Response of the Bronchial Epithelium to Challenge in Severe Equine Asthma

by

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Severe equine asthma (recurrent airway obstruction, heaves) is an inflammatory lung disease affecting horses chronically exposed to adverse environments. Genetic predisposition to the disease is reported to be heterogeneous across families, but clinical signs during exacerbation are remarkably similar. In order to identify commonly expressed genes and pathways in horses with asthma, we sequenced RNA from endobronchial biopsies collected from control and affected horses before and after an asthmatic stimulus. Differential expression analysis of the bronchial epithelium yielded 111 genes significantly different between asthmatic and non-asthmatic horses. Gene set and network analyses identified overrepresentation of upregulated genes involved in neutrophil migration and chemotaxis, immune and inflammatory responses, secretion, blood coagulation and apoptosis. Downregulated genes were overrepresented in rhythmic processes, referring to physiological rhythm. Network analysis identified MMP4, MMP1, IL8 and TLR4 as pivotal molecules, suggesting they have key roles in disease. Furthermore, in asthmatic horses, a large number of significantly upregulated genes contained E2F binding motifs, and were associated with cell cycle, neutrophilic response, hedgehog signaling, hemostasis and coagulation. These genes and motifs were not detected at similar frequencies in non-asthmatic horses. RNA-Seq was also used to identify genetic variants potentially linked to disease mechanisms. Single base mutations in parkin co-regulated (PACRG) and rotatin (RTTN) were identified as more prevalent in asthmatic compared to non-asthmatic horses, and confirmed with Sanger sequencing. PACRG and RTTN are essential proteins in motile and primary cilia development and function, befitting further investigation of cilia morphology in asthmatics. As such, cilia and microvilli were evaluated by electron microscopy, and ultrastructural abnormalities consisting of cilia of abnormal shape and size and branching
microvilli of heterogeneous length were identified. These changes are hypothesized to be a consequence of chronic inflammation and also to contribute to progressive deterioration of bronchial epithelial function. Abnormal cilia are likely to reduce mucociliary clearance and hedgehog signaling, which in turn links gene expression and variants to physiological abnormalities in the bronchiolar epithelium. In summary, studies in this thesis have revealed multiple unique aspects of the bronchial epithelial response in asthmatic relative to non-asthmatic horses. The epithelial barrier is of paramount importance to modulate the host response to environmental agents, but other components of the lung not examined in this thesis also contribute to development of asthma.
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DECLARATION OF WORK PERFORMED
I declare that all work presented in this thesis was completed by myself under the supervision of Dr. Dorothee Bienzle and an advisory committee composed of Dr. Brandon Lillie and Dr. Lewis Luken, with the following exceptions:

• Dr. Dorothee Bienzle assisted with data analysis, microscopic review, and manuscript preparation.
• Dr. Cameron Ackerley (Department of Physiology and Experimental Medicine, University of Toronto) processed fixed tissue specimen, acquired electron microscopy images and assisted with image analysis.
• Dr. Anibal Armien (Department of Veterinary Population Medicine, University of Minnesota) reviewed selected electron microscopy images.
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<td>airway surface liquid</td>
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<tr>
<td>ASM</td>
<td>airway smooth muscle</td>
</tr>
<tr>
<td>ASMC</td>
<td>airway smooth muscle cell</td>
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<tr>
<td>ATI</td>
<td>alveolar type I</td>
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<tr>
<td>ATII</td>
<td>alveolar type II</td>
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<td>bronchoalveolar lavage fluid</td>
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<td>B-cell lymphoma</td>
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<td>BCV</td>
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<td>cDNA</td>
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<td>extracellular matrix</td>
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<td>ERM</td>
<td>ezrin, radixin and moesin</td>
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<td>GOBP</td>
<td>Gene Ontology for biological processes</td>
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<td>GSEA</td>
<td>Gene Set Enrichment Analysis</td>
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<td>sonic hedgehog</td>
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<td>IFT</td>
<td>intraflagellar transport</td>
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<td>interleukin</td>
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<td>InsP₃R</td>
<td>Inositol-1,4,5-trisphosphate receptor</td>
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<table>
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<th>Abbreviation</th>
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<tr>
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<td>matrix metalloproteinase</td>
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<td>neutrophil extracellular trap</td>
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<td>NGS</td>
<td>next-generation sequencing</td>
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<td>PANTHER</td>
<td>Protein ANalysys TThrough Evolutionary Relationships</td>
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<tr>
<td>PCD</td>
<td>primary ciliary dyskinesia</td>
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<tr>
<td>PCL</td>
<td>periciliary liquid</td>
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<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
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<td>PFT</td>
<td>pulmonary function testing</td>
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<td>pI</td>
<td>isoelectric point</td>
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<tr>
<td>qPCR</td>
<td>quantitative real-time PCR</td>
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<td>QTLs</td>
<td>quantitative trait loci</td>
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<tr>
<td>RAO</td>
<td>recurrent airway obstruction</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
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<td>RYR</td>
<td>ryanodine receptor</td>
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<tr>
<td>SCGB</td>
<td>secretoglobin</td>
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<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
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<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>STRING</td>
<td>Search Tool for the Retrieval of Interacting Genes/Proteins</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNFRSF</td>
<td>tumor necrosis factor receptor superfamily</td>
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<tr>
<td>TSLP</td>
<td>thymic stromal lymphopoietin</td>
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<tr>
<td>TWEAKR</td>
<td>tumor necrosis factor-like weak inducer of apoptosis receptor</td>
</tr>
<tr>
<td>VDCC</td>
<td>voltage-dependent Ca^{2+} channel</td>
</tr>
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<td>WB</td>
<td>western blot</td>
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CHAPTER 1

Introduction

Lung: components, structure and function

In mammalian species, lungs serve to exchange gas between cells and the environment as required to sustain the metabolic needs of active respiration. The respiratory tract comprises five distinct regions: the nose, trachea, bronchi, bronchioles and alveoli. Each region has specific cell populations, structures and functions (summarized in Table 1.1).

The upper airway, which includes the nasal cavity, the trachea and the main stem of the bronchi, comprises the cartilaginous part of the airway and is mostly lined by basal and ciliated epithelial cells, goblet cells and secretory club cells. The bronchioles are populated by secretory club cells, ciliated cells and neuroendocrine (NE) cell clusters, while the alveoli are composed of alveolar type (AT)I and II cells [1]. Current knowledge suggests that club (Clara) cells, basal cells and ATII cells may be progenitors that give rise to differentiated cell populations in the lung and therefore contribute to repair and maintenance of the epithelium [1,2].

Development of the bronchiolar epithelium

Lung development originates from the endodermal epithelium. Following formation of two embryonic buds, dichotomous branching occurs in four phases: bronchial and respiratory tree development, vascularization and terminal sac formation, terminal sac development, and maturation of alveolar ducts and alveoli [3]. Each phase involves specific interactions between genes, transcription factors, growth factors, extracellular matrix (ECM), and integrin and non-integrin signals [4]. The molecular mechanisms are complex and incompletely understood, but key pathways involve hedgehog (HH), patched, smoothened, epidermal growth factor (EGF), fibroblast growth factor (FGF), fibroblast growth factor receptor (FGFR), sprouty, bone morphogenetic protein 4 (BMP-4), transforming growth factor beta (TGF-β), mothers against decapentaplegic homologs (SMADs), and others [3,5].

In the early stages of epithelial development, cells are thought to be multipotent and to co-express factors such as surfactant proteins (SP)-C, SP-A, club cell (CC)-10 and calcitonin gene-related
peptide (cGRP). These factors become restricted to the specific cell lineages of ATII, club and NE cells, respectively, later in development [4].

**Response of the bronchiolar epithelium to environmental challenges**

**Club, mucous and basal cells**

Airway epithelium is directly exposed to environmental airborne substances such as dust and pathogens, and has a dual barrier and secretion role. In homeostasis the lung is characterized by very gradual epithelial cell turnover, while following injury there is a dramatic increase in cell proliferation. After injury the impaired epithelium engages in a rapid repair/regeneration process consisting of acute inflammation followed by recruitment of immune cells and epithelial cell migration at the injury site [1,6].

Club cells are the major progenitor cell population of the bronchiolar epithelium and have remarkable morphologic diversity across species [7]. They are self-renewing, give rise to differentiated cells, undergo dedifferentiation when activated, and contribute to homeostasis in the bronchial and bronchiolar microenvironment [6,8-11]. Club cells express a specific protein, secretoglobin (SCGB) 1A1, that has anti-inflammatory and possible transcription factor activity and as such contributes to defense and homeostasis of the airway [9,12]. Horses have three copies of the secretoglobin gene, which in other species is found as single copy, and they generate two distinct proteins, SCGB1A1 and SCGB1A1A [13]. In the lung, SCGB1A1 has an anti-inflammatory effect on neutrophils and likely other cells, and it is downregulated in asthmatic horses [9,12-14]. Decreased SCGB1A1 in asthmatic horses suggests there are fewer and/or functionally impaired club cells. However, SCGB1A1 identifies only mature club cells [10], which implies that absence of SCGB1A1 may also be due to incomplete club cell differentiation in the asthmatic lung. Assessment of claudin 10 could be used to investigate this possibility since it identifies immature non-ciliated cells of the airway epithelium [15]. Alternatively, expression of SCGB3A2, which indicates club cell commitment, could be determined [16]. Presence or absence of differentiated club cells may have broader implications since in mice these cells also regulate the circadian rhythm [17]. Genes involved in circadian rhythm regulation were differentially expressed in horses with asthma following challenge [18], and *SCGB1A1* expression was significantly decreased in the corresponding samples [13]. Hence, absent or immature club cells may impair the normal circadian rhythm and prevent re-establishment of homeostasis following an
inflammatory stimulus in horses. It is unclear whether all club cells have similar function, or whether there are subgroups with different characteristics [8]. C-C motif chemokine 11 (CCL11), a potential negative regulator of neutrophil recruitment [19], was identified as expressed largely by club cells in the lung, [20] concurrent with the HH ligand sonic HH (SHH) [21]. Early NOTCH pathway activation is also necessary for proper club cell differentiation [22] and is an interesting target for further investigation of differentiation pathways in the asthmatic lung.

Other cell populations thought to participate in repair and regeneration of the bronchiolar epithelium include airway smooth muscle cells (ASMCs), NE cells, and distal airway stem cells (DASCs) [23-25]. However, since depletion of NE cells did not impair the repair process, their role in epithelial injury may be minimal [24].

The ECM also regulates progenitor cell behavior by providing growth factors and stem cell specific molecules, and by directing interactions between the ECM and stem cells involving integrins. Therefore, homeostasis of the ECM is essential for proper progenitor cell regulation [1]. Epithelial responses to chronic injury differ from those of acute injury, as discussed below in the context of equine and human asthma.

Cilia
Cilia are ancient cellular organelles present on almost all vertebrate cells and involved in various processes in embryos and adults. There are three types of cilia: nodal cilia, immotile primary cilia and motile cilia [26,27]. This review focuses on the structure and function of motile and immotile primary cilia.

The main cilium is formed by the axoneme, a structure comprised of nine microtubule doublets, called outer doublets, one central microtubule pair for motile cilia, tektins [28] and protofilament ribbon proteins [29]. Motile cilia have a “9 + 2” microtubule arrangement that includes nine outer doublets and one inner doublet, and can be found in the airway, the olfactory bulb, the inner ear and the reproductive tract. The primary purpose of motile cilia is to generate directional flow of fluid or particles, which is relevant in the airway with regards to clearance of accumulated mucus and airborne particles [26]. Immotile primary cilia have a “9 + 0” microtubule pattern with nine microtubule outer-doublets but no inner doublet, and they function in cell signaling and environmental sensing but not fluid movement [26] (Figure 1.1).

The cilium has three main components: the basal body, the transition zone and the main core (Figure 1.2). During cilium formation, the mother centriole with membrane vesicles migrates
to the cell surface and there matures into a basal body comprised of mother and daughter centrioles. The mother centriole triplet microtubule structure comprises A, B and C tubules. The mother centriole also possesses centriolar appendages that will mature into transition fibers and serve as an anchor to the plasma membrane of the cell. The microtubule structures then rise from the basal body while the membrane vesicles form the ciliary membrane. Microtubules rise from the distal region of the basal body, called the transition zone, to the tip of the cilia. Each doublet is formed of tubules A and B, the latter being incomplete and composed of fewer filaments [26,27].

Motile cilia include nexin links between microtubule outer doublets, dynein arms acting as motors for the bending motion characteristic of this type of cilium, and radial spokes [30]. Radial spokes are composed of 22 polypeptides partially pre-assembled in the cell body prior to transport to the ciliary tip, integration into the axoneme and complete assembly [31]. The formation of cilia is a complex cellular process dependent on numerous, consecutive and accurately timed transcription factor activities. In mice, the forkhead box J1 (FOXJ1) transcription factor is required for formation of motile cilia in airways [32,33], and the same transcription factor is also thought to be required in other mammals [34]. Transformation related protein 73 (TAp73) is a cross-species conserved central transcriptional integrator of the complex process of multiciliogenesis within cells, and absence of TAp73 entirely abrogates formation of cilia [35].

Orientation and position of the basal body are crucial for correct placement of microtubule doublets [27]. Elongation of cilia requires transport of specific proteins to the tip of the cilium and back. This process is called intraflagellar transport (IFT) and will be discussed in more detail below.

**Intraflagellar transport**

The cilium itself is unable to synthesize the proteins necessary for its formation and maintenance [36]. IFT is the process of transporting non-membrane-bound components from the cell body to the ciliary distal tip and back, to allow cilia assembly and protein turnover [37]. Since the cilium arises from the cell surface, components have to be exported progressively to the tip during formation. The main components of IFT in ciliated cells are outlined in Figure 1.3.

The canonical anterograde IFT train is powered by hetero-trimeric kinesin-II and enters the cilium loaded with IFT complexes A and B. First, IFT trains are assembled near the basal body. IFT complex A and B proteins bind to the kinesin II motor along with inactive cytoplasmic dynein 2, Bardet-Biedl octameric protein complex (BBsome) and axonemal precursor proteins. This IFT
train then moves along the cilium to the ciliary tip where the IFT train is unloaded. Axonemal proteins are released, the active kinesin motor is inactivated by phosphorylation, the cytoplasmic dynein 2 motor is activated, and the retrograde IFT train is remodeled and loaded with axonemal turnover proteins for transport to the cell body and recycling.

According to current knowledge, IFT has two components: anterograde and retrograde IFT that transports components to the tip of the cilium and back to the cell body, respectively. IFT trains are comprised of IFT proteins going up (anterograde) the B tubule and down (retrograde) the A tubule using motor proteins [38]. The IFT process is necessary during the formation of the cilia and to maintain its length [39]. Despite the dynamic nature of the cilium and persistence of active IFT after initial formation, in general cilium length remains constant [27].

**Intra-ciliary composition**
The composition of cilia is important for appropriate functions. The exact mechanisms regulating which proteins enter and exit the cilium remain to be precisely defined, but the transition zone is thought to play a central role in this process [26,40]. The transition zone and transition fibers together form the ciliary gate. The transition fibers link the B tubule to the plasma membrane. Furthermore, the transition zone is attached to the plasma membrane by Y-links that also act as a diffusion barrier [26,40].

**Genetics of ciliopathies**
Ciliopathies are diseases caused by dysfunction of motile or immotile cilia, and are increasingly recognized in conditions in humans [40]. Current knowledge suggests a central role for FOXJ1 and regulatory Factor X (RFX) transcription factors in airway ciliogenesis [41], and several genes involved in cilium formation, function and IFT processes have been identified.

To date, a total of 187 genes have been directly linked to 35 known ciliopathies. Many ciliopathies have marked genetic heterogeneity, such as primary ciliary dyskinesia (PCD), the most common ciliopathy resulting from impaired motility in motile cilia [40]. PCD is most often the result of an abnormality affecting the motility apparatus, and is classified as first- or second-order depending on whether the ciliopathy stems from defects in ciliary or non-ciliary proteins, respectively [40]. First- and second-order ciliopathy-associated proteins of the motile cilium have been identified in the inner and outer dynein arms, the central pair, nexin-dynein regulatory complex, radial spoke, cytoplasm, transition zone, centriolar satellites, and proteins involved in
regulation of centrioles and transcription in multiciliated cells [40]. For a complete review, please refer to [40].

*Mucociliary clearance*

When mucus and particulates accumulate in the airway, efficient clearance is crucial to prevent airway obstruction and impaired breathing. Mucus accumulation is a feature of many lung diseases, including asthma. Several factors can lead to abnormal mucous accumulation most notably ciliopathies (altering mucociliary clearance mechanisms) leading to abnormal ciliary beating and defective ion transport and mucous cell metaplasia. The latter can be caused by mucous cell metaplasia leading to unbalanced serous cell secretion, decreased water secretion, and increased viscosity [42].

The three components of the mucociliary clearance unit are the ciliated cells (beating cilia), the periciliary liquid (PCL), keeping the mucus at appropriate distance from the epithelium, and the mucus itself [43]. The purpose of ciliary beating is to move the mucus and undesirable particles anterograde in the airway. In humans, ciliated cells typically represent between 70 and 80% of the epithelial cells of the small airways, and have 200-300 cilia and numerous microvilli per cell [36,44]. Ciliated cells of the airway play a crucial role in maintaining mucus flow as well as sensing and responding to extracellular signals [45].

Known factors impacting ciliary beating frequency include luminal fluid tonicity and mechanical stress, post-translational modifications of axonemal components, cyclic adenosine monophosphate (cAMP), Ca$^{2+}$ and nitric oxide concentration [41,46]. Current models of airway ciliary beating define two main stages: the recovery stroke and the effective stroke [47]. In the normal airway epithelium, cilia move in a slightly desynchronized manner in a motion called “metachronal wave” [48,49]. This allows for unobstructed motion of all cilia and more efficient movement of the above mucus layer. The synchronization of individual cilia is thought to result from hydrodynamic interaction between neighbouring cilia [50]. In other words, the flow generated by a cilium beating will synchronize the beating of the neighbouring cillum.

The second and third components of the mucociliary clearance unit are the PCL, which provides a low viscosity layer for ciliary motion and a lubricated layer for efficient movement of the mucus layer, and the mucus itself, trapping airborne substances and protecting the epithelium from desiccation and pathogens. Together, the PCL and mucus form the airway surface liquid (ASL) [51,52]. Specifically, the composition and height of the PCL determine the efficacy of
mucociliary transport and may be influenced by ion transport. Optimal ion composition is crucial for proper lung health. Sodium, chloride and potassium transport channels are key regulators of PCL composition, and these in turn can also be modulated by extracellular signals such as Ca$^{2+}$ and cAMP concentration [52]. Another hypothesis suggests that ASL height is regulated by passive forces [43]. The definite mechanism of ASL regulation remains to be determined.

**Hedgehog pathway**

The primary cilium is central in vertebrate HH pathway regulation, which requires functional cilia with unaltered IFT machinery and basal body for proper signal transduction [53,54]. The HH pathway is also crucial in vertebrate development, and was shown to regulate epithelial quiescence, regeneration and tissue homeostasis in the adult mouse lung [21]. The HH ligand SHH largely co-localizes with SCGB1A1-expressing club cells, and HH activation is thought to control cell cycle progression [21]. Interestingly, differential expression of the HH receptor patched 1 (PTCH1) was observed in asthmatic horses compared to normal horses after challenge [18], and significant enrichment of HH and cell cycle gene sets was observed in the same asthmatic horses [55], suggesting a link between HH signaling and dysregulated cell cycling in the asthmatic lung. Other proteins potentially co-localized with SCGB1A1 such as CCL11 were also downregulated in asthmatic horses (data not shown), strongly inferring the absence of mature functional club cells in the equine asthmatic lung. Primary cilia may participate in the pathogenesis of severe equine asthma through HH signaling.

**Bitter taste receptor signaling in the lung**

Taste receptor type 2 (T2R) have been detected in the nose, trachea and bronchi, and are expressed in a variety of cells including chemosensory, epithelial, ciliated and smooth muscle cells [45,56-58]. T2R signaling in bronchoconstriction and bronchodilation has been well documented. Several T2Rs were expressed in airway smooth muscle (ASM), and activation of these receptors triggered bronchorelaxation in mouse trachea and human bronchi [56,58]. The exact mechanism is poorly understood but may follow from an increase in intracellular Ca$^{2+}$ concentration, activation of K$^+$ channels and membrane hyperpolarization [56], or inhibition of L-type voltage-dependent Ca$^{2+}$ channels (VDCCs) [59]. Motile cilia of the lower airway epithelium express several T2Rs but not taste receptor type 1 (T1Rs), and exposure to bitter compounds increased intracellular calcium and ciliary beating frequency [45,57]. This suggests T2R-related signaling may participate in airway
defense against harmful substances, linking ciliated cells with microvilli signaling [45,60]. T1R receptors are expressed specifically in larger airways, and are therefore less likely to impact the pathogenesis of severe equine asthma [57].

Ion exchange transporters were differentially expressed in horses with asthma compared to non-asthmatic horses. SLC46A2 (ENSECAG00000018603), SLC7A11 (ENSECAG00000022696), SLC7A5 (ENSECAG00000009215), SLC4A11 (ENSECAG00000020277) were significantly upregulated in horses with severe asthma following an asthmatic stimulus compared to non-asthmatic horses, while SLC4A5 (ENSECAG00000014976), SLC16A10 (ENSECAG00000020313), and SLC2A4RG (ENSECAG00000020369) were downregulated [18]. Specific function, location and expression of these solute carriers on the surface of epithelial, ciliated and smooth muscle cells are unknown, but would be an area interesting for further investigation.

**Microvilli**
As mentioned, microvilli are numerous on the surface of ciliated cells [36], but also characterize all differentiated and resting cells, and play a vital role in differentiated cell functions such as regulation of substrate transport and energy metabolism, ion flux, calcium signaling, volume regulation, chemosensing, xenobiotic handling and electromagnetic field perception. Functional membrane proteins detected include sodium, potassium, chloride, calcium and large anion channels, as well as Na⁺/H⁺ exchangers, Na⁺ pumps, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, nitric oxide synthase and cytochrome P420/P450 [61].

