ray BS, Irvin CG, Gelfand EW. Aerosolized antigen exposure without adjuvant causes increased IgE production and increased airway responsiveness in the mouse. *J Allergy Clin Immunol* 1992;89:1127–38.

246. Korsgren M, Erjefält JS, Korsgren O, Sundler F, Persson CG. Allergic eosinophil-rich inflammation develops in lungs and airways of B cell-deficient mice. *J Exp Med* 1997;185:885–92.

247. Bracken SJ, Adami AJ, Szczepanek SM, Ehsan M, Natarajan P, Guernsey LA, Shahriari N, Rafti E, Matson AP, Schramm CM, Thrall RS. Long-Term Exposure to House Dust Mite Leads to the Suppression of Allergic Airway Disease Despite Persistent Lung Inflammation. *Int Arch Allergy Immunol* 2015;166:243–58.

248. Johnson JR, Wiley RE, Fattouh R, Swirski FK, Gajewska BU, Coyle AJ, Gutierrez-Ramos J-C, Ellis R, Inman MD, Jordana M. Continuous Exposure to House Dust Mite Elicits Chronic Airway Inflammation and Structural Remodeling. *Am J Respir Crit Care Med* 2004;169:378–385.

249. Bassirpour G, Zoratti E. Cockroach allergy and allergen-specific immunotherapy in asthma. *Curr Opin Allergy Clin Immunol* 2014;14:535–541.

250. Durham CG, Schwiebert LM, Lorenz RG. Use of the cockroach antigen model of acute asthma to determine the immunomodulatory role of early exposure to gastrointestinal infection. *Methods Mol Biol* 2013;1032:271–86.

251. Kurup VP, Mauze S, Choi H, Seymour BW, Coffman RL. A murine model of allergic bronchopulmonary aspergillosis with elevated eosinophils and IgE. *J Immunol* 1992;148:3783–8.

252. Kurup VP, Choi H, Murali PS, Coffman RL. IgE and eosinophil regulation in a murine model of allergic aspergillosis. *J Leukoc Biol* 1994;56:593–8.

253. Fan M, Jamal Mustafa S. Role of adenosine in airway inflammation in an allergic mouse model of asthma. *Int Immunopharmacol* 2006;6:36–45.

254. PAUWELS RA, BRUSSELLE GJ, KIPS JC. Cytokine Manipulation in Animal Models of Asthma. *Am J Respir Crit Care Med* 1997;156:S78–S81.

255. Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J Exp Med* 1996;183:195–201.

256. Fish SC, Donaldson DD, Goldman SJ, Williams CMM, Kasaian MT. IgE generation and mast cell effector function in mice deficient in IL-4 and IL-13. *J Immunol* 2005;174:7716–24.

257. Holt P, Rose A, Batty J, Turner K. Induction of adjuvant-independent IgE respones in inbred mice: primary, secondary, and persistent IgE responses to ovalbumin and ovomucoid. *Int Arch Allergy Immunol* 1981;65:42–50.

258. Webb DC, Mahalingam S, Cai Y, Matthaei KI, Donaldson DD, Foster PS. Antigen-specific production of interleukin (IL)-13 and IL-5 cooperate to mediate IL-4R??-independent airway hyperreactivity. *Eur J Immunol* 2003;33:3377–3385.

259. Shapiro SD. The use of transgenic mice for modeling airways disease. *Pulm Pharmacol Ther* 2008;21:699–701.

260. Bonamichi-Santos R, Aun M V, Agondi RC, Kalil J, Giavina-Bianchi P. Microbiome and Asthma: What Have Experimental Models Already Taught Us? *J Immunol Res* 2015;2015:614758.

261. Nials AT, Uddin S. Mouse models of allergic asthma: acute and chronic allergen challenge. *Dis Model Mech* 2008;1:213–20.

262. Boyce JA, Austen KF. No audible wheezing: nuggets and conundrums from mouse asthma models. *J Exp Med* 2005;201:1869–73.

263. Yeh Y-C, Yen H-R, Jiang R-S, Wang R-C, Huang W-C, Chen S-C, Lin B-S, Liang K-L. Dose-response relationship of specific allergen exposure-induced immunological tolerance: a mouse model. *Int Forum Allergy Rhinol* 2015;5:784–93.