Typical microvillar structure is outlined in Figure 1.4. Very little is currently known about the phenotype and function of microvilli in pulmonary health and disease, but microvilli have tissue-specific expression of some proteins such as ezrin, radixin and moesin (ERM) [62]. Villin and fimbrin were detected in airway core microvilli and their rootlets, but the brush cell phenotype seemed to be heterogeneous even within the trachea [63,64]. Brush cells have been described throughout the airways, but appear to be less prevalent in smaller airways [63,64]. They are considered likely to be a subpopulation of solitary chemosensory cells able to sense and respond to a variety of environmental stimulus [60,64]. Brush cells are cholinergic and express the bitter taste receptor transduction system [64].

In the lung, microvillar phenotype and function have largely been investigated through observation of brush cells, but findings are likely also applicable to microvilli of ciliated and club
cells. Our group identified abnormal microvilli on bronchial and bronchiolar ciliated cells. An abnormal microvillar phenotype has not been described in asthma, and the mechanisms governing microvillar growth, length and location are still largely undefined. There is no evidence to date that abnormal microvilli contribute to disease states in the lung, but considering the striking morphologic differences between asthmatic and non-asthmatic microvilli, and their likely role in chemosensing and ion transport, we hypothesize that microvilli respond and contribute to the chronic inflammation observed in the equine asthmatic lung.

**Calcium signaling**

Calcium signaling is crucial in many cellular processes but is also very complex [65]. Many organelles and cell types participate in calcium signaling, including microvilli and primary cilia [61,66]. Calcium signaling is part of processes such as cell proliferation, differentiation, transcription factor activation and others. Inositol-1,4,5-trisphosphate receptor (InsP$_3$R) and ryanodine receptor (RYR) families are the most well known channels for Ca$^{2+}$ trafficking, and Ca$^{2+}$ itself can regulate these channels [67,68]. An imbalance between extra- and intracellular Ca$^{2+}$ can have dramatic consequences such as promoting cancer, as reviewed in detail in [65]. Of particular interest, microvillar remodeling could have a role in extracellular ion imbalance since microvilli contain a large number of ion channels on their surface and also have an F-actin cytoskeleton, which is an important calcium reservoir [61].

Hence, we hypothesize that cellular remodeling resulting in abnormal structure of bronchial and bronchiolar cilia and microvilli contributes to imbalanced Ca$^{2+}$ signaling, as also observed in some other diseases [69]. In fact, airway hyperresponsiveness and inflammation were decreased in an allergic asthma mouse model following exposure to calcium receptor antagonists [70]. We suggest calcium antagonism might also be investigated in adult-onset non-allergic asthma in humans, and equine severe asthma.

**Pathogenesis of severe equine asthma**

Severe equine asthma, formerly called recurrent airway obstruction (RAO, heaves), is a chronic inflammatory lung disease affecting mature horses similar to human asthma. It is characterized by excessive mucus production, neutrophilic influx, bronchiolar hyperreactivity, bronchospasm, and remodeling of the bronchiolar wall epithelium [71,72]. Typical diagnostic tests include physical examination, airway assessment by endoscopy, measurement of lung function, and analysis of
bronchoalveolar lavage fluid (BALF). The full clinical phenotype of severe asthma presents in older horses [72]. Initially, remission of clinical signs occurs when affected horses are moved to a low dust environment [73]. However, airway obstruction due to recurrent or persistent inflammation, mucous hypersecretion, fibrosis and eventual smooth muscle hyperplasia result in irreversible airway remodeling [72,74,75].

The relevancy of animal models to study asthma is highly controversial [76] but there is currently no accepted animal model for human asthma other than mice. Mice are thoroughly characterized, inexpensive to keep, have a short reproductive cycle and there are many transgenic models, which makes them an attractive animal model. However, asthma in mice differs fundamentally from asthma in humans: mice have a short lifespan preventing study of chronic airway disease, and they do not naturally exhibit airway hyperresponsiveness, do not respond to histamine and do not develop airway remodeling [77]. Therefore, mice have been questioned as an appropriate model for asthma. Suggested alternative models include rats [78], guinea pigs [79], rabbits [80], dogs [81], sheep [82], primates [83], cats [84], and horses [85]. However, only cats and horses have a naturally occurring form of asthma [84]. In vitro and in silico modeling were also suggested as alternative approaches [86,87], but such models have limited applicability due to the complexity of cellular and molecular interactions at play in asthma [86]. Current models and methods used by researchers in the field of asthma have been insufficient for major advances in understanding and treating the disease. Asthma in humans is a heterogeneous and complex disease [88], and naturally occurring animal models better recapitulate the complexity of human asthma and allow greater flexibility in experimental designs.

Asthma in horses has been suggested as a good model of human asthma [85,89-91]. Exacerbation of clinical signs is typically induced though exposure to moldy/dusty hay or inhaled suspension of hay dust, lipopolysaccharide and mold [92]. However, the exact antigens responsible for triggering asthma in horses remain to be identified, and exacerbation protocols vary although standardization has been proposed [92]. Since the inflammatory/immune response depends on genetic predisposition of the host and the nature of the antigens, and horses with asthma are sensitive to a wider range of antigens than horses without asthma [93-96], minimizing experimental variables in regard to antigen exposure and type of assessment would improve comparison across studies.
Limited ability to repair or regenerate
Homeostasis is altered in disease. In asthma, it is postulated that initial damage to the epithelium triggers secretion of cytokines and chemokines, which attracts and activates leukocytes. Activated leukocytes in turn also secrete cytokines and chemokines, engaging in positive-negative feedback regulation with epithelial cells. In theory, appropriate epithelial cell responses consist of repair and regeneration, and downregulation of inflammation with re-establishment of homeostasis. However, in asthma it is likely that chronic activation of leukocytes leads to persistent inflammation and production of mediators that contribute to the complex process of airway remodeling. Although defining specific roles of mediators and cells in asthma is challenging, the epithelium is likely a central player.

Chronic inflammation of the airway leads to irreversible remodeling resulting in lung dysfunction. During regeneration or repair, it is essential that progenitor cells proliferate and give rise to the appropriate number and type of differentiated cells [1]. Impaired repair is a characteristic of asthma in horses and humans [72, 97]. It has been proposed that irreversible lung change results from stem cell exhaustion, and excessive epithelial cell proliferation, metaplasia, and hypoplasia [1]. In equine asthma, there is to date no evidence of progenitor cell exhaustion, but dysregulation of the cell cycle was observed in the epithelium [55] and blood leukocytes [98]. We hypothesize that dysregulated differentiation is a potential contributor to ASMC remodeling, goblet cell metaplasia and lack of mature club cells. We further hypothesize that asthmatic horses have a genetic and/or epigenetic predisposition that impairs the efficacy or magnitude of their response to environmental antigens.

Genetics of severe equine asthma
Development of equine asthma involves interaction of genetic and environmental factors [99]. Asthma is a complex multifactorial and multigenic disease with risk factors such as genetic background, environment, age and sex [99-101] contributing to a susceptible phenotype. Severe equine asthma and human adult-onset asthma also have a complex mode of inheritance with both recessive and dominant heritability components, and genetic heterogeneity [102,103]. A polymorphism in the IL4 receptor α chain (IL4RA) was the first genetic marker associated with human asthma [104]. In humans, IL4 was implicated in driving Th2 immune responses [105], switching of B-cell antigen production from IgG to IgE [106,107], activating mucin gene
transcription [108], recruiting mast cells [109] and remodeling of airways [110]. IL4 was identified as a key effector molecule in human allergic asthma, and might also be involved in the initial hypersensitivity response as well as in subsequent inflammation, mucous hypersecretion and airway remodeling [111]. In horses, numerous single nucleotide polymorphisms (SNPs) were detected in the IL4R gene, including several in the transcribed region resulting in modifications in the protein [112,113]. However, equine asthma is not characterized by an early hypersensitivity reaction [72], and neither equine nor human adult onset-asthma include a consistent allergic or Th2 component. Therefore, neither has been associated with IL4 and other Th2 cytokines.

A SNP genotype association study was performed in asthmatic horses to study the IL21R. Although the gene included 17 SNPs none significantly related to maternal alleles. Since asthma equally affects horses of both sexes [101], variants in the IL21R are unlikely causally associated with equine asthma [114].

Microsatellite markers on equine chromosome 13 (ECA13q13) were linked with severity of asthma clinical signs in one cohort of horses [115]. This finding agreed with whole genome scans of asthmatic horses that indicated quantitative trait loci (QTLs) on 11 chromosomes (ECA6, 7, 12, 13, 14, 15, 16, 17, 21, 25, and 26). Of these, regions on ECA13, 15 and 21 were most significantly associated with asthma \( (p < 0.05) \) at the genome-wide level [116]. These three regions contain the \( IL4R, IL21R, \) chemokine (C-C motif) ligand 24 (\( CCL24 \)), \( IL27, \) prostaglandin E receptor 4 (\( PTGER4 \)), phosphodiesterase 4D (\( PDE4D \)), suppressor of cytokine signaling 5 (\( SOCS5 \)) and \( IL7 \) genes [113,114,116,117], which are potential asthma predisposing genes by linkage analysis [116]. Ingenuity Pathway Analysis (IPA) software was used to explore interactions between these genes and proteins previously identified by mass spectrometry (MS) in BALF of horses with asthma [117]. Proteins derived from \( SOCS5, IL7R, PTGER4 \) and \( PDE4D \) were predicted to directly interact with \( IL4R, \) forkhead box P3 (\( FOXP3 \)) and arrestin beta 1 (\( ARRB1 \)), respectively, and \( ARRB1 \) was predicted to interact with both \( PTGER4 \) and \( PDE4D \). In addition \( IL4R, IL21R \) and \( CCL24 \) were involved in 56, 33 and 18, respectively, indirect interactions with proteins identified in BALF. Up- and downregulation of \( IL21R \) and \( IL4R \) was identified as having the most wide-ranging effects on genes linked to asthma [117]. Predictions using IPA are interesting but are based on data from humans, mice and rats, and cell culture experiments. Hence, they should be considered cautiously when analyzing data from horses, and further assessed in the context of asthma.

To date, genes and their products that could potentially contribute to predisposition of
horses to asthma have largely been predicted based on small sample numbers rather than large-scale analyses or actual testing. Thus, the genetic basis of equine asthma remains poorly characterized. Epigenetic modification is an additional mechanism important in regulating gene expression. Altered post-transcriptional regulation, in particular DNA methylation, has been associated with asthma in humans [118] and might even affect asthma predisposition in utero [119]. Post-transcriptional regulatory mechanisms have not been investigated in equine asthma.

Innate and adaptive immunity

Childhood asthma typically has three defining clinical features: airway hyperresponsiveness (AHR), eosinophilic airway inflammation and reversible airway narrowing [120]. Pathogenically, childhood allergic asthma is characterized by an early type I hypersensitivity reaction, mast cell recruitment, IgE production and bronchospasm, followed by a type IV hypersensitivity reaction with CD4+ T-cell, basophil, macrophage and eosinophil airway infiltration [121]. In equine asthma, there is no early type I hypersensitivity reaction, and airway infiltrates consist mainly of neutrophils rather than eosinophils [72]. The role of eosinophils as the primary driver of the pathogenesis of asthma is subject of debate, along with the underlying assumption that asthma is an allergy-driven disease [122]. Eosinophilia has been a defining feature of human asthma since the first evidence of eosinophil-predominant inflammation in chronic asthma [123]. Eosinophil infiltration into the airway lumen is not a characteristic of all types of asthma [122], and acute bronchiolar eosinophilia in itself does not trigger symptoms of asthma [124]. In fact, neutrophil infiltration was suggested to be a better indicator of asthma severity, and neutrophils are the predominant cell type in acute severe asthma of older adults [125,126]. Non-eosinophilic asthma was associated with neutrophil infiltration and increased IL8 expression [127], which are also features of asthma in horses [128].

Whether there is a causal allergic component of asthma in horses is unclear [120]. Several types of human asthma are characterized by a rapid hypersensitivity response following challenge, which occurs either late or not at all in horses with asthma [129,130]. The presence and role of IgE in equine asthma is controversial. Horses with asthma react to a greater range of allergens compared to horses without asthma, but the concentration of IgE in BALF and serum does not vary significantly between affected and unaffected horses [95,96,131]. The IgE response likely depends on the nature of the allergens and genetics of the host [94-96], which adds additional factors to an already complex pathogenesis. Mast cells are considered to play an important role in
asthma. Significantly more chymase-positive mast cells were observed in the wall of bronchi and bronchioles of horses with asthma than horses without asthma [131]. Mast cells are important in allergen-induced inflammation, and express the high-affinity IgE receptor (FceRI), which binds circulating IgE and triggers degranulation [132]. IgE-bound antigens might contribute to the mast cell activation and recruitment observed in asthma [133]. Involvement of mast cells in the pathogenesis of asthma is supported by presence of chymase-positive cells in proportion to airway infiltration with neutrophils and lymphocytes [131]. However, if asthma does not include a typical allergic type I hypersensitivity response, mast cell and IgE would have a limited role in the disease.

The second phase of the asthmatic response in humans is characterized by type IV hypersensitivity reaction with CD4+ T-cells, basophils, macrophages and eosinophils [121]. In horses, neutrophil recruitment happens concomitantly with increased CD4+ T cells in BALF [134,135]. Involvement of T-lymphocytes in equine and human asthma is well established and considered an important contributor to neutrophil recruitment [72], however, the role of cytokines is controversial since Th1-predominant, Th2-predominant and Th1/Th2 mixed responses have all been described. A Th2 cytokine profile with increased IL4 and IL5 and decreased IFN-γ mRNA expression in BALF of horses with asthma was initially described [136,137], and suggested an allergic reaction. Similarly, thymic stromal lymphopoietin (TSLP), a cytokine involved in the differentiation of Th2 cells and allergic responses, was upregulated in horses with asthma but not in non-asthmatic horses after exposure to moldy hay [138]. However, in another study, IFN-γ and IL17 were upregulated in cells from the BAL of horses with asthma [128,139]. The link between neutrophil recruitment and a Th2 phenotype is also controversial. IL8 and macrophage inflammatory protein-2 (MIP-2) are potent neutrophil activators [140], and neutrophils from equine asthmatics secreted IL8 [141]. IL8 is thought to be a key player in neutrophil chemotaxis observed in severe equine asthma [9] although the exact mechanisms are poorly understood. It was suggested that IL4 upregulates IL8 and tumor necrosis factor alpha (TNFα) in horse neutrophils, and in turn, inhibits IL1β [142]. However, IL4-mediated inflammation involving IL8 and IL1β is poorly characterized, and results regarding expression of IL8 and IL1β in vitro were disputed [143,144]. Moreover, significant upregulation of IL1β, IL8, TLR4, TNFα, TGFβ1 and NF-kB mRNA was detected in horses with asthma compared to non-asthmatic horses, and an increase, although not significant, was detected for IL17 and INF-γ [145]. These findings also suggest a heterogeneous cytokine profile. It is unclear whether the Th1/Th2 discrepancy is biologically meaningful or the result of variable protocols and samples. Although a Th2 phenotype is accepted
as an important part of most human asthma [97], involvement of IFN-γ [146] implies a mixed cytokine profile. Hence, the cytokine profile of severe equine asthma remains to be clarified, and likely depends on more uniform experimental criteria and assessment of distinct types of leukocytes, epithelial and other cells.

Metalloproteinases (MMPs) are proteolytic enzymes that degrade the ECM and have essential roles in many inflammation-related pathways and interactions. The enzymes may also have a role in equine and human asthma [147,148]. In particular MMP9 has been well characterized in human asthma as a marker of leukocyte infiltration and disease severity [148]. Although MMP9 participates in inflammation and remodeling, the exact mechanism and interactions appear to be very complex and remain undefined [148]. Interestingly, inhibition of IL13, a Th2 cytokine, was reported to abrogate MMP9 activity in a mouse model of asthma [149], suggesting a Th2 component in disease progression. MMP8 might also be important and is thought to be involved in airway inflammation and neutrophil clearance [150]. Both MMP8 and MMP9 may be produced by neutrophils, and MMP8 was upregulated in horses with asthma [148,151,152].

Accumulation of airway mucus is a characteristic of equine asthma that may result from goblet cell metaplasia [153], overexpression of mucin genes [154] released in response to neutrophil-induced inflammation [155] and/or increased mucus viscoelasticity [156]. Mucin 5AC (MUC5AC) is the principal mucin in human asthma [157]. Expression of MUC5AC, apoptosis inhibitor B-cell lymphoma (Bcl-2) and calcium-activated chloride channel regulator 1 (CLCA1) has been suggested to characterize mucus accumulation in horses with asthma, but findings are not unequivocal [154,158,159]. The expression of Bcl-2 [160] was investigated in bronchial mucous cells for a potential role in metaplasia and deregulation of apoptosis in horses with asthma [155]. Although significantly more mucous cells expressed Bcl-2 in asthmatic compared to non-asthmatic horses, the absolute number of mucous cells was similar. Hence, the role of Bcl-2 in equine asthma remains unknown [155]. No specific cytokine profile was associated with overproduction of mucus but IL13 and INF-γ were not upregulated [158]. Assessment of the quantity, timing and nature of mucus overproduction is prone to similar constraints as described above, and careful experimental design with in vivo and in situ assessment is required for full understanding.

Deregulation of apoptosis in neutrophils, macrophages and other cells is also hypothesized to be part of the pathogenesis of asthma [161]. Death of neutrophils by neutrophil extracellular trap (NET) formation (NETosis) was recently described in vivo after BALF analysis of horses with
asthma [9]. NETosis is a form of cell death involving the release of DNA and antimicrobial proteins that trap and kill pathogens. NETs are considered a defense mechanism but were also observed in models of sterile acute lung injury [162]. NETosis has been observed in several lung inflammatory diseases [163,164] and may play a role in asthma [9]. Multiple inflammatory mediators and pathogens may induce NETosis [164], but the precise triggers remain poorly characterized [163]. Apoptosis of CD4+ and CD8+ T cells may also affect equine asthma progression [165].

Finally, as the most abundant immune cell in the lung, macrophages play a key role in pulmonary defense [166]. Macrophages were reported to have dysfunctional phagocytosis in asthma [167], and are thought to play a key role in the pathogenesis of asthma in horses and humans [166,168] but their role is not entirely clear.

The pathogenesis of severe equine asthma involves a great number of cytokines and factors at different stages of disease, with a certain degree of heterogeneity between individuals. A large-scale study of gene expression, pathways and splicing variants might increase understanding of cytokine patterns, and potentially identify key splice variants characteristic for asthma in horses.

Horses with asthma have altered SCGB1A1A to SCGB1A1 expression, and SCGB1A1 has greater anti-inflammatory effect on neutrophils [9,169]. In humans, a polymorphism in SCGB1A1 has variably been associated with susceptibility to asthma [170,171]. In a recent meta-analysis it was suggested that this polymorphism-derived risk of developing asthma is also dependent on ethnic background [171]. SCGB1A1 downregulation has been associated with decreased lung function in humans [172]. In horses, SCGB1A1 is significantly down regulated in the bronchiolar epithelium of asthmatic compared to non-asthmatic horses [12,169], but the causes of this effect are unclear. Since horses are exposed to barn environments for years before developing asthma, hypotheses are that club cells may be forced into repeated dedifferentiation cycles following epithelial injury leading to two possible consequences: loss of ability to terminally differentiate into mature club cells, or cellular senescence and death of progenitor cells. Loss of ability to terminally differentiate is consistent with the observations that epithelial cells can de-differentiate to form de novo tissues [173] and that dedifferentiated club cells do not express SCGB1A1 [10]. Lung epithelial progenitor or stem cells contribute minimally to lung homeostasis [174].

The expression profile of particular genes of the bronchiolar epithelium of horses with asthma is likely variable across individuals and time. However, considering the homogeneity of clinical signs in affected horses, common pathways and networks are also likely, though the
specific immune profile can generally not be predicted, Although several candidates were suggested, no single causal gene has been identified in asthma to date. Recent theories on asthma pathogenesis hypothesized that deregulation of the epithelium is a trigger of the inflammatory cascade rather than inflammation being the primary cause. In fact, airway remodeling can be observed at the onset of asthma in the absence of inflammation [97]. Exhaustion of club cells and inability to undergo proper differentiation following chronic injury are hypotheses that may explain downregulation of SCGB1A1 in horses with asthma. Chronic exposure to environmental airborne substances might lead to dedifferentiation of club cells into progenitor cells and preclude secretion of SCGB1A1 with subsequent unopposed inflammation [10,175].

A diagram of the postulated mechanisms leading to the development of severe equine asthma is in Figure 1.5.

**Transcriptome sequencing**

Next generation sequencing (NGS) technology was first introduced in 2005 with the Roche 454 system, and has since seen rapid deployment in a wide range of applications [176]. In addition to DNA sequencing, NGS technology has been applied to transcriptome sequencing (RNA-Seq), exome or targeted DNA sequencing, and high throughput analysis of transcription factors (CHIP-Seq). Expertise in statistical analysis and downstream bioinformatic tools are evolving rapidly along with the technology, which is most often utilized for discovery rather than diagnostic purposes.

The field of RNA-Seq in particular has seen great advances in recent years and has mostly replaced microarrays for large-scale gene expression analysis. RNA-Seq allows analysis of genome-wide gene expression for identification of active networks and pathways, molecular markers, new isoforms and differential splicing events, etc. New analytic methods are continuously being developed to provide improved tools for optimal analysis. The two main analytic methods are based on gene- or transcript-specific expression. RNA-Seq allows for detection of genes expressed throughout the genome without prior knowledge of their identity. This detection method represents an improvement compared to microarrays because of the ability to discover genes not previously linked to disease, conditions or mechanisms. RNA-Seq also allows for discovery of new genes and transcripts, improved and enriched genome annotation, detection of novel variants, and quantitative allele and gene expression. For quantitative gene expression, RNA-Seq represents a major improvement compared to qPCR since expression of all
coding exons instead of a small fraction of the gene is detected, and detection is independent of slight sequence variation. Sequence variation can be problematic with qPCR if primer-binding sites are affected. Additionally, qPCR usually requires reference genes for expression estimation, but consistent expression of any genes is increasingly recognized as unrealistic.