264. Jungsuwadee P, Benkovszky M, Dekan G, Stingl G, Epstein MM. Repeated Aerosol Allergen Exposure Suppresses Inflammation in B-Cell-Deficient Mice with Established Allergic Asthma. *Int Arch Allergy Immunol* 2004;133:40–48.

265. McMillan SJ, Lloyd CM. Prolonged allergen challenge in mice leads to persistent airway remodelling. *Clin Exp Allergy* 2004;34:497–507.

266. Malm-Erjefält M, Persson CGA, Erjefält JS. Degranulation status of airway tissue eosinophils in mouse models of allergic airway inflammation. *Am J Respir Cell Mol Biol* 2001;24:352–359.

267. Kim C-K, Callaway Z, Kim DW, Kita H. Eosinophil degranulation is more important than eosinophilia in identifying asthma in chronic cough. *J Asthma* 2011;48:994–1000.

268. Temelkovski J, Hogan SP, Shepherd DP, Foster PS, Kumar RK. An improved murine model of asthma: selective airway inflammation, epithelial lesions and increased methacholine responsiveness following chronic exposure to aerosolised allergen. *Thorax* 1998;53:849–856.

269. Bryan SA, O'Connor BJ, Matti S, Leckie MJ, Kanabar V, Khan J, Warrington SJ, Renzetti L, Rames A, Bock JA, Boyce MJ, Hansel TT, Holgate ST, Barnes PJ. Effects of recombinant human interleukin-12 on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet (London, England)* 2000;356:2149–53.

270. O'Byrne PM, Inman MD, Parameswaran K. The trials and tribulations of IL-5, eosinophils, and allergic asthma. *J Allergy Clin Immunol* 2001;108:503–508.

271. Hylkema MN, Hoekstra MO, Luinge M, Timens W. The strength of the OVA-induced airway inflammation in rats is strain dependent. *Clin Exp Immunol* 2002;129:390–6.

272. Liu S, Chihara K, Maeyama K. The contribution of mast cells to the late-phase of allergic asthma in rats. *Inflamm Res* 2005;54:221–8.

273. De Alba J, Raemdonck K, Dekkak A, Collins M, Wong S, Nials AT, Knowles RG, Belvisi MG, Birrell MA. House dust mite induces direct airway inflammation in vivo: implications for future disease therapy? *Eur Respir J* 2010;35:1377–87.

274. Misawa M, Takenouchi K, Abiru T, Yoshino Y, Yanaura S. Strain difference in an allergic asthma model in rats. *Jpn J Pharmacol* 1987;45:63–8.

275. Hylkema MN. The strength of the OVA - induced airway inflammation in rats is strain dependent. 2002;129:390–396.

276. RATNER B, JACKSON HC, GRUEHL HL. RESPIRATORY ANAPHYLAXIS. *Am J Dis Child* 1927;34:23.

277. Kallós P, Kallós L. Experimental asthma in guinea pigs revisited. *Int Arch Allergy Appl Immunol* 1984;73:77–85.

278. Smith N, Johnson FJ. Early- and late-phase bronchoconstriction, airway hyper-reactivity and cell influx into the lungs, after 5???-adenosine monophosphate inhalation: Comparison with ovalbumin. *Clin Exp Allergy* 2005;35:522–530.

279. Sundstrom E, Lastbom L, Ryrfeldt A, Dahlen SE. Interactions among three classes of mediators explain antigen-induced bronchoconstriction in the isolated perfused and ventilated guinea pig lung. *J Pharmacol Exp Ther* 2003;307:408–418.

280. Richardson J, Beland J. Nonadrenergic inhibitory nervous system in human airways. *J Appl Physiol* 1976;41:764–771.

281. Canning BJ. Reflex regulation of airway smooth muscle tone. *J Appl Physiol* 2006;101:971–985.

282. Canning BJ, Chou Y. Using guinea pigs in studies relevant to asthma and COPD. *Pulm Pharmacol Ther* 2008;21:702–720.

283. Hutson PA, Holgate ST, Church MK. The effect of cromolyn sodium and albuterol on early and late phase bronchoconstriction and airway leukocyte infiltration after allergen challenge of nonanesthetized guinea pigs. *Am Rev Respir Dis* 1988;

284. Ressmeyer AR, Larsson AK, Vollmer E, Dahlèn SE, Uhlig S, Martin C. Characterisation of guinea pig precision-cut lung slices: comparison with human tissues. *Eur Respir J* 2006;28:603–11.

285. Ellis JL, Undem BJ, Kays JS, Ghanekar S V, Barthlow HG, Buckner CK. Pharmacological examination of receptors mediating contractile responses to tachykinins in airways isolated from human, guinea pig and hamster. *J Pharmacol Exp Ther* 1993;267:.

286. Ndukwu IM, White SR, Leff AR, Mitchell RW. EP1 receptor blockade attenuates both spontaneous tone and PGE2-elicited contraction in guinea pig trachealis. *Am J Physiol* 1997;273:L626-33.

287. Clarke DL, Belvisi MG, Smith SJ, Hardaker E, Yacoub MH, Meja KK, Newton R, Slater DM, Giembycz MA. Prostanoid receptor expression by human airway smooth muscle cells and regulation of the secretion of granulocyte colony-stimulating factor. *Am J Physiol Lung Cell Mol Physiol* 2005;288:L238-50.

288. McKenniff M, Rodger IW, Norman P, Gardiner PJ. Characterisation of receptors mediating the contractile effects of prostanoids in guinea-pig and human airways. *Eur J Pharmacol* 1988;153:149–59.

289. Andrew DK, Schellenberg RR, Hogg JC, Hanna CJ, Pare PD. Physiological and Immunological Effects of Chronic Antigen Exposure in Immunized Guinea Pigs. *Int Arch Allergy Appl Immunol* 1984;75:208–213.

290. Moise NS, Wiedenkeller D, Yeager AE, Blue JT, Scarlett J. Clinical, radiographic, and bronchial cytologic features of cats with bronchial disease: 65 cases (1980-1986). *J Am Vet Med Assoc* 1989;194:1467–73.

291. Padrid P, Snook S, Finucane T, Shiue P, Cozzi P, Solway J, Leff AR. Persistent airway hyperresponsiveness and histologic alterations after chronic antigen challenge in cats. *Am J Respir Crit Care Med* 1995;151:184–93.

292. Sasaki H, Yanai M, Shimura S, Okayama H, Aikawa T, Sasaki T, Takishima T. Late Asthmatic Response to *Ascaris* Antigen Challenge in Dogs Treated with Metyrapone. *Am Rev Respir Dis* 1987;136:1459–1465.

293. Redman TK, Rudolph K, Barr EB, Bowen LE, Muggenburg BA, Bice DE. Pulmonary immunity to ragweed in a Beagle dog model of allergic asthma. *Exp Lung Res* 27:433–51.

294. Redman TK, Rudolph K, Barr EB, Bowen LE, Muggenburg A, Bice DE. Pulmonary immunity to ragweek in a beagle dog model of allergic asthma. *Exp Lung Res* 2001;27:433–451.

295. Collie D, DeBoer D, BA M, Bice D. Evaluation of association of blood and bronchoalveolar eosinophil numbers and serum total immunoglobulin E concentration with the expression of nonspecific airway reactivity in dogs. *Am J Vet Res* 1997;58:34–39.

296. Osborne ML, Evans TW, Sommerhoff CP, Chung KF, Hirshman CA, Boushey HA, Nadel JA. Hypotonic and isotonic aerosols increase bronchial reactivity in basenji-greyhound dogs. *Am Rev Respir Dis* 1987;135:345–9.

297. Hirshman CA, Malley A, Downes H. Basenji-Greyhound dog model of asthma: reactivity to Ascaris suum, citric acid, and methacholine. *J Appl Physiol* 1980;49:953–957.

298. Hirshman CA, Downes H. Airway responses to methacholine and histamine in basenji greyhounds and other purebred dogs. *Respir Physiol* 1986;63:339–46.