In RNA-Seq, however, detection is biased toward more highly expressed genes, and the nature of the technology only allows for sequencing of short reads (<150 base pairs). This can be problematic for genes with long repetitive sequences, for duplicated genes with large identical portions and for genes with one or multiple pseudogenes. In these cases, PCR and Sanger sequencing may be better options to determine the true identity of sequences. RNA-Seq also relies heavily on proper sample preparation and bioinformatic analysis. Mammalian RNA-Seq datasets can be voluminous, requiring large amount of data storage space and high-level computing resources. High-throughput bioinformatic analysis usually entails the use of specialized software that may not exist for non-model species, therefore requiring scientists to devise new software or program existing software. Defining the optimal approach for analysis of these large datasets remains a challenge. Recent years have seen the development of new algorithms, methods and software, but ongoing developments are likely to yield better organized and understandable information in these datasets. More importantly, a deeper understanding of the multiple highly complex layers of gene regulation in mammals and other species will contribute to improving bioinformatic analysis. Until then, qPCR, conventional PCR, and Sanger sequencing remain useful tools.

Gene expression analysis of RNA-Seq data can be done based on two distinct methods: count-based or transcript-based. The raw count-based analytic method for gene-specific expression analysis is described in detail by Anders et al. [177], and the transcript-specific analysis by Trapnell et al. [178]. Briefly, raw count-based analysis relies on the sequence reads aligning to a particular gene. Each sequence is equivalent to one count, and analysis compares the number of counts between different conditions assuming a negative binomial distribution. EdgeR [179-181] and DESeq [182] are among the most popular tools for statistical analysis of raw-count based RNA-Seq analysis. This approach is most useful when comparing genes across different conditions because it precludes direct adjustment of the counts, which is required when comparing genes of different length and GC composition. Offset matrices can, however, be adjusted with software such as EDASeq [183] and CQN [184]. Exon usage analysis can also be performed using raw counts with tools such as DEXSeq [185], allowing for detection of alternative splicing events.
or differential exon expression. Transcript-based analysis is most useful when comparing expression of different genes, or when attempting to estimate expression of different transcripts. Cufflinks [186], a software for transcript assembly, and Cuffdiff [178], the associated statistical software for differential transcript expression, have been widely used for such analysis and include adjustment for parameters such as gene length. Cufflinks and Cuffdiff are the preferred methods for transcript-based differential gene expression with RNA-Seq.

The aims of this thesis were to identify genes and pathways differentially expressed between asthmatic and non-asthmatic horses following an asthmatic challenge, and to identify potentially predisposing genetic factors. The results presented provide a much clearer picture of the numerous molecular mechanisms affecting the epithelium in severe asthma, and of the physiological consequences of chronic inflammation in the lung. In the long-term, our findings will hopefully guide the development of efficacious and targeted treatments for horse and human asthmatics.

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Figure 1.1. Internal structure and components of motile and non-motile cilia. Modified from [27,34,187]
Figure 1.2. Internal structure and components of motile cilia.
Modified from [26,27,34,187].
Figure 1.3. Intraflagellar components of cilia.
Modified from [27,34,187].
Figure 1.4. Microvillar structure and components.
Modified from [188].
Figure 1.5. Hypothetical pathogenesis of severe equine asthma.

(A) 1 - An asthmatic challenge upregulates inflammatory and chemotactic genes (TNFRSF12A, IL8 and C5AR1) in bronchial epithelial cells, which attracts neutrophils through the CXCR2 receptor into the airway lumen and creates an inflammatory environment. A lack of fully functional anti-inflammatory mechanisms leads to ongoing neutrophil recruitment by neutrophils and epithelial cells, augmentation of inflammation, oxidative stress and nutrient depletion in the microenvironment. 2 - Subsequently, neutrophil extracellular traps (NETs) are formed (PADI4)
leading to release of inflammatory factors and serine proteases and further promotion of inflammation, coagulation and hemostasis. Inflammatory factors and serine proteases reach blood vessels surrounding bronchioles, and trigger expression of THBS1 and PLEK, which may activate or interact with the PDGFR signaling pathway. This promotes coagulation, hemostasis and vascular smooth muscle cell (SMC) and myofibroblast migration toward the epithelium. The disturbed bronchial microenvironment results in an increase in Hedgehog signaling through PTCH1, potential PIK3IP1 downregulation, and FOXM1 and CDC25A upregulation. HH signaling promotes FOSL1 expression and repression of NOTCH1 signaling, disrupting normal differentiation of the epithelium. This hypothetical cascade of events triggers transformation of mature epithelial cells into a squamous metaplastic phenotype [KRT6A, SPRRs and TGM1].

Transformed epithelial cells and progenitor cells in a disturbed microenvironment with coagulation and hemostatic factors tend to undergo epithelial-to-mesenchymal transition (EMT) under control of HH, and to differentiate into smooth muscle cells. Lack of club cells within the asthmatic airway epithelium may result from NOTCH1 repression and dysregulated differentiation of epithelial progenitor cells (upregulation of SFN, FOSL1, CDC25A, and downregulation of PIK3IP1). On the other hand, dysregulated differentiation might also contribute to airway remodeling, following migration and differentiation of epithelial cells, myofibroblasts and vascular SMCs. Heterogeneity in SMCs may account for airway hyperreactivity. (B) In horses without asthma, following initial epithelial inflammation, SCGB1A1 decreases neutrophil and epithelial cell activation, and prevents build-up of reactive oxygen species and therefore inflammation. This allows for re-establishment of homeostasis, proper differentiation of epithelial progenitor cells, and normal function of epithelium. Homeostasis is also maintained through circadian rhythm regulation, which is hypothesized as dysregulated in horses with asthma.
<table>
<thead>
<tr>
<th>Cell type</th>
<th>Functions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Club (Clara) cell</td>
<td>Secretory cell serving as progenitor cell; functions in airway epithelial repair, homeostasis and circadian rhythm regulation in the lung</td>
<td>[9,11,13,17,189]</td>
</tr>
<tr>
<td>Goblet cell</td>
<td>Mucin secretion; airborne substance entrapment and transportation</td>
<td>[190]</td>
</tr>
<tr>
<td>Basal cell</td>
<td>Multipotent stem cell capability; epithelial maintenance and homeostasis</td>
<td>[191]</td>
</tr>
<tr>
<td>Neuroendocrine (NE)</td>
<td>Progenitor cell function (club cells); epithelial repair and regeneration</td>
<td>[7]</td>
</tr>
<tr>
<td>Ciliated cell</td>
<td>Transport of mucus and chemosensation</td>
<td>[45,192]</td>
</tr>
<tr>
<td>Type I alveolar cell (ATI)</td>
<td>Gas exchange, ion and water flux</td>
<td>[193]</td>
</tr>
<tr>
<td>Type II alveolar cell (ATII)</td>
<td>Progenitor of type I alveolar cells, production of surfactant</td>
<td>[194]</td>
</tr>
<tr>
<td>Secretoglobin family 1A member 1 (SCGB1A1) and surfactant protein C (SFTPC) co-expressing cells</td>
<td>Progenitor for airway and alveoli</td>
<td>[2]</td>
</tr>
</tbody>
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CHAPTER 2

Impaired response of the bronchial epithelium to inflammation characterizes severe equine asthma

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Abstract
Severe equine asthma is a naturally occurring lung inflammatory disease of mature animals characterized by neutrophilic inflammation, bronchoconstriction, mucus hypersecretion and airway remodeling. Exacerbations are triggered by inhalation of dust and microbial components. Affected animals eventually are unable of aerobic performance. In this study transcriptomic differences between asthmatic and non-asthmatic animals in the response of the bronchial epithelium to an inhaled challenge were determined.

Paired endobronchial biopsies were obtained pre- and post-challenge from asthmatic and non-asthmatic animals. The transcriptome, determined by RNA-Seq and analyzed with edgeR, contained 111 genes differentially expressed (DE) after challenge between horses with and without asthma, and 81 of these were upregulated. Genes involved in neutrophil migration and activation were in central locations in interaction networks, and related gene ontology terms were significantly overrepresented. Relative abundance of specific gene products as determined by immunohistochemistry was correlated with differential gene expression. Gene sets involved in neutrophil chemotaxis, immune and inflammatory responses, secretion, blood coagulation and apoptosis were overrepresented among upregulated genes, while the rhythmic process gene set was overrepresented among downregulated genes. MMP1, IL8, TLR4 and MMP9 appeared to be the most important proteins in connecting the STRING protein network of DE genes.

Several differentially expressed genes and networks in horses with asthma also contribute to human asthma, highlighting similarities between severe human adult and equine asthma. Neutrophil activation by the bronchial epithelium is suggested as the trigger of the inflammatory cascade in equine asthma, followed by epithelial injury and impaired repair and differentiation. Circadian rhythm dysregulation and the sonic hedgehog pathway were identified as potential novel contributory factors in equine asthma.

Keywords
Asthma – Bronchus - Differential expression analysis – High-throughput nucleotide sequencing – Horse
**Background**

Severe equine asthma, formerly termed recurrent airway obstruction (RAO) or heaves, is a naturally occurring chronic lung inflammatory disease of horses exposed to airborne molds and particulate material [1]. The condition develops with repeated inhalation of molds and/or dusty air in barns or on pasture in hot and humid climates, as well as in environments with high concentration of fungal spores or grass pollen grains [2]. Once sensitized, affected horses cough and have nasal discharge associated with progressive airway obstruction from a neutrophilic exudate, mucus hyperproduction, airway hyperreactivity and bronchospasm. Recurrent episodes of inflammation lead to smooth muscle hyperplasia, fibrosis and irreversible airway remodeling [3-6]. Severe equine asthma is responsive to environmental improvement and anti-inflammatory and bronchodilatory therapy, but is considered to be irreversible once airway remodeling has started [3]. Nevertheless, affected animals may have periods of clinical remission followed by periods of exacerbation over months to years.

Development of severe equine asthma likely involves genetic predisposition in addition to environmental triggers [7]. Findings have suggested that inheritance patterns are complex, implying genetic heterogeneity [8]. A significant association between susceptibility and paternal haplotype was proposed [9,10], and specific regions on equine chromosomes 13 and 15 containing genes such as the interleukin 4 (IL4) and IL21 receptors were associated with increased risk of developing severe asthma in some equine kindreds [10,11]. Copy number loss of a region on chromosome 5 including the gene NME7 involved in ciliary function was more frequent in asthmatic than non-asthmatic horses [12]. However, strong evidence for a role of specific gene variations remains sparse.

The airway epithelium is the first barrier to inhaled substances, and includes multiple cell types such as ciliated and non-ciliated (club or Clara) cells, mucus producing goblet cells and precursor cells. It is thought that in severe equine asthma airway epithelial cells bind inhaled microbial components via pattern recognition receptors (PRR), which initiates an innate immune response with synthesis of inflammatory cytokines and chemokines [13]. In turn, inflammation of the epithelium results in generation of reactive oxygen metabolites, exosomes and proteases that injure epithelial cells and induce proliferation of airway smooth muscle cells, goblet cell hyperplasia, epithelial cell metaplasia and cell death [5,14,15]. In order for the epithelium to resume specialized barrier functions, cells need to regenerate with precise migration, proliferation
and differentiation. Club cells, in particular, are markedly reduced in equine asthmatic airways resulting less anti-inflammatory secretoglobin 1A1 (SCGB1A1) in airway secretions [16,17]. It is postulated here that repeated epithelial cell inflammation and injury results in progressively impaired regeneration of a fully functional epithelial barrier.

There are many proposed classification schemes for human asthma. According to most schemes, severe equine asthma is most similar to severe human adult or late onset asthma, which is distinct from childhood, allergic, exercise-induced and some other forms of human asthma [1,18-20]. Phenotypes of human adult asthma are categorized according to age at onset, clinical characteristics, type of airway inflammation and response to therapy [19]. Severe human adult asthma is associated with airflow obstruction and most often neutrophilic inflammation, although eosinophilic and pauci-granulocytic inflammation is also observed [21]. Neither severe human adult asthma nor severe equine asthma is typically dominated by a Th2 immune response [3,20]. It is difficult to investigate the pathogenesis of asthma in humans, and many inferences are based on nasal or sputum rather than bronchial or bronchiolar samples since the latter are difficult to obtain. Mice systemically sensitized to foreign antigen and then challenged by inhalation are widely used as models of human asthma, but recapitulate neither remission/exacerbation nor neutrophilic inflammation [22].

We hypothesized that the bronchial epithelial response to an inhaled challenge is different in asthmatic and non-asthmatic individuals. To address this hypothesis we designed a paired pre- and post-challenge study that accounts for individual variability in genetically heterogeneous animals, and obtained bronchial biopsy samples from affected and control animals that were processed for RNA sequencing and results analyzed.

**Methods**

**Animals and procedures**

Details of study design and analysis are presented in Figure 2.1. Six horses with and seven horses without asthma had similar mean ages of 15 and 12 years (p =0.352, unpaired t test), respectively, and each group included a variety of breeds. All were maintained for >6 months outdoors prior to study. Horses with historical asthma had been affected for 2 to 6 years, and were free of clinical respiratory disease during at least 6 months prior to study. All animals were placed in a dust-free indoor environment for 24 hours, and thereafter physical examination, pulmonary function test (PFT) and bronchoalveolar lavage (BAL) were performed. During physical examination,
respiratory rate, nasal discharge, presence and severity of expiratory lift, nasal flaring, tracheal sounds, bronchial tones, crackles, wheezes, cough and chest resonance were assessed according to a preset scale yielding a clinical score between 0 and 26. Pulmonary function data were derived from integration and analysis of airflow data and corresponding transpulmonary pressure. For PFT, non-sedated horses were restrained in stocks and fitted with a mask attached to a heated pneumotachograph. Airflow data were captured and fed through a transducer to integrate the flow signal and derive volume measurements. An esophageal balloon catheter was placed mid thorax and attached to a transducer at the proximal end to estimate pleural pressure. Volume and pressure data were analyzed via respiratory loop analysis to derive values for pleural pressure (PpI), dynamic compliance (Cdyn) and lung resistance (RL). During bronchoscopy, the appearance of the upper airways, trachea and main bronchi were visualized, and scored for presence and degree of erythema, edema, secretions, hemorrhagic exudate, and cough reflex. An endoscopic score between 0 and 15 was derived from these parameters. Then, the bronchoscope was gently lodged in a 3rd to 5th generation bronchus, and two sequential aliquots of 200 mL of warmed saline were infused and re-aspirated. An aliquot of BAL fluid was analyzed by total nucleated cell counting and 200-cell differential counting of stained cytocentrifuge preparations. Between two and eight endoscopic biopsies were obtained for RNA-Seq and histopathology. Horses were then exposed to dusty hay until respiratory impairment was apparent in asthmatic horses (range 1 to 3 days, average 2.2 days). Non-asthmatic horses were exposed to dusty hay for 3 days. At this time clinical examination, respiratory function testing and BAL were repeated. BAL and endoscopic biopsies were obtained from a contralateral lung lobe. At exacerbation, mean clinical and bronchoscopic scores in asthmatic horses had increased from a mean of 2.7 to 13.3, and from 2.2 to 9.7, respectively. Non-asthmatic horses had mean clinical and bronchoscopic scores of 1.6 and 2.6 prior to challenge, and 0.4 and 1.9 post-challenge, respectively (Fig. 2.3). The average change in PpI was 7.92 cm H2O in asthmatic horses, and -0.82 cm H2O in non-asthmatic horses.

All procedures were approved by the Institutional Animal Care Committee of the University of Guelph (protocol R10-031) and conducted in compliance with Canadian Council on Animal Care guidelines. Changes in pulmonary function and BAL fluid composition between the two groups following an asthmatic challenge were analyzed by taking the differences between “after” and “before” values for each horse and testing with Welch’s t test for significant association with presence of asthma.
Changes in pulmonary function and BAL fluid (BALF) composition between the two groups following an asthmatic challenge were analyzed by taking the differences between “after” and “before” values for each horse and testing with Welch’s t test for significant association with presence of asthma. Graphs and p-values were generated using Prism 6.0a (GraphPad, La Jolla, CA) and unpaired t-tests with correction for multiple comparisons by the Holm-Sidak method with alpha =5.000%. Significance threshold was set at \( p < 0.05 \).

RNA extraction, library preparation and sequencing
Total RNA was extracted from endobronchial biopsies (Qiagen, Toronto, ON). Quality and concentration of RNA were determined with the Bioanalyzer RNA Nanochip (Agilent, ON) and gel electrophoresis. Only samples with RNA integrity number >6.9 and little to no degradation apparent on electrophoretograms were accepted. RNA-Seq unstranded library preparation and sequencing were performed at The Centre for Applied Genomics (TCAG; Toronto, ON) using the Illumina TruSeq RNA sample preparation and sequencing protocol following the manufacturer’s guidelines (Illumina, San Diego, CA). Briefly, for each sample, approximately 1 µg of non-degraded, high quality total RNA was enriched for poly-A RNA, fragmented into 200 to 300 bases, and converted to double stranded cDNA libraries. Illumina adapters were ligated to the ds-cDNA and PCR-amplified for 14 cycles. Barcoded primers were then added to each sample to allow sequencing in the same lane and detection of individual samples in the sequence data. Final RNA libraries were quantified (KAPA Library Quantification kit, Kapa Biosystems, Wilmington, MA) prior to pooling and sequencing. Illumina flow cell was prepared and sequenced on an Illumina HiSeq 2500 instrument in 5 lanes following the manufacturer’s instructions to generate paired-end reads of 100-bases.

Genome-guided RNA alignment
Raw read quality was assessed using FastQC software version 0.10.1 [23] and aligned to the horse reference genome [24] (Ensembl v70) with STAR version 2.4 [25]. The STAR_pass2 alignment protocol was followed including these adaptations: horse Ensembl version 70 GTF annotation file for first- and second-pass, and the junction SJ.tab file generated by STAR for the second-pass after non-canonical junctions were removed. Default settings were used except for: --runThreadN 8 --outFilterScoreMinOverLread 0.5 --outFilterMatchNminOverLread 0.5. Read counts were
Differential gene expression

Differential expression (DE) analysis was performed in R, version 3.2.1 [27], with the edgeR package version 3.10.2 [28-30]. A paired DE analysis was performed to assess changes between groups (asthmatics versus non-asthmatics) and within groups (before versus after challenge). EdgeR analysis was based on section 3.5 of the edgeR user’s guide (last revised April 10, 2017). Briefly, the minimum count number was set at 1 read per million in at least 3 samples. Normalization factors and effective library size were applied, duplicates were removed and dispersion was estimated using the “estimateGLMCommonDisp”, “estimateGLM TrendedDisp” and “estimateGLM TagwiseDisp” functions. The model matrix was designed as: ~group+group:horse+group:challenge, where “group” refers to non-asthmatic and asthmatic groups, “horse” refers to each individual horse, and “challenge” refers to samples before and after the asthmatic challenge. Fit of the generalized linear model and tests for differences in expression were performed with the “glmFit” and “glmLRT” functions, respectively and the following contrast was used to compare asthmatic and non-asthmatic horses: glmLRT(fit, contrast=c(0,0,0,0,0,0,0,0,0,0,0,-1,1)). GC content bias was assessed using EDAseq [31], but need for normalization was not indicated. Statistical significance was set at a false discovery rate (FDR) <0.05.

Immunohistochemistry

The protein product of four genes with significant up- or downregulation was assessed by immunohistochemistry (IHC). Confirmation of protein expression for a group of genes was deemed sufficient as a proxy to confirm the correctness of sequencing, alignment and statistical workflow. The genes were selected based on significant differential expression between asthmatic and non-asthmatic horses, availability of cross-reactive antibodies and potential roles in asthma pathogenesis. Antibodies were initially tested in Western blots with equine tissue samples to verify that a single protein product of appropriate size was detected (data not shown). Tumor necrosis factor receptor superfamily member 12A (TNFRSF12A or TWEAKR, tumor necrosis factor-like weak inducer of apoptosis receptor), patched-1 (PTCH1), cell division cycle 25 homolog A (CDC25A) and interleukin 8 (IL8) proteins were assessed in biopsies fixed in formalin and
routinely sectioned and processed. Antibody reactivity was first assessed by Western blot (WB) analysis against horse serum or lung protein extracts (Suppl. Fig. 2.1). Proteins were separated in 12% (w/v) SDS-polyacrylamide gels (TGX Stain-Free FastCast premixed acrylamide solutions; Bio-Rad, Mississauga, ON) under reducing conditions. Proteins were then electro-transferred to PVDF membranes using the Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked in 5% BSA solution before immunoblotting with polyclonal rabbit anti-human TWEAKR (Biorbyt, Berkeley, CA), PTCH1 (C-terminal region; Aviva Systems Biology, San Diego, CA) and CDC25A (Abcam, Toronto, ON), and polyclonal rabbit anti-horse IL8 (MyBioSource, Inc., San Diego, CA). Membranes were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (DAKO, Mississauga, ON) and exposed with Clarity Western ECL Substrates (Bio-Rad). Images were captured with a ChemiDoc imaging system (Bio-Rad). If bands of expected size were present, antibodies were applied in immunohistochemistry (IHC) to 3-5 µm thick sections placed on charged glass slides, de-paraffinized in xylene, rehydrated in alcohol, incubated with dual endogenous enzyme blocker and serum-free protein blocker (both DAKO). Slides were then sequentially incubated with the above primary antibodies, Envision Dual Link System-HP (DAKO) and Nova Red chromogen (Vector Laboratories, Burlingame, CA), and counterstained with hematoxylin. Negative control sections for each IHC analysis were prepared in the same manner except the primary antibody was omitted.