299. Kirschvink N, Reinhold P. Use of Alternative Animals as Asthma Models. *Curr Drug Targets* 2008;9:470–484.

300. Abraham WM, Delehunt JC, Yerger L, Marchette B. Characterization of a late phase pulmonary response after antigen challenge in allergic sheep. *Am Rev Respir Dis* 1983;128:839–44.

301. Abraham WM, Sielczak MW, Wanner A, Perruchoud AP, Blinder L, Stevenson JS, Ahmed A, Yerger LD. Cellular markers of inflammation in the airways of allergic sheep with and without allergen-induced late responses. *Am Rev Respir Dis* 1988;138:1565–71.

302. Abraham WM, Sielczak MW, Wanner A, Perruchoud AP, Blinder L, Stevenson JS, Ahmed A, Yerger LD. Cellular markers of inflammation in the airways of allergic sheep with and without allergen-induced late responses. *Am Rev Respir Dis* 1988;138:1565–71.

303. Lanes S, Stevenson JS, Codias E, Hernandez A, Sielczak MW, Wanner A, Abraham WM. Indomethacin and FPL-57231 inhibit antigen-induced airway hyperresponsiveness in sheep. *J Appl Physiol* 1986;61:864–72.

304. Freitag A, Watson RM, Matsos G, Eastwood C, O'Byrne PM. Effect of a platelet activating factor antagonist, WEB 2086, on allergen induced asthmatic responses. *Thorax* 1993;48:594–8.

305. Kuitert LM, Hui KP, Uthayarkumar S, Burke W, Newland AC, Uden S, Barnes NC. Effect of the platelet-activating factor antagonist UK-74,505 on the early and late response to allergen. *Am Rev Respir Dis* 1993;147:82–6.

306. Fornhem C, Kumlin M, Lundberg JM, Alving K. Allergen-induced late-phase airways obstruction in the pig: mediator release and eosinophil recruitment. *Eur Respir J* 1995;8:1100–9.

307. Jones TR, McAuliffe M, McFarlane CS, Piechuta H, Macdonald D, Rodger IW. Effects of a selective phosphodiesterase IV inhibitor (CDP-840) in a leukotriene-dependent non-human primate model of allergic asthma. *Can J Physiol Pharmacol* 1998;76:210–7.

308. Johnson HG, Stout BK. Late Phase Bronchoconstriction and Eosinophilia as well as Methacholine Hyperresponsiveness in <i>Ascaris</i>-Sensitive Rhesus Monkeys Were Reversed by Oral Administration of U-83836E. *Int Arch Allergy Immunol* 1993;100:362–366.

309. Gundel RH, Wegner CD, Letts LG. Antigen-induced Acute and Late-phase Responses in Primates. *Am Rev Respir Dis* 1992;146:369–373.

310. Schelegle ES, Gershwin LJ, Miller LA, Fanucchi M V., Van Winkle LS, Gerriets JP, Walby WF, Omlor AM, Buckpitt AR, Tarkington BK, Wong VJ, Joad JP, Pinkerton KB, Wu R, Evans MJ, Hyde DM, Plopper CG. Allergic Asthma Induced in Rhesus Monkeys by House Dust Mite (Dermatophagoides farinae). *Am J Pathol* 2001;158:333–341.

311. Plopper CG, Joad JP, Miller LA, Schelegle ES, Fanucchi M V, Van Winkle LS, Tyler NK, Avdalovic M V, Evans MJ, Lasley WL, Buckpitt AR, Pinkerton KE, Tarkington BK, Davis S, Nishio SJ, Gershwin LJ, Wu R, Hyde DM. Lung effects of inhaled corticosteroids in a rhesus monkey model of childhood asthma. *Clin Exp Allergy* 2012;42:1104–18.

312. Turner CR, Andresen CJ, Smith WB, Watson JW. Characterization of a primate model of asthma using anti-allergy/anti-asthma agents. *Inflamm Res* 1996;45:239–45.

313. Gundel RH, Wegner CD, Torcellini CA, Clarke CC, Haynes N, Rothlein R, Smith CW, Letts LG. Endothelial leukocyte adhesion molecule-1 mediates antigen-induced acute airway inflammation and late-phase airway obstruction in monkeys. *J Clin Invest* 1991;88:1407–11.

314. Roeder T, Isermann K, Kabesch M. *Drosophila* in Asthma Research. *Am J Respir Crit Care Med* 2009;179:979–983.

315. Roeder T, Isermann K, Kallsen K, Uliczka K, Wagner C. A Drosophila Asthma Model – What the Fly Tells Us About Inflammatory Diseases of the Lung. *Adv Exp Med Biol* 2012. p. 37–47.

316. Wagers SS, Haverkamp HC, Bates JHT, Norton RJ, Thompson-Figueroa JA, Sullivan MJ, Irvin CG. Intrinsic and antigen-induced airway hyperresponsiveness are the result of diverse physiological mechanisms. *J Appl Physiol* 2007;102:221–30.

317. Reddy AT, Lakshmi SP, Reddy RC. Murine model of allergen induced asthma. *J Vis Exp* 2012;e3771.doi:10.3791/3771.

318. HAMELMANN E, SCHWARZE J, TAKEDA K, OSHIBA A, LARSEN GL, IRVIN CG, GELFAND EW. Noninvasive Measurement of Airway Responsiveness in Allergic Mice Using Barometric Plethysmography. *Am J Respir Crit Care Med* 1997;156:766–775.

319. Johnson JR, Wiley RE, Fattouh R, Swirski FK, Gajewska BU, Coyle AJ, Gutierrez-Ramos J-C, Ellis R, Inman MD, Jordana M. Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling. *Am J Respir Crit Care Med* 2004;169:378–85.

320. Sundström E, Låstbom L, Ryrfeldt A, Dahlén S-E. Interactions among three classes of mediators explain antigen-induced bronchoconstriction in the isolated perfused and ventilated guinea pig lung. *J Pharmacol Exp Ther* 2003;307:408–18.

321. Griffiths-Johnson DA, Karol MH. Validation of a non-invasive technique to assess development of airway hyperreactivity in an animal model of immunologic pulmonary hypersensitivity. *Toxicology* 1991;65:283–94.

322. Abraham WM, Ahmed A, Cortes A, Sielczak MW, Hinz W, Bouska J, Lanni C, Bell RL. The 5-lipoxygenase inhibitor zileuton blocks antigen-induced late airway responses, inflammation and airway hyperresponsiveness in allergic sheep. *Eur J Pharmacol* 1992;217:119–26.

323. Bischof RJ, Snibson K, Shaw R, Meeusen ENT. Induction of allergic inflammation in the lungs of sensitized sheep after local challenge with house dust mite. *Clin Exp Allergy* 2003;33:367–75.

324. Iwashita K, Kawasaki H, Sawada M, In M, Mataki Y, Kuwabara T. Shortening of the induction period of allergic asthma in cynomolgus monkeys by Ascaris suum and house dust mite. *J Pharmacol Sci* 2008;106:92–9.

325. LOWELL FC. OBSERVATIONS ON HEAVES. AN ASTHMA-LIKE SYNDROME IN THE HORSE. *J Allergy* 35:322–30.

326. Derksen FJ, Robinson NE, Armstrong PJ, Stick JA, Slocombe RF. Airway reactivity in ponies with recurrent airway obstruction (heaves). *J Appl Physiol* 1985;58:598–604.

327. Couëtil LL, Cardwell JM, Gerber V, Lavoie J-P, Léguillette R, Richard EA. Inflammatory Airway Disease of Horses--Revised Consensus Statement. *J Vet Intern Med* 2016;30:503–15.

328. Magno M. Comparative anatomy of the tracheobronchial circulation. *Eur Respir J Suppl* 1990;12:557s–562s; discussion 562s–563s.

329. Derksen FJ, Olszewski MA, Robinson NE, Berney C, Hakala JE, Matson CJ, Ruth DT. Aerosolized albuterol sulfate used as a bronchodilator in horses with recurrent airway obstruction. *Am J Vet Res* 1999;60:689–93.