Protein network and gene ontology analysis
Gene products were searched for known and predicted protein interactions in Cytoscape version 3.4.0 [32] using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database [33] and string-db plugin [34] within Cytoscape. Horse Ensembl ID were converted to human ID using Biomart [35,36] and to gene symbols directly in Cytoscape through the STRING database. When multiple horse Ensembl IDs had identical human symbols, redundant symbols were removed. The confidence score cut-off applied for interactions was 0.4 (medium confidence). Single nodes, doublets and triplets detached from the main network cluster were removed, and network analysis was performed. Node color and size were determined based on betweenness centrality (BC) and degree, respectively. Confidence of interactions was displayed with different intensity of edge color.
Gene ontology (GO) overrepresentation analysis of biological function was performed with Protein ANalysis THrough Evolutionary Relationships (PANTHER) software version 10.0 [37] with significance threshold set at $p < 0.05$ (including Bonferroni adjustment). The analysis was performed using annotations for Homo sapiens by converting the equine gene symbols into human gene symbols prior to analysis. Species to be analyzed was then identified as human, and information on protein function was assigned to candidates according to prediction in NCBI or UniProt databases.

Results

Induction of asthma

Following exposure to inhaled challenge material, severe bronchoconstriction and profound airway secretions were apparent in asthmatic but not non-asthmatic horses (Fig. 2.2A), and cell concentration and the proportion of neutrophils were increased in bronchoalveolar lavage (BAL) fluid (Fig. 2.2B). Bronchial biopsies showed submucosal edema and an influx of leukocytes (Fig. 2.2C). Changes in BAL fluid cell and tissue composition in non-asthmatics were mild or absent. As a group, asthmatic horses had significantly higher bronchoscopic scores, pleural pressure, BAL nucleated cell concentration and percent neutrophils, and significantly lower dynamic compliance after asthmatic challenge than non-asthmatics (Fig. 2.3).

Differential expression analysis

The results of mapped RNA-Seq reads for individual horses are summarized in Table 1.1. Analysis identified 111 genes differentially expressed (DE) between horses with and without asthma as a result of challenge (Fig. 2.4A). Significantly differentially expressed genes pertain to the epithelium and include keratin-related genes (identified as potential keratin [KRT] 6 based on human homologues ENSG00000185479, and KRT17), genes linked to matrix metalloproteinases (MMPs), inflammation (Toll-like receptor 4 (TLR4), and others), neutrophil chemotaxis (IL8, C-X-C chemokine receptor type 2 (CXCR2)), coagulation and hemostasis (such as plekstrin (PLEK)), cell proliferation (CDC25A), apoptosis (such as BCL2 interacting killer [BIK]) and others. Six of 30 downregulated genes function in regulation of the circadian rhythm.

The relationship between paired samples from individual animals is shown in a multidimensional scaling (MDS) plot with the distance between pairs of samples corresponding to the average root mean square of the largest log2FC (leading logFC, Fig. 2.4B). Post-challenge
samples from asthmatic animals were distinctly distant from those of pre-challenge asthmatic and non-asthmatic animals. This implies that within the asthmatic group differential expression of genes was greater than biological variance, and that most of the DE genes originated from asthmatic animals. The biological coefficient of variance (BCV) was calculated to determine how much the variance in counts exceeded that which would arise from Poisson counts alone [29]. The BCV for RNA-Seq analysis of genetically identical organisms is typically around 0.1 [29] while in this study the BCV was 0.23 (data not shown). This high value reflects the biological variance as expected from outbred individuals, and also emphasizes the benefit of a paired sample design to correct for inter-individual variation.

A heat map of counts per million (CPM) of DE genes for each sample (Fig. 2.5) shows that there is a wide distribution of change in expression (logFC), an inverse relationship of CPM with log FC, and consistency of change across individuals. The magnitude of the log fold change of significantly upregulated genes in asthmatic horses after challenge ranged from 0.6 to 5.6 (Fig. 2.6), and that of significantly downregulated genes from -0.62 to -2.2 (Fig. 2.7).

Protein expression
Specific RNA-Seq results were further investigated in biopsy tissues by IHC. Expression of TNRFSF12A, CDC25A and IL8 mRNA was markedly increased in asthmatic and decreased in non-asthmatic horses after challenge, while PTCH1 mRNA was slightly decreased (Fig. 2.8A). Immunohistochemical results representative of each group showed more intense reactivity for TNRFSF12A, CDC25A and IL8 protein in tissues from asthmatic than non-asthmatic horses after challenge (Fig. 2.8B). TNRFSF12A staining was moderately intense throughout the epithelium of asthmatics after challenge and only present in individual epithelial and sub-epithelial cells from non-asthematics. CDC25A reactivity was intense in epithelium of asthmatic animals, and less prominent in tissue from non-asthmatic animals, in particular after challenge. IHC results for IL8 also showed marked increase after challenge in asthmatic but not non-asthmatic animals. PTCH1 staining was less abundant in asthmatic than non-asthmatic animals after challenge.

Gene ontology analysis
PANTHER analysis of GO overrepresentation for biological processes (GOBP) using the Homo sapiens database identified significantly overrepresented gene sets among up- and downregulated genes, listed in Table 2.2 with associated gene names. The most specifically involved gene sets
concerned neutrophil chemotaxis (GO:0030593), immune response (GO:0006955), inflammatory response (GO:0006954), secretion (GO:0046903), positive regulation of blood coagulation (GO:0030194), positive regulation of apoptotic signaling pathway (GO:2001235), positive regulation of response to external stimulus (GO:0032103) and regulation of immune system process (GO:0002682) for upregulated genes, and rhythmic process (GO:0048511) for downregulated genes.

Upregulated genes shared between the most specific gene-sets are shown in Table 2.3 along with evidence for their association with asthma and their known functions. S100 calcium binding protein A9 (S100A9) was the only gene that contributed to all gene sets, and has been associated with asthma in mice [38]. All genes associated with 4 or more gene sets have also been associated with asthma in humans or mice and include S100A9, thrombospondin 1 (THBS1), TLR4, IL8, complement component 5a receptor 1 (C5AR1), MMP9, NLR family, pyrin domain containing 12 (NLRP12) and triggering receptor expressed on myeloid cells 1 (TREM1) [20,38-48]. Other genes associated with 3 or fewer gene sets have also been associated with asthma such as plasminogen activator, urokinase receptor (PLAUR) and serpin family E member 2 (SERPINE2), and several additional genes were first identified here.

Among downregulated genes, only the rhythmic process (GO:0048511) gene set was overrepresented. This gene set includes genes associated with asthma in humans and mice such as adrenoceptor beta 2 (ADRB2), nuclear receptor subfamily 1 group D member 2 (NR1D2) and period circadian clock 3 (PER3), as well as genes that have not previously been linked to asthma such as D site of albumin promoter (albumin D-box) binding protein (DBP), circadian-associated repressor of transcription (CIART or CHRONO, ChIP-derived repressor of network oscillator) and thyrotrophic embryonic factor (TEF).

Protein network analysis
Protein products of genes DE between groups in response to challenge identified multiple interactions with medium to high confidence (scores ranging from 0.4 to 1). The main protein interaction cluster derived from the 111 DE genes contained 51 nodes, each representing one protein and connected by 113 edges (Figure 2.9). MMP1, IL8 and TLR4, followed by MMP9, had highest scores for betweenness centrality (BC), indicating they are most important for connections with other proteins. IL8, TLR4 and MMP9 had the highest number of direct connections (degree). S100A9, associated with all overrepresented gene sets (Table 2.3), is connected to the network
through its predicted interaction with TLR4. In addition, MMP1 and THBS1 each connect two genes (Table 2.3) with potential role in severe equine asthma though not yet associated with asthma in humans or mice.

**Discussion**

The goal of this study was to identify bronchial epithelial genes and pathways associated with severe asthma in horses. Although predisposition for development of severe asthma in horses is thought to be hereditary, inheritance is incompletely defined and thought to be complex [7]. Analysis of the bronchial epithelium aimed to capture the *in situ* genetic changes that characterize the pathogenesis of severe equine asthma, an approach difficult to apply in other species. Although components of the lung such as bronchioles, alveoli, interstitium and leukocytes are also affected in asthma, they were not specifically evaluated in this study. Biopsies consisted predominantly of epithelium, which in itself is a variable tissue composed of ciliated columnar cells, goblet cells, and in smaller bronchi occasional club cells. Subepithelial components of biopsies included cells such as leukocytes and fibroblasts, and extracellular matrix such as collagen and edema. Hence, cells other than epithelium contributed some RNA to analysis, which is a limit in this study. Nevertheless, assessment of *in situ* samples from a naturally occurring inducible model of asthma is previously unreported, and yielded unprecedented insight. Pre- and post-challenge bronchial biopsies from asthmatic and non-asthmatic horses were obtained, the transcriptome was derived from high throughput sequencing, and results were analyzed with a paired design to account for individual variation. Both groups of animals were of similar age, and as expected, the response to the inhaled challenge consisted of bronchoconstriction, marked airway neutrophilic inflammation, mucus accumulation and impaired airflow in asthmatic but not non-asthmatic animals.

RNA-Seq data were analyzed using edgeR software. EdgeR and DESeq [49] are among the most widely used tools for RNA-Seq analysis using raw counts. Both software tools use comparable methods with the exception of count normalization and dispersion estimation methods [50]. DESeq tends to be more conservative and edgeR more sensitive to outliers [51], but they yield overall highly similar results [51,52]. Regardless of the method used, considering the genetic variability among horses, dispersion estimation would be more precise if data from a larger sample of individuals were analyzed. Therefore, within a limited sample size, the potential for false-positive results warrants caution for interpretation of individual findings.
Overall, the analysis identified 111 DE genes, which is a number similar to that identified in comparable studies in humans [53]. IHC analysis of representative bronchial biopsies confirmed that epithelial cell gene expression was representative of observed differential gene expression results. Transcriptome analysis of paired lung adenocarcinoma and non-neoplastic samples from non-smoking and smoking patients yielded 175 DE genes [53], while comparison of RNA-Seq results of single bronchial biopsies from human asthmatics and healthy controls yielded 46 DE genes [53]. The latter study compared the bronchial transcriptome of asthmatics and non-asthmatics, rather than the difference in the response to an asthmatic challenge, as we did here. The workflow included different sequencing and statistical analyses, and the design did not account for inter-individual variability [54]. Nonetheless, similar to our results, up- and downregulation of solute carrier (SLC) genes and upregulation of an integrin-coding gene was detected. However, B-cell lymphoma 2 (BCL2) expression was lower, and SCGB1A1 was higher in asthmatics compared to control, which contrasts with results in severe asthma in horses [17,55] and may be due to different experimental design and different phases of disease being assessed.

Gene ontology and network analysis were subsequently used to characterize the DE genes. Since limited annotation of the horse genome constrains species-specific gene network and gene set analyses, human databases were utilized to expand interpretation of the findings. This approach yielded results consistent with prior association in asthma, but specific function of such gene products in horses remains to be ascertained. Finally, expression of the protein product of 4 DE genes was investigated immunohistochemically. Semi-quantitative assessment affirmed a link between gene and protein expression but factors such as RNA transcript stability and cell-to-cell variability in gene expression are incompletely accounted for with this approach. Hence, linked rather than individual genes should convey greater confidence for a role in asthma pathogenesis.

Several genes within overrepresented gene sets have been linked to asthma in humans. S100A9 was the only gene shared across all gene sets, and is a calcium-binding protein highly conserved across species. S100A9 and related S100 proteins are highly expressed by neutrophils, and activate innate immune responses via interaction with TLR4 [56]. S100 proteins have also recently been identified to interact with airway epithelial cells to induce MUC5AC, the most abundant airway mucin [56]. Although MUC5AC was not differentially expressed in our study, it is known to be promoted by exposure to cytokines in both horses and humans in a concentration- and time-dependent manner [57,58]. Our sampling time points may not have captured peak expression in all horses necessary to reach significance in our stringent statistical analysis.
Overexpression of *MUC5A* in horses with severe asthma [59] suggests a possible link of *S100A9* with mucus hyperproduction.

Network analysis of genes DE between asthmatic and non-asthmatic horses intimated MMP1, MMP9, TLR4 and IL8 as responsible for many interactions, and therefore to link and influence several asthmatic pathways and processes. *MMP9* was present in multiple significantly overrepresented GO gene sets. In addition, it had high BC and degree (number of direct connections) in the STRING network, meaning it accounted for many direct and indirect interactions within the network. MMP9 is increased in human asthma [60], has anti-apoptotic effects in kidney injury and neutrophils [61,62] and may be a link between inflammation and tissue remodeling [60,63]. MMP9 also links serglycin (SRGN) to the main interaction cluster. SRGN is a proteoglycan that forms complexes with proMMP9 [64,65] and is expressed in a variety of hematopoietic and non-hematopoietic cells [66]. Presence in immature granules has suggested a role in neutrophil differentiation [67], which could also influence neutrophil function in the context of granule release and cell death associated formation of neutrophil extracellular traps (NETs), also called NETosis.

Neutrophil infiltration was present in all asthmatic horses, consistent with the overrepresented neutrophil chemotaxis (GO:0030593) gene set. All genes within this gene set were previously associated with asthma pathogenesis except for *CSF3R*. CSF3 regulates production, differentiation and function of granulocytes, and overexpression is consistent with neutrophilic inflammation in equine severe asthma [68]. IL8 had among the highest BC and degree indicating a central role for linking components of the network. IL8 is a potent neutrophil attractant in the lung [69] and signals through CXCR1 and CXCR2 [70]. Increased *IL8* expression likely initiates and perpetuates neutrophil influx into the airways, but IHC also identified epithelial cells as a source of IL8. Secretion of IL8 by human epithelial cells can be promoted by exposure to TWEAK and activation of its receptor TNFRSF12A [71]. TWEAK is upregulated in multiple tissues with inflammation, and associated with tissue changes such as remodeling [72]. Hence it may be plausible that epithelial cells upregulate *TNFRSF12A* early in response to challenge, which in turn enhances IL8 production and maintains neutrophilic inflammation, leading to eventual proteolytic and oxidative injury.

It has been reported that asthmatic horses have dysregulated apoptosis of BAL but not peripheral blood leukocytes [73,74]. Conversely, higher expression of immediate early response 3 gene (*IER3*) identified in another study suggested dysregulated apoptosis in peripheral blood.
mononuclear cells of asthmatic horses [75]. Hence, the importance of leukocyte apoptosis in asthma of horses is unresolved. Significant overrepresentation of the apoptotic signaling pathway (GO:2001235) gene set was identified in tissue biopsies in this study, which included some extravasated leukocytes. This gene set included S100A9, oncostatin M (OSM), THBS1, TNFRSF12A, stratifin (SFN), plasminogen activator urokinase receptor (PLAUR) and MMP9. Other genes, such as BIK, a pro-apoptotic protein [76], had lower expression in asthmatic compared to non-asthmatic horses. BIK interacts with BCL2 and may protect airway mucous cells from apoptosis during remission from asthmatic exacerbation [77], [55]. Although BCL2 was not DE, this may be a factor of the timing of biopsies and the lower proportion of mucous versus ciliated epithelial cells. Formation of neutrophil extracellular traps (NETs), another form of induced cell death, is prominent in BAL of horses with severe asthma [78]. The mechanism of NET formation is incompletely defined, but peptidyl arginine deiminase type IV (PADI4), differentially expressed in asthmatic and non-asthmatic horses, contributes through citrullination of histones [79]. PADI4 expressed during NET formation may also promote coagulation through the release of serine proteases [80]. The positive regulation of blood coagulation (GO:0030194) gene set was overrepresented and included S100A9, PLEK, THBS1 and TLR4. Chronic upregulation of coagulation [81] and systemic inflammation were reported in horses with severe asthma [82], and activation of the coagulation cascade [81] together with impaired epithelial repair [83] are features of human asthma. Hence, several lines of evidence suggest concurrence of hemostatic, coagulative and tissue repair processes with neutrophil activation in severe asthma. Furthermore, apoptosis and NETosis appear to be component of asthma as suggested by differential expression and linkage of genes in these pathways.

Among the DE genes are several of potential interest that are not part of overrepresented gene sets or interaction networks. Differences in cell cycle-related gene expression in peripheral blood mononuclear cells (PBMCs) have been reported in asthmatic horses [75]. CDC25A, a cell cycle-related gene, was not part of any network, but was highly DE and likely influences cell cycle and differentiation in bronchial epithelium during inflammation, as it does in other contexts such as neoplasia [84,85]. Genes such as ENSECAG00000014899 and ENSECAG00000017229 (potential orthologs of human KRT6 genes), KRT17 and ENSECAG00000007450 (potential ortholog of human SPRR1A/B) and transglutaminase 1 (TGM1) were not identified in network interactions, but are likely to function in squamous metaplasia [86]. Recent reports implicate hedgehog (HH) pathway-associated molecules in lung disease of humans [87].
polymorphisms (SNPs) in patched-1 (*PTCH1*), a DE gene, and hedgehog-interacting protein (*HHIP*), involved in the hedgehog pathway, have been associated with lung function in humans [88,89]. In conjunction, *PTCH1, HHIP* and family with sequence similarity 13, member A (*FAM13A*) predicted lung function abnormalities in an asthmatic cohort [88].

Five differentially expressed genes linked to regulation of the circadian clock were consistently downregulated in asthmatic animals: *CIART (CHRONO), PER3, DBP, TEF, ADRB2* and *NR1D2*. *CIART* is part of a transcriptional repressor of the mammalian clock, and contributes to a suppressive glucocorticoid response that is dependent on physiological stress [90]. *PER3* is expressed in a circadian pattern in the brain suprachiasmatic nucleus and also in peripheral tissues [91]. Changes in this group of genes may indicate disrupted circadian rhythm in the asthmatic lung. *NR1D2* and *PER3* have been associated with asthma in mice through bioinformatics analysis of genes and pathways [92]. *ADRB2* has been directly linked to circadian leukocyte recruitment [93]. In addition, in mice club cells may have a role in the circadian regulation of the lung through rhythmic *CXCL5* (orthologue gene to *CXCL6* in horses and humans) responses and loss of this regulation leads to aberrant neutrophil influx [94]. *SCGB1A1* is considered a key molecule for homeostasis in the lung, and club cells and *SCGB1A1* are reduced in horses with severe asthma [78]. Club cell depletion may result from impaired epithelial precursor cell recruitment and differentiation, and trigger further dysregulated pulmonary circadian rhythm.

**Conclusion**

There were pronounced differences in the epithelial response to challenge in asthmatic and non-asthmatic horses. Genes identified include many with prior association in asthma, and novel genes that potentially link pathogenic mechanisms. For candidate genes of interest, further functional characterization should be undertaken. For example, a protein-protein interaction assay in BALF using recombinant versions of protein of interest might be informative. In addition, investigation of epigenetic markers may further characterize environmental influences on genes.

**Declarations**

**Ethics approval**

All procedures were approved by the Institutional Animal Care Committee of the University of Guelph (protocol R10-031) and conducted in compliance with Canadian Council on Animal Care guidelines. Animals used in this research belong to the University of Guelph.
Consent for publication
Not applicable

Availability of data and materials
The raw sequences have been submitted to NCBI sequence read archive (SRA) on May 2nd, 2017, and are publicly available under study PRJNA384774 (SRP106023).

Competing interests
The authors declare that they have no competing interests.

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Authors’ contributions
LT, OC, MEC, LV, ADM and DB participated at the conception and design of the work and acquisition of data. LT and DB performed the analysis and interpretation of data, and drafting of the work. SA critically revised and participated to the analysis and interpretation of data. All authors reviewed and approved the final version of the manuscript.

Acknowledgements
Not applicable

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different gene expression profiles between smoking and nonsmoking patients. Tumour Biol. 2015;36:8993-9003.


56. Kang JH, Hwang SM, Chung IY. S100A8, S100A9 and S100A12 activate airway epithelial cells to produce MUC5AC via extracellular signal-regulated kinase and nuclear factor-κB pathways. Immunology. 2015;144:79-90.


76. Chinnadurai G, Vijayalingam S, Rashmi R. BIK, the founding member of the BH3-only family proteins: mechanisms of cell death and role in cancer and pathogenic processes. Oncogene.


Figure 2.1. Outline of study design and analysis.
Figure 2.2. Endoscopic appearance of bronchi, BAL lavage cell yield and histological appearance of bronchial biopsies.

Asthmatic horses had bronchoconstriction and increased secretions in airways after asthmatic challenge, while changes in horses without asthma consisted of mild bronchoconstriction (A). In asthmatic horses, cell concentration and proportion of neutrophils was increased in BAL fluid after challenge (B), and in bronchial biopsies epithelial basophilia and influx of submucosal leukocytes was evident (C).
Figure 2.3. Bronchoscopic score, BAL and respiratory measurements in asthmatic and non-asthmatic horses.

The individual change from pre- to post-challenge bronchoscopic score, pleural pressure, alveolar leukocyte concentration, proportion of neutrophils, and dynamic compliance differed significantly between asthmatic and control horses, while the change in lung resistance did not have a uniform pattern.
Figure 2.4. Differential gene expression in asthmatic compared to non-asthmatic horses after challenge.

EdgeR smear plot showing the log2 fold-change (FC, y-axis) versus the average log2 count per million (CPM, x-axis) of the change in gene expression due to challenge in all horses (A). Horizontal blue lines delineate 1-fold change and each point represents one gene. Differentially expressed genes are indicated in red (FDR <0.05). Genes with positive log2FC were upregulated in asthmatic compared to non-asthmatic horses, while genes with negative FC were downregulated. The majority of genes expressed differentially between the two groups are upregulated. Leading logFC plot (B) shows individual horses with asthma before (black) and after (red) challenge, and horses without asthma before (blue) and after (green) challenge. No clustering was observed for non-asthmatic horses, but post-challenge samples from asthmatic horses are located distant from other samples.
Figure 2.5. Heat map of differentially expressed genes in asthmatic compared to non-asthmatic horses after challenge.

A heat map of differentially expressed genes significantly up-(A) and downregulated (B) between asthmatic and non-asthmatic horses after challenge shows consistent change across individuals. Genes with positive log2FC were upregulated in asthmatic compared to non-asthmatic horses, while genes with negative FC were downregulated. Level of expression is expressed as cpm and ordered from highest (top) to lowest log2 fold-change (logFC, bottom). Significance threshold was set at FDR <0.05.
Figure 2.6. Differentially expressed genes with positive log fold-change in asthmatic compared to non-asthmatic horses.

Stacked bar chart of positive log2 fold-change (logFC) for genes differentially expressed between asthmatic and non-asthmatic horses in response to challenge.  

a ENSECAG00000014899, b ENSECAG00000017229
Figure 2.7. Differentially expressed genes with negative log fold-change in asthmatic compared to non-asthmatic horses.

Stacked bar chart of negative log2 fold-change (logFC) for genes differentially expressed between asthmatic and non-asthmatic horses in response to challenge.
Figure 2.8. Selected gene and protein expression from asthmatic and non-asthmatic, horses before and after an asthmatic horses.

Gene (A) and protein expression (B) of TNFRSF12A, PTCH1, CDC25A and IL8 in bronchial biopsies from horses with and without asthma. TNFRSF12A, CDC25A and IL8 mRNA, expressed as counts per million (CPM), was upregulated in asthmatic and downregulated in non-asthmatics following challenge, while PTCH1 was slightly downregulated in asthmatics and upregulated in non-asthmatics. (B) IHC results approximated gene expression with a relative increase in
TNFRSF12A, CDC25A and IL8 immunoreactivity and decrease in PTCH1 immunoreactivity in asthmatic animals.
Figure 2.9. Network of genes differentially expressed between asthmatic and non-asthmatic horses after challenge.

The network cluster is derived from 111 genes and contains 51 nodes each representing a protein, and 113 edges each representing an interaction between two proteins. Node color from white to green represents lowest to highest betweenness centrality (BC). The size of each node corresponds to the degree (number of connections). The color of edges represents the confidence of the interaction ranging from 0.4 (medium confidence, light gray) to 1 (highest confidence, black). Nodes with red borders have negative fold-change.
Supplementary figure 2.1. Antibody reactivity validation with immunoblots.

Immunoblots assessing antibody reactivity for (A) TNFRSF12A, (B) PTCH1, (C) CDC25A and (D) IL8. Only antibodies yielding a single band of expected size were used in subsequent immunohistochemical assays.
Table 2.1. Summary of RNA-seq reads mapped to the horse genome.

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## Table 2.2. Significantly over-represented biological processes identified by GO analysis.

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<td>S100A9, PLEK, SERPINB2, THBS1, TLR4, TNFRSF12A, PLAUR, SERPINE2</td>
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<td>Myeloid leukocyte migration</td>
<td>GO:0097529</td>
<td>7</td>
<td>5.81E-04</td>
<td>S100A9, TREM1, CSF3R, CXCR2, LGALS3, CXCL8, C5AR1</td>
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<tr>
<td>Movement of cell or subcellular component</td>
<td>GO:0006928</td>
<td>19</td>
<td>7.57E-04</td>
<td>S100A9, OLR1, TREM1, S100A2, NLRP12, THBS1, TNFRSF12A, CSF3R, MMP1, PLAUR, PTGS2, CXCR2, SLC7A5, SLC7A11, MMP9, ITGAX, LGALS3, CXCL8, C5AR1</td>
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<td>Leukocyte chemotaxis</td>
<td>GO:0030595</td>
<td>7</td>
<td>2.35E-03</td>
<td>S100A9, TREM1, CSF3R, CXCR2, LGALS3, CXCL8, C5AR1</td>
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<tr>
<td>Response to stimulus</td>
<td>GO:0050896</td>
<td>48</td>
<td>3.75E-03</td>
<td>CAMP, CLEC4E, S100A9, PLEK, SERPINB2, ORM1, OLR1, TREM1, FCAR, MRGPRX2, OSM, NLRP12, THBS1, TLR4, TNFRSF12A, MMP3, CDC25A, SFN, CSF3R, KRT17, CYCS, PTX3, FOSL1, HCAR3, PLAUR, SERPINE2, IL1RN, NFE2, YARS, SRGN, PADI4, PTGS2, CXCR2, SLC7A11, AQP9, RETN, LILRB3, MMP9, ITGAX, CD300A, LGALS3, HOMER1, CXCL8, CD300LB, CCRL2, C5AR1, RGS18, APOBEC3B</td>
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<tr>
<td>Regulation of multicellular organismal process</td>
<td>GO:0051239</td>
<td>26</td>
<td>3.84E-03</td>
<td>CAMP, CLEC4E, S100A9, PLEK, SERPINB2, ORM1, NLRP12, THBS1, TLR4, TNFRSF12A, SFN, FOSL1, SRGN, PTGS2, RETN, LILRB3, MMP9, SLC46A2, LGALS3, CXCL8, C5AR1</td>
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<tr>
<td>Single-organism cellular process</td>
<td>GO:0044763</td>
<td>59</td>
<td>7.87E-03</td>
<td>CAMP, CLEC4E, S100A9, PLEK, MMP8, ORM1, OLR1, TREM1, DHRS9, MRGPRX2, OSM, SYNJ2, S100A2, NLRP12, PLBD1, THBS1, TLR4, TNFRSF12A, TUBA1C, MMP3, CDC25A, SFN, CSF3R, KRT17, CYCS, MMP1, FOSL1, HCAR3, PHLD2A, PLAUR, SERPINE2, IL1RN, NFE2, YARS, SLC4A11, SRGN, PADI4, PTGS2, CXCR2, SLC7A5, SLC7A11, AQP9, ARG2, RETN, LILRB3, MMP9, ITGAX, CD300A, LGALS3, HOMER1, CXCL8, MFSD2A, CCR2, C5AR1, TGM1, SPRR1B, RGS18, TUBA4A, TREML2</td>
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<td>Secretion</td>
<td>GO:0046903</td>
<td>12</td>
<td>1.01E-02</td>
<td>PLEK, ORM1, TREM1, MRGPRX2, THBS1, TLR4, SERPINE2, IL1RN, SRGN, PTGS2, AQP9, TUBA4A</td>
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<tr>
<td>Regulation of body fluid levels</td>
<td>GO:0050878</td>
<td>11</td>
<td>1.28E-02</td>
<td>S100A9, PLEK, SERPINB2, THBS1, TLR4, SFN, PLAUR, SERPINE2, NFE2, PTGS2, SLC7A11</td>
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<td>Positive regulation of cell death</td>
<td>GO:0010942</td>
<td>12</td>
<td>1.33E-02</td>
<td>S100A9, OSM, NLRP12, THBS1, TNFRSF12A, MMP3, SFN, CYCS, FOSL1, PLAUR, PTGS2, MMP9</td>
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<tr>
<td>Positive regulation of hemostasis</td>
<td>GO:1900048</td>
<td>4</td>
<td>1.51E-02</td>
<td>S100A9, PLEK, THBS1, TLR4</td>
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<td>Positive regulation of hemostasis</td>
<td>GO:0030194</td>
<td>4</td>
<td>1.51E-02</td>
<td>S100A9, PLEK, THBS1, TLR4</td>
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83
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<tr>
<th>Process</th>
<th>GO: Number</th>
<th>Count</th>
<th>P-value</th>
<th>Genes</th>
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<td>Positive regulation of coagulation</td>
<td>GO:0050820</td>
<td>4</td>
<td>1.77E-02</td>
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<td>Cell chemotaxis</td>
<td>GO:0060326</td>
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<td>Positive regulation of apoptotic signaling</td>
<td>GO:2001235</td>
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<td>2.35E-02</td>
<td>S100A9, OSM, THBS1, TNFRSF12A, SFN, PLAUR, MMP9</td>
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<tr>
<td>Single-organism process</td>
<td>GO:0044699</td>
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<td>2.73E-02</td>
<td>CAMP, CLEC4E, SI0O0A9, PLEK, MMP8, ORM1, OLR1, TREM1, DHRS9, MRGPRX2, OSM, SYNJ2, S100A2, NLRP12, PLBD1, THBS1, TLR4, TNFRSF12A, TUBA1C, MMP3, CDC25A, SFN, CSF3R, KRT17, CYCS, PTX3, MMP1, FOSL1, HCRA3, PHLD2A, PLAUR, SERPINE2, IL1RN, NFE2, YARS, SLC4A11, SRGN, PADI4, PTGS2, CXCR2, SLC7A5, HIGD1A, SLC7A11, AQP9, ARG2, RE TN, LILRB3, MMP9, ITGAX, CD300A, SLC46A2, LGALS3, HOMER1, CXCL8, MFSD2A, CCRL2, C5AR1, TGM1, SPRR1B, RGS18, TUBA4A, TREML2</td>
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<tr>
<td>Regulation of response to external stimulus</td>
<td>GO:0032101</td>
<td>13</td>
<td>3.33E-02</td>
<td>S100A9, PLEK, SERPINB2, TREM1, OSM, NLRP12, THBS1, TLR4, PLAUR, SERPINE2, PTGS2, CXCL8, C5AR1</td>
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<td>Positive regulation of response to external stimulus</td>
<td>GO:0032103</td>
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<td>3.60E-02</td>
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<td>Positive regulation of apoptotic process</td>
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<tr>
<td>Regulation of immune system process</td>
<td>GO:0002682</td>
<td>17</td>
<td>4.73E-02</td>
<td>CLEC4E, ORM1, TREM1, THBS1, TLR4, CSF3R, TREML4, CD300LD, LILRB3, CD300A, SLC46A2, LGALS3, SAMS1, CXCL8, CD300LB, C5AR1, TREML2</td>
</tr>
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</table>

**Down-regulated**

| Rhythmic process                           | GO:0048511  | 6     | 3.15E-02| ADRB2, DBP, NR1D2, CIART, TEF, PER3                                       |

a Number of differentially expressed genes  
b Classified as most specific (shaded)
Table 2.3. The function of genes with overrepresented GO terms.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Positive regulation of immune system process</th>
<th>Immune response</th>
<th>Inflammatory response</th>
<th>Secretion</th>
<th>Positive regulation of apoptotic signaling pathway</th>
<th>Neutrophil chemotaxis</th>
<th>Positive regulation of blood coagulation</th>
<th>Association with asthma</th>
<th>Protein function (NCBI or Uniprot; Homo sapiens)</th>
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<td>S100A9</td>
<td>✓</td>
<td>✓</td>
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<td>THBS1</td>
<td>✓</td>
<td>✓</td>
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<td>PLEK</td>
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<tr>
<td>LGALS3</td>
<td>✓ ✓ × × × ✓ ×</td>
<td>Key role in inflammation and airway remodelling in murine models of allergic asthma [95-97]. Decreased in sputum of neutrophilic asthmatics compared to mixed neutrophilic and eosinophilic asthmatics [98]</td>
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<td></td>
<td></td>
<td>Involved in apoptosis, innate immunity, cell adhesion and T-cell regulation</td>
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<tr>
<td></td>
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<td>Antimicrobial activity</td>
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<tr>
<td>OSM</td>
<td>× ✓ × ✓ ✓ ×</td>
<td>Part of IL6 family of cytokines, OSM specifically is increased in asthmatics and correlates with irreversible airway obstruction [99]. OSM was associated with asthma but not atopy in children [100]</td>
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<td></td>
<td>Inhibition of proliferation, regulation of production of other cytokines</td>
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<tr>
<td>PTGS2</td>
<td>× × ✓ ✓ × ×</td>
<td>Polymorphism in the gene associated with asthma and atopy in children [101]</td>
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<td>Prostaglandin biosynthesis</td>
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<td>ORM1</td>
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<tr>
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<td>Acute-phase protein, potential immunosuppressive activity</td>
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<tr>
<td>IL1RN</td>
<td>× ✓ ✓ × × ×</td>
<td>Sputum IL1RN to IL-1β ratio decreased in neutrophilic asthmatics compared to mixed neutrophilic and eosinophilic asthmatics [98]</td>
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<td>EMT in bronchial epithelial cells [47] Modulates IL1-mediated inflammation and inhibits activity of IL1A &amp; B</td>
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<td>CSF3R</td>
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<td>Controls granulocyte production and activity.</td>
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<tr>
<td>CXCR2</td>
<td>× × ✓ × × ✓</td>
<td>Expression increased in sputum of patient with non-eosinophilic asthma [21]. Controversial role in asthma. CXCR2 antagonists alone do not improve clinical signs of asthma but reduce neutrophil number [21]. Combination of CXCR1 and CXCR2 antagonists promising for therapy [102]</td>
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<td>IL8 receptor, neutrophil migration</td>
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<tr>
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<td>Response to pathogens, Antibacterial, regulation of cell chemotaxis and inflammation.</td>
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<tr>
<td>PTX3</td>
<td>× ✓ ✓ × × × ×</td>
<td>Expression increased in bronchial tissues of asthmatics and highly expressed in smooth muscle cells [103]</td>
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<td>Up-regulated in response to inflammation in epithelial cells. Role in angiogenesis and tissue remodelling.</td>
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<tr>
<td>MRGPRX2</td>
<td>× ✓  × ✓ × ✓ × × NA</td>
<td>IgE independent activation of mast-cells, leading to inflammation and smooth muscle cell contraction [104]. Mast cell-specific receptor, mediates allergic reactions.</td>
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<tr>
<td>AQP9</td>
<td>× ✓  × ✓ × ✓ × × NA</td>
<td>Neutrophil regulation [105]; membrane channel</td>
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<td>CLEC4E</td>
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<td>Inflammation</td>
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<td>LILRB3</td>
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<td>Inhibits immune response</td>
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<td>CD300LB</td>
<td>✓ ✓  × × × × × × NA</td>
<td>Expressed by granulocytes [106]. Activates immune receptor</td>
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<td><strong>Specific to one gene set</strong></td>
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<td>TNFRSF12A</td>
<td>× ×  × × × ✓ × × NA</td>
<td>May be involved in angiogenesis, proliferation and cellular adhesion</td>
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<td>SFN</td>
<td>× ×  × × × ✓ × × NA</td>
<td>Regulation of protein kinase C [107] and epithelial cell growth through Akt/mTOR pathway when bound to KRT17 [108]</td>
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<tr>
<td>PLAUR</td>
<td>× ×  × × × ✓ × ×</td>
<td>Increased expression <em>in vitro</em> in bronchial epithelium from asthmatics [109]</td>
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<tr>
<td>OLR1</td>
<td>× ×  ✓ × × × × × NA</td>
<td>Degradation of oxidized low-density lipoprotein</td>
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<tr>
<td>CCRL2</td>
<td>× ×  ✓ × × × × ×</td>
<td>Expressed in lung epithelial cells and up-regulated following LPS exposure. Potential role in initiation of allergic inflammation [110]</td>
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<td>Impaired wound repair process [109]. May be involved in plasminogen activation and ECM degradation</td>
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<td>Unknown function, up-regulated in activated neutrophils</td>
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<tr>
<td>SERPINE2</td>
<td>× × × × ✓ x x x</td>
<td>Urokinase inhibitor [111], overexpression in asthma and down-regulation correlated with improved FEV1 [112]</td>
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* Not available
CHAPTER 3

Airway transcriptome gene-set enrichment analysis in equine severe asthma shows impaired cell cycle and tissue repair

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Abstract
Severe equine asthma is a chronic inflammatory condition of the lower airways similar to adult-onset asthma in humans. Exacerbations are characterized by bronchial and bronchiolar neutrophilic inflammation, mucus hypersecretion, and airway constriction. In this study we aimed to analyze gene interactions in the transcriptomic response of the bronchial epithelium in asthmatic and non-asthmatic animals. We found 2341 and 120 differentially expressed genes in asthmatic and non-asthmatic horses, respectively, after a challenge. In asthmatic horses, transcription factor motifs with the highest enrichment scores for upregulated genes belonged to the E2F transcription factor family, and three genes within the E2F family (E2F2, E2F3 and E2F8) were significantly upregulated. Gene set enrichment analysis identified 587 significantly enriched gene sets linked by 18,777 edges in asthmatic horses, and 171 gene sets linked by 2326 edges in non-asthmatic horses. Gene sets enriched in asthmatic animals were involved in cell cycle, neutrophil migration and chemotaxis, wound healing, hemostasis, coagulation, regulation of body fluid levels and the hedgehog pathway. These results suggest that in asthmatics dysregulated cell proliferation and epithelial healing are fundamental lesions that prevent re-establishment of homeostasis and perpetuate inflammation. Hedgehog and E2F pathways are likely key components of this disease process.

Background
Severe equine asthma (recurrent airway obstruction, heaves) is a naturally occurring lung condition affecting horses that are chronically exposed to airborne environmental dust and microbial components. Features of exacerbated disease include excessive mucus production, cough, neutrophilic airway inflammation, bronchial hyperreactivity, and bronchospasm. Chronic inflammation in the lower airway of affected horses leads to epithelial hyperplasia, smooth muscle hyperplasia and hypertrophy, and fibrosis culminating in irreversible airway remodeling.

The specific pathways underlying the condition are still poorly understood. Genetic predisposition and environmental triggers are thought to be the major factors leading to the development of the disease. Inheritance patterns are complex, implying genetic heterogeneity and suggesting mechanistic variation across different kinships. In humans, childhood asthma associated with atopy was strongly linked with a Th2 response, which prompted investigation of Th2 pathways in severe equine asthma. However, human adult-onset asthma, which resembles
severe equine asthma, has not been consistently linked to a particular Th1 or Th2 response\textsuperscript{9}. Similarly, in horses, findings regarding a particular Th set have been equivocal, pointing toward a mixed response with a biological complexity likely greater than that of the Th1/Th2 paradigm\textsuperscript{10-14}. Other factors such as the heterogeneity of cells and tissues assessed, the degrees of asthmatic exacerbation in subjects, the frequency and timing of sampling in a dynamic response, and the limited number of pathway-specific markers likely contribute to inconsistency in conclusions.

Transcription factors assessed to date in the equine asthmatic inflammatory response include activator protein-1 (AP-1), cyclic AMP response element binding protein (CREB), CAAT/enhancer binding protein (C/EBP), GATA-3 and nuclear factor (NF)-κB\textsuperscript{15-17}. Activity of AP-1 in bronchial brushing (BB) cells and NF-κB in bronchoalveolar lavage (BAL) cells positively correlated with active disease, while CREB activity was higher in BB cells of asthmatic than control horses 2 months after challenge\textsuperscript{15-17}. Differences in target binding of GATA-3 and C/EBP were not detected, and specific targets of transcription factors were not identified. Target enrichment analysis in peripheral blood mononuclear cells (PBMC) from asthmatic horses also suggested hypoxia-inducible factor 1 (HIF-1) as a potential regulator\textsuperscript{18}. Serum response factor (SRF) and its co-factor myocardin (MYOCD) were increased in airway smooth muscle (ASM) cells of peripheral but not central airways of asthmatic horses, and were considered to contribute to ASM hypertrophy\textsuperscript{19}.

The bronchial epithelium has a major role in the development of asthma. In human asthma, the airway epithelium barrier function is physically and functionally impaired, which manifests with disrupted tight junctions, altered innate immune products and compromised regeneration of differentiated cell types\textsuperscript{20,21}. SCGB1A1 production by the bronchial epithelium is one such specialized epithelial function lost in asthmatic horses that indicates absence of mature club (Clara) cells, and therefore limited anti-inflammatory properties of airway secretions\textsuperscript{22,23}. Production of select epithelial cytokines and activity of p65 NF-kappa B homodimer in BAL leukocytes was altered in asthmatic horses\textsuperscript{16,24}, but the airway epithelium has not been analyzed comprehensively.

The evolution of RNA-sequencing (RNA-Seq) technology brought the possibility of large-scale analysis of gene expression, a valuable tool to quantitatively capture unbiased gene expression. In turn, gene set enrichment analysis aims to determine whether defined sets of related or interconnected genes identified by RNA-Seq significantly differ between phenotypes. The relationship of statistically significant RNA-Seq gene expression changes with biological significant meaning remains to be fully explored in many experimental systems, and likely varies
across different genes. Therefore, stringent statistical cutoffs applied to unbiased genome-wide sequencing data may impair the ability to detect biologically meaningful changes. Gene set enrichment performed with software such as Gene Set Enrichment Analysis (GSEA) leverages the unbiased nature of RNA-Seq data by by-passing pre-determined statistical cut-offs at the single gene level, in order to comprehensively identify cellular processes associated with a particular phenotype.

In this study, we investigated changes in gene sets associated with exacerbated equine severe asthma. We hypothesized that homogeneity in disease manifestations stem from expression of common pathways in genetically distinct horses. To address this hypothesis, we collected endobronchial biopsies from asthmatic and non-asthmatic horses following a challenge, and sequenced the transcriptome. We then performed gene set enrichment analysis of RNA-Seq data without preset cut-offs.

**Methods**

**Animals and procedures**

Overall study design is outlined in Figure 3.1. Animal procedures, sample collection and sample processing were as previously described. Briefly, six horses with asthma in remission and seven horses without asthma (mean ages of 15 and 12 years ($p =0.352$, unpaired t test, respectively) were placed in a dust-free indoor environment for 24 hours before exposure to dusty hay until respiratory impairment was apparent in asthmatic horses (range 1 to 3 days, average 2.2 days). Non-asthmatic horses were exposed to dusty hay for 3 days. Physical examination, pulmonary function tests (PFTs) and bronchoalveolar lavage (BAL) were performed before and after exposure to the asthmatic challenge, and endoscopic bronchial biopsies were obtained from a contralateral lung lobe. All procedures were approved by the Institutional Animal Care Committee of the University of Guelph (protocol R10-031) and conducted in compliance with Canadian Council on Animal Care guidelines.