330. Relave F, David F, Leclère M, Alexander K, Hélie P, Meulyzer M, Lavoie JP, Marcoux M. Thoracoscopic lung biopsies in heaves-affected horses using a bipolar tissue sealing system. *Vet Surg* 2010;39:839–46.

331. Relave F, David F, Leclère M, Alexander K, Bussières G, Lavoie JP, Marcoux M. Evaluation of a thoracoscopic technique using ligating loops to obtain large lung biopsies in standing healthy and heaves-affected horses. *Vet Surg* 2008;37:232–40.

332. Naylor JM, Clark EG, Clayton HM. Chronic obstructive pulmonary disease: Usefulness of clinical signs, bronchoalveolar lavage, and lung biopsy as diagnostic and prognostic aids. *Can Vet J = La Rev Vet Can* 1992;33:591–8.

333. Derksen FJ, Scott JS, Miller DC, Slocombe RF, Robinson NE. Bronchoalveolar lavage in ponies with recurrent airway obstruction (heaves). *Am Rev Respir Dis* 1985;132:1066–70.

334. Zosky GR, Sly PD. Animal models of asthma. *Clin Exp Allergy* 2007;37:973–988.

335. Seahorn TL, Beadle RE, McGorum BC, Marley CL. Quantification of antigen-specific antibody concentrations in tracheal lavage fluid of horses with summer pasture-associated obstructive pulmonary disease. *Am J Vet Res* 1997;58:1408–11.

336. Benditt JO. Esophageal and gastric pressure measurements. *Respir Care* 2005;50:68-75-77.

337. Cockcroft DW. Direct Challenge Tests. *Chest* 2010;138:18S–24S.

338. Ferrari CR, Cooley J, Mujahid N, Costa LR, Wills RW, Johnson ME, Swiderski CE. Horses With Pasture Asthma Have Airway Remodeling That Is Characteristic of Human Asthma. *Vet Pathol* 2018;55:144–158.

339. Couëtil LL, Rosenthal FS, Simpson CM. Forced expiration: a test for airflow obstruction in horses. *J Appl Physiol* 2000;88:1870–9.

340. Smith N, Johnson FJ. Early- and late-phase bronchoconstriction, airway hyper-reactivity and cell influx into the lungs, after 5'-adenosine monophosphate inhalation: comparison with ovalbumin. *Clin Exp Allergy* 2005;35:522–30.

341. Zosky GR, von Garnier C, Stumbles PA, Holt PG, Sly PD, Turner DJ. The pattern of methacholine responsiveness in mice is dependent on antigen challenge dose. *Respir Res* 2004;5:15.

342. Durham CG, Schwiebert LM, Lorenz RG. Use of the cockroach antigen model of acute asthma to determine the immunomodulatory role of early exposure to gastrointestinal infection. *Methods Mol Biol* 2013;1032:271–86.

343. Kurup VP, Mauze S, Choi H, Seymour BW, Coffman RL. A murine model of allergic bronchopulmonary aspergillosis with elevated eosinophils and IgE. *J Immunol* 1992;148:3783–8.

344. Andrew DK, Schellenberg RR, Hogg JC, Hanna CJ, Paré PD. Physiological and immunological effects of chronic antigen exposure in immunized guinea pigs. *Int Arch Allergy Appl Immunol* 1984;75:208–13.

345. Shaw DE, Sousa AR, Fowler SJ, Fleming LJ, Roberts G, Corfield J, Pandis I, Bansal AT, Bel EH, Auffray C, Compton CH, Bisgaard H, Bucchioni E, Caruso M, Chanez P, Dahlén B, Dahlen S-E, Dyson K, Frey U, Geiser T, Gerhardsson de Verdier M, Gibeon D, Guo Y-K, Hashimoto S, Hedlin G, Jeyasingham E, Hekking P-PW, Higenbottam T, Horváth I, *et al.* Clinical and inflammatory characteristics of the European U-BIOPRED adult severe asthma cohort. *Eur Respir J* 2015;46:1308–21.

346. Chung KF. Neutrophilic asthma: a distinct target for treatment? *Lancet Respir Med* 2016;4:765–767.

347. Holgate ST, Polosa R. The mechanisms, diagnosis, and management of severe asthma in adults. *Rev 780 www.thelancet.com* 2006;368:.