**RNA-Seq sample preparation and analysis**

Total RNA was extracted from endobronchial biopsies (Qiagen, Toronto, ON). RNA quality and concentration was determined with the Bioanalyzer RNA Nanochip (Agilent, ON) and capillary electrophoresis. RNA-Seq unstranded library preparation and sequencing were performed at The
Centre for Applied Genomics (TCAG; Toronto, ON) using the Illumina TruSeq RNA sample preparation and sequencing protocols (Illumina, San Diego, CA). For each sample, approximately 1 µg of non-degraded, high quality total RNA was enriched for poly-A RNA, fragmented into 200 to 300 bases, and converted to double stranded cDNA libraries. Final RNA libraries were quantified (KAPA Library Quantification kit, Kapa Biosystems, Wilmington, MA) prior to pooling and sequencing. Illumina flow cells were prepared and samples sequenced on an Illumina HiSeq 2500 instrument in 5 lanes following the manufacturer’s instructions to generate paired-end reads of 100-bases.

Raw read quality was assessed using FastQC software version 0.10.1 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and aligned to the horse reference genome42 (Ensembl v70) with STAR version 2.443. The STAR_pass2 alignment protocol was followed including these adaptations: horse Ensembl version 70 GTF annotation file for first- and second-pass, and the junction SJ.tab file generated by STAR for the second-pass after non-canonical junctions were removed. Default settings were used except for: --runThreadN 8 --outFilterScoreMinOverLread 0.5 --outFilterMatchNminOverLread 0.5. Read counts were generated from STAR alignment files using HTSeq version 0.6.1p1 44 with settings -s no -f bam -r name.

Differential expression (DE) analysis was performed in R, version 3.2.1 (www.r-project.org), with the edgeR package version 3.10.245-47. A paired DE analysis was performed to assess changes between groups (asthmatics versus non-asthmatics) and within groups (before versus after asthmatic challenge). EdgeR analysis was based on section 3.5 of the edgeR user’s guide (last revised April 10, 2017). The model matrix was designed as followed: ~group+group:horse+group:challenge, where “group” refers to non-asthmatic and asthmatic groups, “horse” refers to each horse, and “challenge” refers to samples before and after the asthmatic challenge. Fit of the generalized linear model and tests for differences in expression were performed with the “glmFit” and “glmLRT” functions, respectively. GlmLRT(fit, coef="groupNonAsthmatic:postChallenge"), and

\begin{align*}
\text{glmLRT}(\text{fit}, \text{coef}="\text{groupasthmatic:postChallenge}")
\end{align*}

were used to analyze asthmatic challenge effect within the non-asthmatic and asthmatic horse group (before challenge versus after challenge), respectively. Statistical significance was set at false discovery rate (FDR) <0.05.
Gene set enrichment, modules and network analysis

Ranked Gene Set Enrichment Analysis (GSEA) was performed with software version 2.1.1\textsuperscript{25,48}. The human gene set file excluded annotations with evidence codes - 'IEA' (inferred from electronic annotation), 'ND' (no biological data available), 'RCA' (inferred from reviewed computational analysis) and was downloaded from:
Human\_GO\_AllPathways_no\_GO\_iea_January_28_2015_symbol.gmt\textsuperscript{49}.

GSEA pre-ranked analysis (GseaPreranked) was performed using default settings except for “Collapse dataset to gene symbols” set to “False”. Prior to analysis, a ranked list was calculated with each gene assigned a score based on the FDR and the direction of the log fold-change (“+” or “-“). Horse Ensembl IDs were converted to HUGO gene symbols. Non-matching symbols were enriched using the human orthologues when percent identity was above 80% for target and query sequence. Gene sets identified as significant (FDR <0.05, \textit{p} <0.001) with GSEA were visualized using the Enrichment Map plugin available for Cytoscape version 3.4.0\textsuperscript{27,50}. Connected nodes only were included, and gene set clusters were summarized and labeled manually with the WordCloud plugin\textsuperscript{51} for Cytoscape version 3.4.0\textsuperscript{50}.

Transcription factor target enrichment

Transcription factor target enrichment among differentially expressed genes in asthmatic and non-asthmatic horses was performed with Cytoscape (v 3.4.0) in combination with the iRegulon plugin\textsuperscript{52}. Analysis was conducted using the \textit{Homo sapiens} database and default settings. Genes with FDR <0.05 were included in the analysis, and up- and downregulated genes were analyzed separately. Transcription factors of interest with motif normalized enrichment scores (NES) >3 were selected for further analysis.

The datasets generated and analyzed in this study are available in the NCBI Sequence Read Archive at https://www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP106023
Results

Differential expression analysis

Differential expression analysis of the bronchial epithelium transcriptome following challenge (Fig. 1) in horses with (Fig. 3.2A) and without (Fig. 3.2B) asthma yielded 2341 and 120 differentially expressed (DE) genes, respectively. Analysis of DE genes by the distance between each pre- and post-challenge pair corresponding to the average root mean square of the largest log-fold change (leading fold-change) between two samples showed clustering of pre-challenge asthmatic and non-asthmatic samples, and post-challenge asthmatic samples, but post-challenge samples from asthmatics formed a distant non-clustered group (data previously shown)\(^\textsuperscript{26}\). This suggested that gene differential expression was greater than variance within the asthmatic group.

Gene set enrichment analysis

In asthmatic horses, 587 gene sets were significantly enriched and linked by 18,777 edges while in non-asthmatic horses 171 gene sets were significantly enriched and linked by 2326 edges. Results of GSEA (FDR <0.05, \(p <0.001\)) were visualized using the Enrichment Map plugin available for Cytoscape (Figures 3.3 and 3.4). In Figure 3.3 linked gene sets only in horses with asthma are shown. Gene sets involved in cell cycle regulation dominated, and genes involved in inflammation/immune-response, metabolism, extracellular matrix (ECM) degradation, and protein translation and processing (Fig. 3.3) were also enriched. High redundancy of significantly enriched gene sets affected the cell cycle, Toll-like receptor (TLR) pathway, wound healing, glycosylation as well as other inflammatory and defense response pathways. The gene sets generated tight clusters around nodes (red and blue squares). The clusters with edges (green lines) that were difficult to visualize due to the proximity or density of gene sets were circled (Fig. 3.3). In horses without asthma, similar but fewer enriched gene sets were noted (Fig. 3.4). Enriched gene sets suggest activity of related pathways.

Neutrophil migration and chemotaxis

In horses with asthma, significantly upregulated gene sets with lowest rank at max metric, suggesting higher likelihood of involvement in disease process, pertained to granulocyte and neutrophil chemotaxis and migration (Fig. 3.5, Table 3.1). Additional significant gene-sets were related to phases and components of the cell cycle (Table 3.2). In horses without asthma, gene sets with lowest rank at max included those related to mitosis, cytoskeleton, protein binding and
leukocyte migration and chemotaxis (Table 3.3). Gene sets linked to M phase and protein translation were identified as most highly significant (Table 3.4).

Cell cycle, hedgehog and hemostasis-related gene sets
Detailed analysis of cell cycle phases identified significantly enriched gene sets involved in all phases except G1 in horses with asthma, while only those in M phase were significantly enriched in non-asthmatic horses (Fig. 3.6A). High enrichment of cell cycle gene sets in horses with asthma correlated with significantly upregulated expression of M-phase inducer phosphatase 1 (CDC25A) in horses with asthma (data not shown). Forkhead box protein M1 (FOXM1) also was significantly upregulated only in horses with asthma (data not shown). CDC25A and FOXM1 expression correlated with the observed enrichment in cell cycle gene sets (Fig. 3.6B).

Several gene sets interacting with hedgehog (HH) were identified as significantly enriched in horses with but not without asthma (Fig. 3.7): “hedgehog 'on' state” (FDR =0.0172; REACT_268718.1), “hedgehog ligand biogenesis” (FDR=6.14E-05; REACT_264605.1), “Hh ligand biogenesis disease” (FDR =1.92E-05; REACT_263883.1) and “processing-defective Hh variants abrogate ligand secretion” (FDR =7.47E-05; REACT_264623.1). Significant upregulation of hypoxia-inducible factor 1α (HIF1α) and enrichment of the p53-hypoxia pathway (MSIGDB_C2) gene set was detected only in horses with asthma. Similarly, gene sets involved in “response to wounding and hemostasis” (Figure 3.8), were significant only in horses with asthma.

iRegulon
In asthmatic horses, a large number of significantly upregulated genes were identified as potential targets of E2F transcription factors. The ten transcription factor motifs with the highest enrichment score are listed in Tables 3.5 and 3.6 for asthmatic and non-asthmatic horses, respectively. E2F2, E2F3 and E2F8 were significantly upregulated in asthmatic horses, while only E2F1 was significantly downregulated in non-asthmatic horses.

Discussion
The goal of this study was to identify key pathways in the pathogenesis of asthma through identification and analysis of gene sets associated with the response of asthmatic horses to challenge. Bronchial biopsies from asthmatic and non-asthmatic horses were obtained before and after a challenge, the whole transcriptome was sequenced, and gene expression was analyzed for
each group with a paired design to account for individual variation. GSEA was then applied to assess whether genes that were *a priori* assigned to a specific biological process (gene set) were associated with the asthmatic phenotype\(^\text{25}\).

GSEA builds upon quantitative and qualitative gene expression data derived from RNA-Seq to cluster genes based on common functions, locations, pathways, interactions or other connecting properties. Thereby, linked processes that are significantly altered but might not be identified by individual differential gene expression analysis are recognized. Since significant gene sets may include shared and variable numbers of genes, Enrichment Map, a Cytoscape plugin software to visualize GSEA output and to facilitate interpretation of results, was applied. Hence, after differential expression analysis with edgeR, data from each group of animals were further analyzed to identify significantly enriched gene sets and their linked edges. Edges reflect genes shared within a gene set with the thickness of edges corresponding to the number of overlapping genes, and similar gene sets being clustered together\(^\text{27}\). Genes with expression that differed significantly between asthmatic and non-asthmatic horses after challenge were 2341 and 120, respectively. Analysis yielded 587 significantly enriched gene sets linked by 18,777 edges and 171 gene sets linked by 2326 edges, respectively, which was in approximate relation to the number of differentially expressed genes. The enrichment map highlighted many closely positioned groups of gene sets, reflecting a high degree of redundancy of genes within sets. In addition, one or several genes linked most gene sets, which implies that many pathways and networks responsible for specific features of asthma are related.

Neutrophil influx into airways is a hallmark of equine severe asthma\(^\text{28}\). Neutrophil and granulocyte migration and chemotaxis gene sets were ranked at max classification in asthmatic horses. Rank at max classification represents the maximum enrichment score (ES), which in turn is the maximal deviation from zero calculated for each gene descending the ranked list. Therefore, the rank represents the degree of over-representation of a gene set at the top or bottom of the ranked gene list. Enriched genes involved in neutrophil and granulocyte migration and chemotaxis were identified between positions 3 and 16 of the ranked list in asthmatic horses, yielding the highest score of all gene sets (Table 3.1). This indicates that the top genes differentially expressed in the epithelium of horses after asthmatic challenge are highly enriched for neutrophil migration and chemotaxis, which is consistent with the observed pathogenesis, and likely mediated by IL8 and CXCR2, that were also differentially expressed in asthmatic compared to non-asthmatics horses \(^\text{26}\).
It was particularly interesting that gene sets affecting all phases of the cell cycle, except for part of the G1 phase, were significantly enriched in asthmatic horses while only those involved in M phase were significantly enriched in non-asthmatic horses. As apparent in Figure 3.3, proximity visualization placed cell cycle gene sets very close together and linked them by a large number of edges, reflecting a high degree of redundancy. Detailed analysis of individual cell cycle phases (Figure 3.6) showed that gene sets in M, G0 and early G1, and G2 phase were most significant in asthmatic horses. Although the significance of relative differences between groups cannot be derived from this analysis, we hypothesize that processes in G0, early G1 and G2 phase are most altered in asthmatic relative to non-asthmatic horses because release of injurious mediators from granulocytes stimulates induction of cell cycling in the epithelium. In agreement with this hypothesis, CDC25A (ENSECAG0000016336), an M2-inducing phosphatase, was upregulated and differentially expressed in asthmatic horses. Altered cell cycle regulation has previously been reported when peripheral blood mononuclear cells (PBMC) from asthmatic horses were exposed to hay dust extract. In that study changes in PBMC, which are comprised of monocytes and lymphocytes, were assessed, while in our study changes in bronchial epithelium were investigated. Induction of genes associated with cell proliferation in both studies suggests that hay dust extract has a direct effect on PBMC (which may include previously in vivo sensitized lymphocytes), and that bronchial inflammatory cells or inhaled hay dust also stimulate epithelial cell cycling. Significant upregulation of FOXM1, a regulator of G1/S and G2/M cell cycle transition, further confirmed that there was induction and progression of the cell cycle. FOXM1 regulates CDC25A gene transcription by direct promoter binding and indirectly via the E2F transcription factor.

Patched 1 (PTCH1) was significantly downregulated in horses with asthma compared to non-asthmatic horses and HH-related gene sets were enriched. Abnormal activation of HH signaling through aberrant FOXM1 and GLI Family Zinc Finger 1 (Gli1) activation was observed in colorectal cancer cells, and considered essential for cell growth and proliferation. Our data suggest that activation of Hh signaling leads to persistent bronchial cell proliferation, which we hypothesize occurs through FOXM1 and CDC25A activation.

It has been proposed that injury of terminal bronchioles in airway inflammation leads to luminal exudation of plasma and accumulation of fibrinogen, thrombin and mucus in airways, and in turn a pro-coagulant state. Similarly, horses with asthma during active disease were considered to be in a hypercoagulable state and to have systemic inflammation, with
hypercoagulability persisting during remission. We found that gene sets associated with wound healing, hemostasis, blood coagulation and regulation of body fluid levels were significantly associated with the asthmatic phenotype, lending support to linkage and importance of these pathways in equine asthma. Smooth muscle cells of the bronchioles are hypercontractile in human asthmatics, and SRF and its co-factor MYOCD are thought to contribute to airway remodeling in severe equine asthma. Several genes differentially expressed between asthmatic and non-asthmatic horses are involved in wound healing, coagulation, hemostasis and regulation of body fluids, including thrombospondin 1 (THBS1), oncostatin (OSM), pleckstrin (PLEK) and others. Many of these genes have been linked to asthma, but their precise role in pathogenesis remains to be defined.

Upregulated genes in asthmatic horses were highly enriched in E2F-linked transcription factor-binding motifs: E2F8 was most significantly upregulated, followed by E2F2 and E2F3. Members of the E2F transcription factor family are key players in cell cycle regulation. E2F8 is thought to be a transcriptional repressor of DNA-damage responses in cancer, and also to have opposing roles as oncogene or tumor suppressor. E2F2 and E2F3 are transcriptional activators. Nuclear overexpression of E2F3 has been associated with lung cancer, and E2F2 was identified in non-small cell lung carcinoma in humans. In non-asthmatic horses, E2F1, a transcriptional activator, was significantly downregulated. FOXM1 in part activates CDC25A through E2F transcription factor-pathways. Interestingly, in humans, E2F1 inhibition was linked to reduced airway SMC proliferation. Hence, data presented here suggest that E2F8, E2F2 and E2F3 regulate cell proliferation in epithelial cells of asthmatic horses.

Conclusion
In this study key pathways highly altered in severe asthma of horses were identified. Affected animals had comprehensive activation of cell cycle and inflammation/immune response programs in the bronchial epithelium, which implies that impaired regeneration of the epithelial barrier may be the breakpoint event leading to subepithelial tissue remodeling and eventual irreversible lung parenchymal changes.
Acknowledgments

This study was supported with funding from the Natural Sciences and Engineering Research Council of Canada, Equine Guelph, the Ontario Ministry of Agriculture, Food and Rural Affairs and the Ontario Veterinary College Scholarship Program.

Author contributions

LT performed experiments, analysis and interpretation, and wrote the manuscript; OC, MEC, LV, and AD performed experiments and contributed to interpretation; SA contributed to analysis and interpretation; DB conceived the study, contributed to analysis and interpretation, and edited the manuscript.

References


Figure 3.1. Outline of study design and analysis.

Asthmatic and non-asthmatic animals: endobronchial biopsies before and after asthmatic challenge

RNA extraction

Library preparation and Illumina sequencing

Genome-guided alignment

**edgeR – paired samples:**

* Differential treatment response of mRNA expression in asthmatic versus non-asthmatic horses following challenge

Cut-off free gene list

Ranked GSEA analysis using human gene sets

Visualization with Cytoscape Enrichment Map Analysis, FDR <0.05, p-value <0.01

FDR < 0.05

Transcription factor target enrichment analysis with iRegulon and Cytoscape
Figure 3.2. Differential gene expression in asthmatic and non-asthmatic horses after challenge.

EdgeR smear plot showing the log fold-change (FC, y-axis) versus the average log count per million (CPM, x-axis) for the change in gene expression due to asthmatic challenge in horses with asthma (A), and in horses without asthma (B). Red dots represent the genes that differ significantly in horses with asthma before and after challenge (A), and in horses without asthma before and after challenge (B). Horizontal blue lines delineate 1-fold change. Significance set at FDR <0.05. Differentially expressed genes can be observed in both groups, but a greater number were observed in horses with asthma.
Figure 3.3. Significantly enriched gene sets in asthmatic horses.

Clusters of gene sets involved in cell cycle, Toll-like receptor (TLR) pathways, wound healing, glycosylation and other inflammatory and defense responses are apparent in asthmatic horses.

Results of gene set enrichment analysis (GSEA) of horses with asthma visualized with Cytoscape
Enrichment Map. Each node (square) corresponds to a gene set either upregulated (red) or downregulated (blue) in response to asthmatic challenge. Edges (green lines) link sets with overlapping genes, and thickness of lines correlates with the number of genes in common between two sets. Similar gene sets were automatically clustered together. Gene clusters were manually labeled and arranged. Only gene sets with FDR <0.05 and $p < 0.01$ were included in visualizations. Disconnected nodes and small clusters were removed.
Figure 3.4. Significantly enriched gene sets in non-asthmatic horses. Clusters of gene sets involved in cell cycle, mRNA processing, cell metabolism and ECM are apparent, and small aggregates of gene sets with function in leukocyte chemotaxis, peptidase and cilium are apparent in non-asthmatic horses. See legend of Fig. 3 for details.
Figure 3.5. Significant enrichment of neutrophil chemotaxis and migration gene sets in asthmatic horses.

In horses with asthma, gene sets for neutrophil chemotaxis and migration were significantly enriched. The y-axis represents enrichment score (ES) and the x-axis genes (vertical black lines) represented in gene sets. The green line connects points of ES and genes. ES is the maximum deviation from zero as calculated for each gene going down the ranked list, and represents the degree of over-representation of a gene set at the top or the bottom of the ranked gene list. The colored band at the bottom represents the degree of correlation of genes with the asthma phenotype (red for positive and blue for negative correlation). Significance threshold set at FDR <0.05. Gene sets for leukocyte only in horses with asthma.
Figure 3.6. Significant enrichment of cell cycle gene sets in asthmatic horses.
In horses with asthma, gene sets for M, G1, G0 and early G1, S and G2-M phases of the cell cycle were significantly enriched, while in horses without asthma only gene sets for M phase of the cell cycle were significantly enriched (A). See legend of Fig. 3.5 for details.
Figure 3.7. Significant enrichment of hedgehog signaling gene sets in asthmatic horses.

In horses with asthma, Hedgehog-associated pathways were significantly enriched. See legend of Fig. 3.5 for details.
Figure 3.8. Significant enrichment of wound healing, hemostasis, blood coagulation and regulation of body fluid gene sets in asthmatic horses.

In horses with asthma, gene sets associated with wound healing, hemostasis, blood coagulation and regulation of body fluid were significantly enriched. See legend of Fig. 3.5 for details.
Table 3.1. Top upregulated gene-sets in horses with asthma following challenge, ranked according to lowest rank at maximum score.

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<td>Neutrophil migration - go:1990266</td>
<td>15</td>
<td>1.93</td>
<td>9.06E-04</td>
<td>16</td>
</tr>
<tr>
<td>Myeloid leukocyte migration - go:0097529</td>
<td>31</td>
<td>1.80</td>
<td>5.83E-03</td>
<td>16</td>
</tr>
<tr>
<td>Leukocyte chemotaxis - go:0030595</td>
<td>46</td>
<td>1.69</td>
<td>2.03E-02</td>
<td>16</td>
</tr>
<tr>
<td>Positive regulation of cytokine secretion - go:0050715</td>
<td>33</td>
<td>1.61</td>
<td>4.11E-02</td>
<td>68</td>
</tr>
<tr>
<td>Negative regulation of inflammatory response - go:0050728</td>
<td>30</td>
<td>1.67</td>
<td>2.56E-02</td>
<td>134</td>
</tr>
<tr>
<td>Cell separation after cytokinesis - go:0000920</td>
<td>18</td>
<td>1.73</td>
<td>1.35E-02</td>
<td>140</td>
</tr>
<tr>
<td>Chemokine receptor binding - go:0042379</td>
<td>22</td>
<td>1.95</td>
<td>5.97E-04</td>
<td>164</td>
</tr>
</tbody>
</table>

\(^\wedge\) Normalized enrichment score
\(^*\) False discovery rate
Table 3.2. Top upregulated gene-sets in horses with asthma following challenge, ranked according to FDR.

<table>
<thead>
<tr>
<th>Name of gene set</th>
<th>Size of gene set</th>
<th>NES^</th>
<th>FDR*</th>
<th>Rank at max</th>
</tr>
</thead>
<tbody>
<tr>
<td>M phase - react_910.4</td>
<td>213</td>
<td>2.68</td>
<td>&lt;0.001^a</td>
<td>1633</td>
</tr>
<tr>
<td>Cell cycle, mitotic - react_152.7</td>
<td>373</td>
<td>2.67</td>
<td>&lt;0.001^a</td>
<td>1633</td>
</tr>
<tr>
<td>Mitotic metaphase and anaphase - react_150314.2</td>
<td>150</td>
<td>2.64</td>
<td>&lt;0.001^a</td>
<td>1624</td>
</tr>
<tr>
<td>Cell cycle - react_115566.4</td>
<td>430</td>
<td>2.63</td>
<td>&lt;0.001^a</td>
<td>1633</td>
</tr>
<tr>
<td>Mitotic nuclear division - go:0007067</td>
<td>126</td>
<td>2.61</td>
<td>&lt;0.001^a</td>
<td>1016</td>
</tr>
<tr>
<td>Mitotic anaphase - react_1275.3</td>
<td>149</td>
<td>2.60</td>
<td>&lt;0.001^a</td>
<td>1624</td>
</tr>
<tr>
<td>Nuclear division - go:0000280</td>
<td>168</td>
<td>2.59</td>
<td>&lt;0.001^a</td>
<td>1016</td>
</tr>
<tr>
<td>Separation of sister chromatids - react_150471.2</td>
<td>141</td>
<td>2.58</td>
<td>&lt;0.001^a</td>
<td>1624</td>
</tr>
<tr>
<td>Mitotic prometaphase - react_682.3</td>
<td>96</td>
<td>2.54</td>
<td>&lt;0.001^a</td>
<td>1515</td>
</tr>
<tr>
<td>Chromosome segregation - go:0007059</td>
<td>129</td>
<td>2.54</td>
<td>&lt;0.001^a</td>
<td>1186</td>
</tr>
</tbody>
</table>

^Normalized enrichment score
* False discovery rate
^a Exact FDR value not detected (<1/maximum number of permutations)
Table 3.3. Top upregulated gene-sets in non-asthmatic horses following challenge, ranked according to lowest rank at maximum score.

<table>
<thead>
<tr>
<th>Name of gene set</th>
<th>Size of gene set</th>
<th>NES*</th>
<th>FDR*</th>
<th>Rank at max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitotic cytokinesis - go:0000281</td>
<td>25</td>
<td>1.99</td>
<td>2.91E-04</td>
<td>286</td>
</tr>
<tr>
<td>Negative regulation of protein binding - go:0032091</td>
<td>34</td>
<td>2.005</td>
<td>2.39E-04</td>
<td>288</td>
</tr>
<tr>
<td>Cytoskeleton-dependent cytokinesis - go:0061640</td>
<td>30</td>
<td>1.96</td>
<td>8.31E-04</td>
<td>288</td>
</tr>
<tr>
<td>Negative regulation of binding - go:0051100</td>
<td>58</td>
<td>1.87</td>
<td>7.87E-03</td>
<td>288</td>
</tr>
<tr>
<td>Aurora a signaling - pathway interaction database</td>
<td>25</td>
<td>1.84</td>
<td>1.36E-02</td>
<td>288</td>
</tr>
<tr>
<td>Regulation of protein binding - go:0043393</td>
<td>71</td>
<td>1.77</td>
<td>3.87E-02</td>
<td>288</td>
</tr>
<tr>
<td>Condensed nuclear chromosome - go:0000794</td>
<td>29</td>
<td>1.75</td>
<td>4.49E-02</td>
<td>288</td>
</tr>
<tr>
<td>Microtubule cytoskeleton organization involved in mitosis - go:1902850</td>
<td>24</td>
<td>1.75</td>
<td>4.35E-02</td>
<td>291</td>
</tr>
<tr>
<td>Condensed chromosome, centromeric region - go:0000779</td>
<td>26</td>
<td>1.94</td>
<td>1.46E-03</td>
<td>319</td>
</tr>
<tr>
<td>Regulation of leukocyte chemotaxis - go:0002688</td>
<td>48</td>
<td>1.82</td>
<td>1.98E-02</td>
<td>347</td>
</tr>
</tbody>
</table>

* Normalized enrichment score  
* False discovery rate
Table 3.4. Top upregulated gene-sets in non-asthmatic horses following challenge, ranked according to FDR.

<table>
<thead>
<tr>
<th>Name of gene set</th>
<th>Size of gene set</th>
<th>NES^</th>
<th>FDR^</th>
<th>Rank at max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Translation - react_1014.4</td>
<td>118</td>
<td>2.20</td>
<td>&lt; 0.001^a</td>
<td>1771</td>
</tr>
<tr>
<td>Cap-dependent translation initiation - react_2099.1</td>
<td>88</td>
<td>2.16</td>
<td>&lt; 0.001^a</td>
<td>1771</td>
</tr>
<tr>
<td>3'-utr-mediated translational regulation - react_1762.2</td>
<td>80</td>
<td>2.15</td>
<td>&lt; 0.001^a</td>
<td>1503</td>
</tr>
<tr>
<td>M phase - react_910.4</td>
<td>213</td>
<td>2.15</td>
<td>&lt; 0.001^a</td>
<td>1647</td>
</tr>
<tr>
<td>l13a-mediated translational silencing of ceruloplasmin expression - react_79.2</td>
<td>80</td>
<td>2.15</td>
<td>&lt; 0.001^a</td>
<td>1503</td>
</tr>
<tr>
<td>Eukaryotic translation initiation - react_2159.5</td>
<td>88</td>
<td>2.14</td>
<td>&lt; 0.001^a</td>
<td>1771</td>
</tr>
<tr>
<td>Protein targeting to er - go:0045047</td>
<td>87</td>
<td>2.14</td>
<td>&lt; 0.001^a</td>
<td>1869</td>
</tr>
<tr>
<td>Mitotic prometaphase - react_682.3</td>
<td>96</td>
<td>2.13</td>
<td>&lt; 0.001^a</td>
<td>1249</td>
</tr>
<tr>
<td>Gtp hydrolysis and joining of the 60s ribosomal subunit - react_2085.2</td>
<td>81</td>
<td>2.13</td>
<td>&lt; 0.001^a</td>
<td>1771</td>
</tr>
<tr>
<td>Cotranslational protein targeting to membrane - go:0006613</td>
<td>85</td>
<td>2.11</td>
<td>&lt; 0.001^a</td>
<td>1869</td>
</tr>
</tbody>
</table>

^ Normalized enrichment score
^* False discovery rate
^a Exact FDR value not detected (<1/maximum number of permutations)
Table 3.5. Most enriched transcription factor motifs among significantly up and downregulated genes in horses with asthma following challenge.

<table>
<thead>
<tr>
<th>Transcription factor motif ID</th>
<th>NES^</th>
<th>Transcription factors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Upregulated genes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transfac_pro-M00920</td>
<td>8.72</td>
<td>E2F7,TFDP1,<strong>E2F3</strong>,E2F4,E2F1,TFDP3,E2F5,<strong>E2F2</strong></td>
</tr>
<tr>
<td>Transfac_pro-M00919</td>
<td>8.72</td>
<td>E2F1,TFDP1, <strong>E2F3</strong>,E2F4,TFDP3,E2F7,<strong>E2F2</strong>,E2F5</td>
</tr>
<tr>
<td>Swissregulon-E2F1..5.p2</td>
<td>8.63</td>
<td><strong>E2F2</strong>,E2F4,E2F1,E2F5,<strong>E2F3</strong>,TFDP1,E2F7,TFDP3</td>
</tr>
<tr>
<td>Homer-M00032</td>
<td>8.56</td>
<td>E2F7,E2F4,E2F6,E2F1</td>
</tr>
<tr>
<td>Transfac_pro-M00740</td>
<td>8.30</td>
<td>E2F1,<strong>E2F3</strong>,E2F4,E2F5,<strong>E2F2</strong>,TFDP1,E2F7,TFDP3</td>
</tr>
<tr>
<td>Transfac_pro-M00738</td>
<td>8.29</td>
<td>E2F4,E2F1,E2F5,<strong>E2F3</strong>,<strong>E2F2</strong>,TFDP1,E2F7,TFDP3</td>
</tr>
<tr>
<td>Jaspar-PF0069.1</td>
<td>8.25</td>
<td>E2F4,E2F7,E2F6,E2F1,<strong>E2F8</strong></td>
</tr>
<tr>
<td>Homer-M00028</td>
<td>8.18</td>
<td>E2F1,E2F4,<strong>E2F3</strong>,E2F7,TFDP1,E2F5,<strong>E2F2</strong>,TFDP3</td>
</tr>
<tr>
<td>Transfac_pro-M00939</td>
<td>8.15</td>
<td>E2F1,TFDP1,E2F4,E2F3*,TFDP3,E2F7,E2F5,<strong>E2F2</strong></td>
</tr>
<tr>
<td>Transfac_public-M00050</td>
<td>8.11</td>
<td>E2F4,TFDP1,<strong>E2F3</strong>,E2F1,TFDP3,E2F7,E2F5,<strong>E2F2</strong></td>
</tr>
<tr>
<td><strong>Downregulated genes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transfac_pro-M02790</td>
<td>5.24</td>
<td>RFX7,<strong>RFX5</strong></td>
</tr>
<tr>
<td>Transfac_pro-M02789</td>
<td>4.69</td>
<td>RFX4,<strong>RFX5</strong>,RFX7</td>
</tr>
<tr>
<td>Taipale-SGTTGCYARGCAACS-RFX4-DBD</td>
<td>4.58</td>
<td>RFX4,RFX3,<strong>RFX2</strong>,RFX1</td>
</tr>
<tr>
<td>Taipale-SGTTGCYARGCAACS-Rfx2-DBD</td>
<td>4.57</td>
<td>RFX3,<strong>RFX2</strong>,RFX4,<strong>RFX1</strong></td>
</tr>
<tr>
<td>Taipale-GTAAACAW-FOXO3-full</td>
<td>4.57</td>
<td>FOXO3,FOXO2,FOXO1,FOXF2,FOXD1,FOXO4**,FOXO6**,FOXA1,FOX2,FOXJ1,FOXJ2,FOXI1,FOXK1</td>
</tr>
<tr>
<td>Swissregulon-FOX_D1-D2..p2</td>
<td>4.47</td>
<td>FOXD1,FOX2,FOXO1,HLTF,FOXO4**,FOXO6**,FOXA2,FOXJ2,FOXO3,FOXA2,FOXA1,FOXI1,FOX2,FOXO4**,FOXK1</td>
</tr>
<tr>
<td>Taipale-SGTTGCYARGCAACS-RFX2-DBD</td>
<td>4.26</td>
<td><strong>RFX2</strong>,RFX3,RFX4</td>
</tr>
<tr>
<td>Yetfasco-501</td>
<td>4.26</td>
<td>N/A</td>
</tr>
<tr>
<td>Yetfasco-1452</td>
<td>3.80</td>
<td>HLF,<strong>TEF</strong>,NFIL3,<strong>DBP</strong>,ATF2</td>
</tr>
<tr>
<td>Homer-M00165</td>
<td>3.80</td>
<td><strong>RFX5</strong>,<strong>RFX1</strong>,RFX7</td>
</tr>
</tbody>
</table>

^ Normalized enrichment score
* Transcription factors significantly upregulated following challenge
# Transcription factors significantly downregulated following challenge
Table 3.6. Most enriched transcription factor motifs among significantly up- and downregulated genes in horses without asthma following challenge.

<table>
<thead>
<tr>
<th>Transcription factor motif ID</th>
<th>NES</th>
<th>Transcription factors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Upregulated genes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>jaspar-MA0314.1</td>
<td>6.01</td>
<td>NFYB, POLE3, NFYC, NFYA</td>
</tr>
<tr>
<td>yetfasco-1536</td>
<td>5.73</td>
<td>NFYB, POLE3, NFYA, NFYC</td>
</tr>
<tr>
<td>homer-M00123</td>
<td>5.58</td>
<td>RELA, NFKB2, REL, NFKB1, BCL3, OVOL2, STAT6, EBF1</td>
</tr>
<tr>
<td>taipale-RRGGTCAAAGTCCRN N-HNF4A-DBD</td>
<td>5.49</td>
<td>HNF4A, NR2F1, NR2F2, HNF4G, RXRG, PPARG, RXRB, RXRA, NR2C2</td>
</tr>
<tr>
<td>jaspar-MA0316.1</td>
<td>5.27</td>
<td>NFYC, POLE4, NFYA, NFYB</td>
</tr>
<tr>
<td>transfac_public-M00288</td>
<td>5.27</td>
<td>NFYB, NFYA, POLE3, NFYC</td>
</tr>
<tr>
<td>elemento-CTGGGCCA</td>
<td>5.23</td>
<td>N/A</td>
</tr>
<tr>
<td>yetfasco-1537</td>
<td>5.19</td>
<td>NFYC, POLE4, NFYA, NFYB</td>
</tr>
<tr>
<td>jaspar-MA0060.1</td>
<td>5.09</td>
<td>NFYA, NFYB, NFYC, YBX1, POLE3, POLE4</td>
</tr>
<tr>
<td>transfac_public-M00287</td>
<td>4.91</td>
<td>NFYA, NFYB, NFYC, YBX1, POLE3, POLE4</td>
</tr>
<tr>
<td><strong>Downregulated genes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taipale-SGTTGCYARGCAACS-RFX4-DBD</td>
<td>5.77</td>
<td>RFX4, RFX3, <strong>RFX2</strong>#, RFX1</td>
</tr>
<tr>
<td>Transfac_pro-M00481</td>
<td>5.67</td>
<td>AR, PGR, NR3C1, NR3C2</td>
</tr>
<tr>
<td>Factorbook-NR3C1</td>
<td>5.39</td>
<td>NR3C1, AR, PGR, NR3C2, HSF1</td>
</tr>
<tr>
<td>Yetfasco-658</td>
<td>5.38</td>
<td>N/A</td>
</tr>
<tr>
<td>Transfac_pro-M02789</td>
<td>5.17</td>
<td>RFX4, RFX5, RFX7</td>
</tr>
<tr>
<td>Taipale-RRGWACANNNTGTW CYY-AR-DBD</td>
<td>5.15</td>
<td>AR, NR3C1, NR3C2, PGR</td>
</tr>
<tr>
<td>Transfac_pro-M02465</td>
<td>4.96</td>
<td>HMGB4, TOX, TOX2, TOX4, TOX3, TBP, TBPL2, TBPL1</td>
</tr>
<tr>
<td>Transfac_pro-M02790</td>
<td>4.82</td>
<td>RFX7, RFX5</td>
</tr>
<tr>
<td>Transfac_pro-M01201</td>
<td>4.77</td>
<td>AR, NR3C1, PGR, NR3C2</td>
</tr>
<tr>
<td>Swissregulon-AR.p2</td>
<td>4.75</td>
<td>AR, PGR, NR3C1, NR3C2, HSF1</td>
</tr>
</tbody>
</table>

^ Normalized enrichment score
* Transcription factors significantly upregulated following challenge
# Transcription factors significantly downregulated following challenge
CHAPTER 4

Variant analysis of RNA sequences in severe equine asthma

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**Corresponding author: dbienzle@uoguelph.ca

Tessier L, Côté O, Dorothee B. Variant analysis of RNA sequences in severe equine asthma. Under review, \textit{PeerJ} no. 22266
Abstract

**Background.** Severe asthma is a chronic inflammatory disease of the lung in horses similar to low-Th2 late-onset asthma in humans. The disease in horses has complex inheritance including both dominant and recessive patterns that remain ill defined. This study aimed to determine the utility of RNA-Seq to call gene variants and to identify mutations potentially linked to disease.

**Methods.** RNA-Seq data were generated from endobronchial biopsies collected from 6 asthmatic and 7 non-asthmatic horses before and after challenge (26 samples total). Sequences were aligned to the equine genome with Spliced Transcripts Alignment to Reference software. Read preparation for variant calling was performed with Picard tools and Genome Analysis Toolkit (GATK). Coverage was visualized using Integrative Genomic Viewer software and variants were called and filtered using GATK and Ensembl Variant Effect Predictor (VEP) tools. Novel variant selection by VEP was based on score of <0.01 predicted with Sorting Intolerant From Tolerant (SIFT) software, missense nature, location within the protein coding sequence and presence in all asthmatic but not all non-asthmatic individuals. For selected mutations, the effect of predicted variants on protein function was assessed with Polymorphism Phenotyping (PolyPhen) 2 and Screening for Non-Acceptable Polymorphism (SNAP) 2 softwares. RNA-Seq predicted variants were confirmed in all horses, and investigated in an additional 4 asthmatic and 7 non-asthmatic individuals with PCR and Sanger sequencing. Gene alignment and three-dimensional protein structures were predicted with Geneious software.

**Results.** Level of expression across the genome showed a similar pattern in all individuals. RNA-Seq variant calling and filtering identified with highest confidence mutations in *PACRG* and *RTTN*. Sanger sequencing confirmed that the *PACRG* variant was appropriately identified in all 26 samples while the *RTTN* variant was identified correctly by RNA-Seq in 24 of 26 samples. Both mutations were predicted to result in loss of function by SIFT and PolyPhen2, and to be non-neutral by SNAP2. Amino acid substitutions projected no change of hydrophobicity and isoelectric point in PACRG, a change of both properties for RTTN; and a slight change in three-dimensional structure for PACRG and RTTN. For PACRG, samples from additional individuals confirmed a trend for the heterozygous genotype in asthmatics, while the RTTN homozygous mutant phenotype was more prevalent in the asthmatic compared to non-asthmatic group.

**Discussion.** RNA-Seq was sensitive and specific for calling gene variants in this disease model. Precision and consistency of allele identification are generally dependent on coverage. However,
even moderate coverage (<10-20 cpm) yielded correct identification in 92% of samples, suggesting RNA-Seq may be suitable to detect variants in low coverage samples. The impact of amino acid alterations in PACRG and RTTN proteins are unknown at this point, but their role in the structure and function of cilia may warrant further investigation.

**Keywords**
Horse, asthma, RNA-Seq, variant, mutation, GATK

**Background**
Severe equine asthma (recurrent airway obstruction, heaves) is a chronic inflammatory lung disease caused by inhalation of environmental dust and microbial components [1]. Exacerbation of the disease triggers excessive mucus production, cough, neutrophilic airway inflammation, bronchial hyperreactivity, and bronchospasm. Recurrent exacerbations induce smooth muscle hyperplasia and hypertrophy, fibrosis and eventual irreversible airway remodeling [2-5].

Asthma in humans is recognized to be a heterogeneous disease that is classified based on genetic, molecular and clinical features [6,7]. Severe equine asthma is most similar to human severe, late-onset asthma characterized by absence of Th2 cytokines, and presence of neutrophilic inflammation and bronchial neutrophil chemokines [6,8]. Severely asthmatic horses do not have a hypersensitivity response [2] and efforts to associate equine asthma with a Th2 cytokine profile have yielded inconsistent or inconclusive results [9-17]. Mechanisms leading to the development of both severe equine asthma and late-onset low-Th2 severe asthma in humans remain largely undefined.

Interactions between genes and environmental factors have been recognized to contribute to development of equine asthma for many years [18]. Genetic factors likely reside in multiple genes, and may be influenced by age and sex [18-20]. Several susceptibility gene variants, haplotypes and regions have been associated with human asthma [21-34], but no specific markers have been identified in the late-onset low-Th2 sub-phenotype [6,8]. Similarly, genetic markers of equine severe asthma were identified in certain families, but were not significantly associated across different families and genetic backgrounds [20,35-38].

As landmarks in epithelial-environmental interaction, cilia are highly specialized cellular projections. Most vertebrate cells have a single non-motile (‘primary’) cilium that transduces
signals from the environment or other cells, while motile cilia occur in multiples on specialized cells of the respiratory tract, oviduct and central nervous system ventricles [39,40]. Motile cilia directionally propel cells or extracellular fluid through “metachronal wave” beating movements [41,42]. The ability of motile cilia to beat in a synchronized manner requires specialized proteins that are absent in non-motile primary cilia, but otherwise both types of cilia have similar internal architecture. The main part of the cilium is the axoneme, which is comprised of nine outer microtubule doublets, one central microtubule pair (in motile multiple cilia) and a multitude of affiliated proteins. Prominent among these are tektins that stabilize microtubules and regulate axoneme length [43], and protofilament ribbon proteins that are essential for sliding of adjacent microtubule doublets to generate ciliary movement [44]. Abnormalities in cilia are now appreciated as cause for the development of respiratory diseases, often through genetic variants associated with a loss of function affecting unique ciliary proteins [39]. Factors that affect beating, synchronization or orientation of motile cilia result in accumulation of mucus in airways [39]. Furthermore, hedgehog (HH) signaling is strongly linked to ciliary function, and many components of the HH signaling pathway localize to cilia [45,46].

Parkin co-regulated (PACRG) is a gene conserved across species [47], and shares a bi-directional promoter with parkin (PARK2) [48]. PACRG is affiliated with axonemal doublet microtubules, and contributes to the signaling pathway that controls dynein-driven microtubule sliding [47,49-51]. A single nucleotide variant (SNV) in PACRG was strongly associated with an increased risk of developing childhood asthma following early-life tobacco smoke exposure [52]. Rotatin (RTTN) is a cillum-associated protein [53] essential for assembly of centrosomes in non-motile and motile cilia [54]. Absence of RTTN, or presence of gene variants that disrupt the interaction of RTTN with SCL/TAL1 interrupting locus (STIL), abrogate proper ciliary development and function [54], and recessive mutations in RTTN are linked to abnormal primary ciliary development in humans [55]. Collectively, these findings incriminate that aspects of ciliary function may be impaired in asthma.