348. Barnes PJ. Pathogenesis of asthma: a review. *J R Soc Med* 1983;76:580–6.

349. Sont JK, Willems LN, Bel EH, van Krieken JH, Vandenbroucke JP, Sterk PJ. Clinical control and histopathologic outcome of asthma when using airway hyperresponsiveness as an additional guide to long-term treatment. The AMPUL Study Group. *Am J Respir Crit Care Med* 1999;159:1043–51.

350. Postea O, Biel M. Exploring HCN channels as novel drug targets. *Nat Rev Drug Discov* 2011;10:903–14.

351. Xue L, Li Y, Han X, Yao L, Yuan J, Qin W, Liu F, Wang H. Investigation of hyperpolarization-activated cyclic nucleotide-gated channels in interstitial cells of Cajal of human bladder. *Urology* 2012;80:224.e13-8.

352. Xiao J, Nguyen T V, Ngui K, Strijbos PJLM, Selmer I-S, Neylon CB, Furness JB. Molecular and functional analysis of hyperpolarisation-activated nucleotide-gated (HCN) channels in the enteric nervous system. *Neuroscience* 2004;129:603–14.

353. Hurtado R, Bub G, Herzlinger D. A molecular signature of tissues with pacemaker activity in the heart and upper urinary tract involves coexpressed hyperpolarization-activated cation and T-type Ca2+ channels. *FASEB J* 2014;28:730–9.

354. Greenwood IA, Prestwich SA. Characteristics of hyperpolarization-activated cation currents in portal vein smooth muscle cells. *Am J Physiol Cell Physiol* 2002;282:C744-53.

355. McGovern AE, Robusto J, Rakoczy J, Simmons DG, Phipps S, Mazzone SB. The effect of hyperpolarization-activated cyclic nucleotide-gated ion channel inhibitors on the vagal control of guinea pig airway smooth muscle tone. *Br J Pharmacol* 2014;171:3633–50.

356. DiFrancesco D, Borer JS. The funny current: cellular basis for the control of heart rate. *Drugs* 2007;67 Suppl 2:15–24.

357. Cates CJ, Cates MJ. Regular treatment with formoterol for chronic asthma: serious adverse events. *Cochrane database Syst Rev* 2012;CD006923.doi:10.1002/14651858.CD006923.pub3.

358. Nelson HS, Weiss ST, Bleecker ER, Yancey SW, Dorinsky PM, SMART Study Group. The Salmeterol Multicenter Asthma Research Trial: a comparison of usual pharmacotherapy for asthma or usual pharmacotherapy plus salmeterol. *Chest* 2006;129:15–26.

359. Hunter C, Bright L, Costa L, Bowser J, Shaw P, Brashier M, Wilson J, CE S. Horses with a spontaneous, non-eosinophilic asthma-like disease demonstrate reversible airway hyper-responsiveness at methacholine doses used to diagnose moderate to severe human asthma. *Am J Respir Crit Care Med* 2013;187:.

360. Seibold MA, Wang B, Eng C, Kumar G, Beckman KB, Sen S, Choudhry S, Meade K, Lenoir M, Watson HG, Thyne S, Williams LK, Kumar R, Weiss KB, Grammer LC, Avila PC, Schleimer RP, Burchard EG, Brenner R. An african-specific functional polymorphism in KCNMB1 shows sex-specific association with asthma severity. *Hum Mol Genet* 2008;17:2681–2690.

361. Vroman H, Bergen IM, van Hulst JAC, van Nimwegen M, van Uden D, Schuijs MJ, Pillai SY, van Loo G, Hammad H, Lambrecht BN, Hendriks RW, Kool M. TNF-α–induced protein 3 levels in lung dendritic cells instruct T H 2 or T H 17 cell differentiation in eosinophilic or neutrophilic asthma. *J Allergy Clin Immunol* 2017;doi:10.1016/j.jaci.2017.08.012.

362. Evans CM, Kim K, Tuvim MJ, Dickey BF. Mucus hypersecretion in asthma: causes and effects. *Curr Opin Pulm Med* 2009;15:4–11.

363. Nguyen LP, Lin R, Parra S, Omoluabi O, Hanania NA, Tuvim MJ, Knoll BJ, Dickey BF, Bond RA. Beta2-adrenoceptor signaling is required for the development of an asthma phenotype in a murine model. *Proc Natl Acad Sci U S A* 2009;106:2435–40.

364. Wu C, Orozco C, Boyer J, Leglise M, Goodale J, Batalov S, Hodge CL, Haase J, Janes J, Huss JW, Su AI. BioGPS: an extensible and customizable portal for querying and organizing gene annotation resources. *Genome Biol* 2009;10:R130.

365. McGovern AE, Robusto J, Rakoczy J, Simmons DG, Phipps S, Mazzone SB. The effect of hyperpolarization-activated cyclic nucleotide-gated ion channel inhibitors on the vagal control of guinea pig airway smooth muscle tone. *Br J Pharmacol* 2014;171:3633–50.

366. Muccitelli RM, Tucker SS, Hay DW, Torphy TJ, Wasserman MA. Is the guinea pig trachea a good in vitro model of human large and central airways? Comparison on leukotriene-, methacholine-, histamine- and antigen-induced contractions. *J Pharmacol Exp Ther* 1987;243:467–73.

367. Steinhoff MS, von Mentzer B, Geppetti P, Pothoulakis C, Bunnett NW. Tachykinins and their receptors: contributions to physiological control and the mechanisms of disease. *Physiol Rev* 2014;94:265–301.

368. Barnes PJ. Neurogenic Airway Inflammation in Animal Models. In: Pierangelo Geppetti PH, editor. *Neurogenic Inflamm* Boca Raton, FL: CRC Press; 1996. p. 174–176.

369. Venugopal CS, Holmes EP, Polikepahad S, Laborde S, Kearney M, Moore RM. Neurokinin receptors in recurrent airway obstruction: a comparative study of affected and unaffected horses. *Can J Vet Res* 2009;73:25–33.

370. Altura BT, Turlapaty PD, Altura BM. Pentobarbital sodium inhibits calcium uptake in vascular smooth muscle. *Biochim Biophys Acta* 1980;595:309–12.

371. Mawby DI, Meric SM, Crichlow EC, Papich MG. Pharmacological relaxation of the urethra in male cats: a study of the effects of phenoxybenzamine, diazepam, nifedipine and xylazine. *Can J Vet Res* 1991;55:28–32.

372. Fox K, Ford I, Steg PG, Tendera M, Ferrari R, BEAUTIFUL Investigators. Ivabradine for patients with stable coronary artery disease and left-ventricular systolic dysfunction (BEAUTIFUL): a randomised, double-blind, placebo-controlled trial. *Lancet (London, England)* 2008;372:807–16.

373. Greene D, Kang S, Kosenko A, Hoshi N. Adrenergic regulation of HCN4 channel requires protein association with β2-adrenergic receptor. *J Biol Chem* 2012;287:23690–7.

374. Haitin Y. A "funny" cyclic dinucleotide receptor. *Nat Chem Biol* 2014;10:413–414.

375. Okabe K, Inoue Y, Kawarabayashi T, Kajiya H, Okamoto F, Soeda H. Physiological significance of hyperpolarization-activated inward currents (Ih) in smooth muscle cells from the circular layers of pregnant rat myometrium. *Pflugers Arch* 1999;439:76–85.

376. Ohkubo T, Kawarabayashi T, Inoue Y, Kitamura K. Differential expression of L- and T-type calcium channels between longitudinal and circular muscles of the rat myometrium during pregnancy. *Gynecol Obstet Invest* 2005;59:80–5.

377. Orsino A, Taylor C V, Lye SJ. Connexin-26 and connexin-43 are differentially expressed and regulated in the rat myometrium throughout late pregnancy and with the onset of labor. *Endocrinology* 1996;137:1545–53.

Online supplement references

1s. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol10:R25.

2s. Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B. Mapping and quantifying mammalian transcriptomes by rna-seq. Nat Methods 2008;5:621-628.