RNA-Seq is a promising approach for calling genetic variants concurrent with analysis of gene and allele-specific expression, alternative splicing, and pathways. In this study we investigated whether single nucleotide variants (SNVs) detected by RNA-Seq were also present in Sanger-sequenced amplicons, and whether specific variants were associated with the equine asthmatic phenotype.
Methods

Animals and procedures

Animal procedures, sample collection and sample processing were as previously described [56]. In brief, 13 horses (six asthmatic horses in remission and seven non-asthmatic horses) with mean ages of 15 and 12 years, respectively (p =0.352, unpaired t test) were transferred indoor in a dust-free environment. After 24 hours, asthmatic horses were exposed to dusty hay until exacerbation was apparent in asthmatic horses (range 1 to 3 days, average 2.2 days), while non-asthmatic horses were exposed for 3 days. Each horses underwent physical examination, pulmonary function test and bronchoalveolar lavage. Sequential endoscopic bronchial biopsy were additionally obtained before and after exposure to the asthmatic challenge from the contralateral lung lobe. Samples from an additional four asthmatics and seven non-asthmatic horses were used for PCR-amplification of specific variant regions and Sanger sequencing. All procedures were approved by the Institutional Animal Care Committee of the University of Guelph (protocol R10-031) and conducted in compliance with Canadian Council on Animal Care guidelines.

RNA-Seq sample preparation and sequence alignment

As previously described [56], total RNA was extracted from endobronchial biopsies (Qiagen, Toronto, ON). Quality and concentration were determined with the Bioanalyzer RNA Nanochip (Agilent, Mississauga, ON) and capillary electrophoresis. Unstranded library preparation and sequencing were performed at The Centre for Applied Genomics (TCAG; Toronto, ON) using Illumina TruSeq protocols (Illumina, San Diego, CA). Per sample, approximately 1 µg of non-degraded RNA was enriched for poly-A RNA, fragmented into 200 to 300 bases, and converted to double stranded cDNA libraries. RNA libraries were then quantified (KAPA Library Quantification kit, Kapa Biosystems, Wilmington, MA) prior to pooling and sequencing. Paired-end reads of 100-bases were sequenced in 5 lanes of an Illumina flow cell using a HiSeq 2500 instrument following the manufacturer’s instructions.

RNA-Seq sequence quality was assessed using the FastQC software version 0.10.1 [57] and aligned to the horse reference genome [58] (Ensembl v70) with STAR version 2.4 [59]. The STAR_pass2 alignment protocol was followed, which included: horse Ensembl version 70 GTF
annotation file for first- and second-pass, and the junction SJ.tab file generated by STAR for the second-pass after non-canonical junctions were removed. Default settings were used except for the following: --runThreadN 8 --outFilterScoreMinOverLread 0.5 --outFilterMatchNminOverLread 0.5.

**Variant calling and filtering**
Read processing, variant calling and initial filtering were performed following the Genome Analysis ToolKit (GATK) best practice guide for variant calling on RNA-Seq, except for the Indel realignment step considering the pass-2 STAR alignment initially performed. Initial read processing was first performed with Picard tools version 1.114 (http://broadinstitute.github.io/picard/) to add read groups and mark duplicates. Split n’ Trim as well as base recalibration were performed using the GATK software version 3.2.2 [60] and the 
-T SplitNCigarReads, -rf ReassignOneMappingQuality, -RMQF 255, -RMQT 60 and -U ALLOW_N_CIGAR_READS options.

Variants were subsequently called using the Haplotype Caller function in GATK with the same genome annotation file used in the read alignment phase and the following options: -recoverDanglingHeads, -dontUseSoftClippedBases, -stand_call_conf 20.0 and -stand_emit_conf 20.0 options. Resultant variants were processed with the variant filtration function of GATK software and the following options: -window 35, -cluster 3, -filterName FS, -filter "FS > 30.0", -filterName QD and -filter "QD < 2.0".

Variants were analyzed individually in each of 26 samples (6 asthmatics and 7 non-asthmatics, before and after asthmatic challenge). Variants of interest were first identified based on presence in pre- and/or post-challenge samples from asthmatic horses. The STATS function in the SeqMule software [61] was used to identify consensus variants within groups with the --c vcf options. Venn diagrams depicting variants shared between and within groups were constructed using the -p --venn options (Suppl. Figure 1). The Ensembl Variant Predictor (EVP) on-line tool [62] was used to further analyze and filter variants. In asthmatic horses, a total of 26,619 pre- and 24,527 post-challenge variants were identified, respectively, while the corresponding numbers were 28,909 and 28,451 for non-asthmatic horses. Approximately 30% of variants were novel and not previously described. The types of variants and their coding region effects are summarized in Suppl. Figures 2 and 3. For further variant selection using VEP
the inclusion criteria were 1) missense mutation in protein-coding sequence; and 2) predicted to cause loss of protein function. The variant effect on protein function was analyzed with the Sorting Intolerant From Tolerant (SIFT) [63-67] tool and the threshold score was set at <0.01. Low confidence loss of function predictions and existing variants were excluded. Ten variants present in all asthmatic horses (before or/and after challenge) but not all non-asthmatic horses were identified. Of these, only two variants were expressed in all samples and were more prevalent in asthmatic compared to non-asthmatics. Effects of these two variants were then investigated with Polymorphism Phenotyping (PolyPhen) 2 [68] and Screening for Non-Acceptable Polymorphism (SNAP) 2 softwares [69-71].

**Sequence alignment and protein secondary structure prediction**

DNA sequence alignment and prediction of secondary structure was performed with Geneious version 10.2.3. Default settings were used for both operations. Hydrophobicity and isoelectric point (pl) were included for each amino acid of the protein sequence.

**PCR**

PCR was performed on DNA extracted from peripheral blood or endobronchial biopsie collected from the 13 previously described horses (6 asthmatics and 7 non-asthmatics) and an additional 4 and 7 asthmatic and non-asthmatic horses respectively. Primers for amplification of variant regions from bronchial cDNA were *PACRG* forward (5’ -CTC TGA ACC TCC GAA ACC GAC-3’) and reverse (5’-CTC CTG GGA TAA CTC ACC ATT C-3’), and *RTTN* forward (5’-TCC TGA GTT GTA TCA AGA AGT G-3’) and reverse (5’-CCA GCC TGC AAT TCC TTT CT-3’). A Taq polymerase PCR kit (Invitrogen, Mississauga, ON) was used for PCR amplifications. Each reaction was performed in a 25 µL final volume, including 5 µL of 10X PCR buffer, 0.2 mM dNTPs, 2 mM MgSO₄, 0.3 µM of each primer, 2 U of Platinum Taq, and 5 µL (100 ng) of template DNA. PCR conditions for amplification were 3 min at 94 °C followed by 35 cycles of 94 °C for 45 s, 60 °C or 58 °C for 30 s for *PACRG* and *RTTN*, respectively, and 72 °C for 90 sec, followed by final elongation for 10 min at 72 °C. Twenty µL of each PCR product was separated by electrophoresis in a 1% agarose gel stained with SYBR Safe (Invitrogen). Amplicons of appropriate size were cut out and DNA extracted.
and purified (QIAquick, Qiagen). Extracted and purified PCR products were Sanger sequenced at Laboratory Services Division (Guelph, ON).

**Results**

**RNA-Seq coverage**

RNA-coverage was visualized with Integrative Genomics Viewer (IGV) software. With the IGV tool large-scale genomic data sets are visualized in real-time over a wide range of resolutions [72]. Coverage (Fig. 4.1) was similar across horses and conditions except for slightly higher coverage in some asthmatic horses following challenge.

**Variant calling and filtering**

The GATK variant calling and filtering workflow yielded 2823 and 1788 variants present specifically in the asthmatic group pre- and post-challenge, respectively (Suppl. Figure 4.1). Of these, 10 were missense mutations, coded for proteins and had SIFT scores <0.01. Variants in PACRG (Fig. 4.2A) and RTTN (Fig. 4.2B) had lowest prevalence in non-asthmatic horses. A missense G/A substitution was detected in the coding sequence of PACRG at position 265,643 (Ensembl sequence ENSECAG00000014308) /264,806 (NCBI accession number 100050378) (Fig. 4.2A). A missense T/A substitution was detected in the coding sequence of RTTN at position 27,190 (Ensembl sequence ENSECAG00000009711)/ 27,871 (NCBI accession number 100052029) (Fig. 4.2B).

**Protein alignment**

In PACRG, the G/A substitution resulted in replacement of valine (V) for methionine (M) at position 182 (Fig. 4.3A). PACRG sequence alignment of wild type (WT) and mutant proteins predicted changes from beta-strand to alpha-helix structure in the mutant protein a few amino acids distant from the site of substitution (182) at positions 187 and 188 (Fig. 4.3B). Hydrophobicity and isoelectric point were expected to remain similar despite the substitution (Geneious). In the RTTN sequence alignment, T/A substitution resulted in replacement of arginine (R) with tryptophan (W) at position 1807 of the ENSECAT00000010304 protein isoform (Ensembl sequence, corresponding to position 1812 of isoform X1 in NCBI [XP_001493238]) in NCBI sequence) (Fig. 4.4A). Sequence alignment of WT and mutant
proteins also indicated a change from alpha helix to beta strand structure near the site of substitution (bp 1807) at position 1816 (Fig. 4.4B). In addition, increased hydrophobicity and decreased pI were projected at the site of substitution (1807) in the mutant compared to the WT protein.

**Expression of PACRG and RTTN**

Expression of *PACRG* in counts-per-million (CPM) was overall higher than that of *RTTN* (Fig. 4.5). *PACRG* CPM ranged from ~30.7 to 66.3 in asthmatic horses (Fig. 4.5A) and ~25.1 to 65.6 in non-asthmatic horses (Fig. 4.5B), while expression of *RTTN* varied from ~11.5 to 24.9 and ~13.1 to 29.9 in asthmatic (Fig. 4.5C) and non-asthmatic (Fig. 4.5D) horses, respectively. Expression of *PACRG* overall was slightly higher in asthmatic than non-asthmatic horses.

**Predicted effect of variant on protein function**

The mutations detected in *PACRG* and *RTTN* and the associated amino acid substitution were predicted to cause loss of protein function when analyzed with SIFT and PolyPhen2 (Suppl. Fig. 4.4A and B). For *PACRG*, the mutation score was 0.993 with sensitivity of 0.70 and specificity of 0.97. For *RTTN*, the score was 0.979 with a sensitivity of 0.76 and a specificity of 0.96. Furthermore, for *PACRG*, V to M substitution at position 182 was predicted to affect protein function with a score of 64 at 80% expected accuracy (Table 1) with SNAP2. For *RTTN*, SNAP2 calculated that the R to W substitution at position 1807 would affect protein function with a score of 81 at 91% expected accuracy (Table 2).

**Sanger sequencing**

*PACRG* variants identified by RNA-Seq were confirmed with Sanger sequencing of PCR amplicons in asthmatic (Fig. 4.6A) and non-asthmatic (Fig. 4.6B) horses. In the asthmatic group, four horses were heterozygous [A/G] and two had homozygous-mutant alleles [A/A] (Fig. 4.6A). In the non-asthmatic group, one horse was heterozygous with alleles [A/G], three horses were homozygous-WT [G/G] and three horses were homozygous-mutant [A/A] (Fig. 4.6B). DNA was not available to confirm the genotype of horse 1. Hence, all genotypes derived from RNA-Seq were identical to Sanger sequencing results.
For RTTN, results of RNA-Seq and Sanger sequencing were very similar (Fig. 4.7). Four asthmatic horses were heterozygous [A/T] and two were homozygous-mutant [A/A] (Fig. 4.7A). In the non-asthmatic group, horse 5 was heterozygous [A/T] and four horses were identified as homozygous-WT [T/T]. However, horse 1 was identified as homozygous before and heterozygous after the asthmatic challenge, while horse 4 was identified as heterozygous before and homozygous after the asthmatic challenge. Based on Sanger sequencing the genotype of horse 4 was homozygous. DNA was not available to confirm the genotype of horse 1. In all asthmatic horses, genotypes were consistent between pre- and post-challenge and sequencing methods.

**Sequence alignment**

Sanger DNA sequences of PACRG from 10 asthmatic horses and 14 non-asthmatic horses (including those that were analyzed by RNA-Seq) were aligned. Among asthmatic horses, eight had the heterozygous [A/G], two had the homozygous mutant [A/A] and none had the homozygous-WT [G/G] genotype. Among non-asthmatic horses, six had the heterozygous [A/G], five had the homozygous mutant [A/A] and three had the homozygous-WT [G/G] genotype (Fig. 4.8A). RTTN alignment in asthmatic horses yielded five heterozygous [A/T], two homozygous mutant [A/A], and three homozygous-WT [T/T] genotypes. In non-asthmatic horses, four had the heterozygous [A/T], 10 had the homozygous-WT [T/T], and no horse had the homozygous mutant [A/A] genotype (Fig. 4.8B).

**Discussion**

The goal of this study was to assess the reliability of an adapted RNA-Seq variant calling workflow compared to Sanger sequencing, and to identify genetic variants of potential interest in asthmatic horses. Variant calling using RNA-Seq reads is a recent practice, and reliability of results is a function of sequencing platform, depth, quality, precision of read mapping and appropriate variant calling and filtering methods. The reliability of identifying genetic variants using RNA-Seq has been considered uncertain. In some reports RNA-Seq was considered useful for identifying genetic variants [73,74] while in others differences between RNA and DNA sequences were found with potentially frequent false results [75-77].
In this study we applied a modification of GATK best practices for variant calling with RNA-Seq, and verified the results with Sanger sequencing. In 24 of 26 samples variants in *PACRG* and *RTTN* were identified by both methods, while two horses’ genotypes were discordant by RNA-Seq with inconsistent genotypes before and after challenge. Sanger sequencing confirmed one of the discordant genotypes, while the other could not be further assessed.

We first visually assessed the overall genome coverage of RNA-Seq using IGV. Although this method has limited precision, there was a similar expression pattern across all horses, conditions and the entire genome, and expression appeared slightly higher in asthmatic horses after challenge. This was expected since differential gene expression analysis previously showed more prominent changes in asthmatic compared to non-asthmatic horses [56]. Two candidate missense variants in the *PACRG* and *RTTN* coding sequence were identified after variant filtering. SIFT was initially applied, followed by PolyPhen2 and SNAP2, to predict the variant effect on protein function. SIFT uses phylogenetic data [63-67], while PolyPhen2 uses structural information and multiple alignments [68] to predict whether or not a mutation may cause loss of function. The two methods often yield similar results, but limited specificity suggests that results should be interpreted with caution [78]. SNAP2, on the other hand, uses evolutionary, structural, solvent-access and annotation information, as well as data from available homologs to predict whether a mutation is likely to have an effect or not on protein function [69-71]. While these three approaches can yield different results [79,80], inferences regarding PACRG and RTTN amino acid substitutions were consistent.

Presence of the mutation was confirmed with PCR and Sanger sequencing in 24 samples. Correlation between RNA-Seq and Sanger sequencing showed that for *PACRG* both alleles of the gene were properly identified in all horses and conditions by the modified GATK workflow. For *RTTN*, two of the samples were misidentified by the workflow with alleles inconsistently identified before and after challenge. Lower expression and therefore lesser sequencing coverage, in particular in post-challenge samples, might have increased the likelihood of error in variant calling. Nonetheless, the vast majority of alleles were identified properly, suggesting that this workflow is suitable for variant calling in RNA-Seq at gene coverage in the 10 to 20 cpm range. Read counts were not filtered by common read counting algorithms, such as HTSeq, and may
have included ambiguous reads warranting filtering for other purposes such as gene expression analysis.

The substitutions identified changed V182M (valine to methionine) and R1807W (arginine to tryptophan) in PACRG and RTTN, respectively. For PACRG, the V->M substitution minimally affected hydrophobicity and pI, while the R->W substitution in RTTN increased hydrophobicity and decreased pI. The mutations were considered to potentially cause loss of function and to have non-neutral effects (Tables 1 and 2). The PACRG mutation could impair or modify ability of the protein to bind interacting partners or form homodimers. For the RTTN mutation, tryptophan is an aromatic, non-polar and hydrophobic amino acid often buried in hydrophobic cores, while arginine is a polar and positively charged amino acid often found on outside chains [81]. A change in the structural stability or binding affinity of the entire protein or the affected residue could impact ciliary structure and function. RTTN interacts with STIL and is essential for proper full-length centriole assembly [54]. The R1807W mutation in the carboxy-terminal region is not immediately at the suggested centrosome-targeting and STIL-binding site [54] but could nevertheless result in defective centrioles and hence cilium structure and function.

Variant sequence determination in 13 RNA-Seq and 11 additional samples showed that 80% of asthmatic animals were heterozygous [G/A] and 20% were homozygous mutant for PACRG, and that no individual had the homozygous-WT genotype (G/G). Conversely, in non-asthmatic animals more than half were homozygous, whether WT or mutant (5 [A/A] and 3 [G/G]). In both groups the frequency of the homozygous-WT genotype was lower than either heterozygous genotype.

For RTTN, 20% of asthmatic horses were homozygous mutant (A/A), 30% were homozygous WT (T/T) and 50% were heterozygous [T/A]. Among non-asthmatics, none was homozygous mutant (A/A), while 71% of horses were homozygous WT (T/T) and 29% were heterozygous. Therefore, the mutation was present in 70% of asthmatic horses and in only 30% of non-asthmatic horses (heterozygous or homozygous mutant). Considering samples from non-asthmatic outnumbered those from asthmatic horses, there appears to be a clear trend for presence of the mutation in the latter group.

The PACRG protein associates with protofilaments [82] of the ciliary axoneme [47,83,84], has a role in ciliary morphogenesis and function [50] and is directly involved in
ciliary motility through control of dynein-driven microtubule sliding [49]. PACRG also has a variety of interacting partners such as microtubules, α- and β-tubulin and meiosis/spermiogenesis associated 1 (MEIG1) protein, heat shock protein (HSP) 70 and HSP 90 [51,85,86]. Impaired function or interaction of PACRG with its partners could weaken or impair ciliary stability and motility. We also suggest the possibility of homodimer formation by PACRG, which could explain the prevalence of the heterozygous genotype observed here and in pulmonary tuberculosis in humans [87]. A genome-wide interaction study also identified a PACRG SNP to be linked to an increased risk of childhood-onset asthma development following early-life exposure to tobacco smoke [52]. SNPs in PACRG also contributed to susceptibility to tuberculosis [88].

PACRG may be linked to HH signaling in mice where patched1 (PTCH1) and PACRG-PARK2 loci are thought to interact and regulate ciliary function in ependymal cells [89]. Interestingly, PTCH1 is differentially expressed in asthmatic compared to non-asthmatic horses following challenge [56], linking PACRG and an asthmatic response to environmental agents with the HH pathway.

The exact nature and function of methionine in protein structure remains incompletely understood, and substitutions involving methionine has been associated with several diseases [90]. Both valine and methionine are hydrophobic residues grouped among the least polar amino acids [91]. Methionine is a sulfur-containing amino acid that is among the most hydrophobic residues and also easily oxidized if exposed [92]. Although V->M substitutions are generally neutral, methionine’s sulfur connected to a methyl group would make it less likely to interact with other proteins [93]. Methionine was overrepresented as a mutant residue in several mutations associated with decrease or loss of function [94], including the human androgen receptor [95]. Although the effect of a V->M substitution is unknown, any change in PACRG structure or binding affinity could impact ciliary function, and may be of great interest in the context of severe asthma.

For RTTN, the R->W substitution altered the hydrophobicity and isoelectric point at position 1807. RTTN is a centrosome-associated protein first discovered for its role in axial rotation and left-right specification in the mouse embryo [53]. R <=> W substitutions were predicted to be the most problematic in the human genome [96], and R->W substitution is generally disfavored in all proteins types [93]. Overrepresentation of mutated arginine was a
prominent feature among disease-causing mutations in a range of diseases [94]. Equine RTTN appears to be highly conserved across individual horses in exons but not introns (unpublished results), which suggests strong evolutionary pressure on the coding sequence and renders mutations in the coding sequence as particularly unusual.

Conclusion
Variants can be confidently called with RNA-Seq results as low as 10-20 CPM. Single point mutations in PACRG and RTTN were more prevalent in asthmatic compared to non-asthmatic horses. The heterozygous mutant genotype of PACRG was more prevalent among asthmatics while the homozygous-WT genotype of RTTN was more prevalent in non-asthmatics. Functional cilia are crucial for lung health, and mutations resulting in impaired function will have a negative impact. The significance of the substitutions in PACRG and RTTN remain to be determined but they are highly suggestive to affect ciliary function. Analysis of variant frequency at a population level and WT and mutant protein-protein interactions, and determining protein crystal structure, may be useful future investigations.

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**Figure 4.1. RNA-Seq coverage visualized with IGV software and illustrated by a heat map graph.**

RNA-Seq coverage visualized with IGV software and illustrated by a heat map graph. White indicates <10 counts, and gradually darkening blue indicates raw counts from 10 (light blue) to a maximum of 5000 (dark blue). Sample origin from asthmatic and non-asthmatic horses pre- and post-challenge is indicated on the y-axis. Chromosomal location (1-31, X) is indicated on the top, and annotations from Refseq (Refseq genes) and Ensembl (EqCab2.70.gtf) on the bottom. Coverage is similar across different samples, with slightly higher coverage in some asthmatic horses post-challenge.
Figure 4.2. Variants in **PACRG** and **RTTN** genes.

Variants in **PACRG** (A) and **RTTN** (B) genes. Diagrams show position of gene (thick green line), mRNA (red line) and coding region (yellow line). Whole gene (upper) and close-up view surrounding the variant (lower) are included with gene sequence at the bottom. Location of the variant is indicated in blue for NCBI and Ensembl databases.
Figure 4.3. Alignment of wild-type (WT) and mutant (mut) PACRG proteins with associated predicted hydrophobicity and isoelectric point (pI).

Alignment of wild type (WT) and mutant (mut) PACRG proteins with associated predicted hydrophobicity and isoelectric point. Replacement of methionine for valine at position 182 changes a beta strand to an alpha helix at position 187 and 188. Alpha helices (pink), coils (gray line), turns (blue arrows) and beta strands (yellow arrows).
Figure 4.4. Alignment of wild-type (WT) and mutant (mut) RTTN protein with associated predicted hydrophobicity and isoelectric point (pI).

Alignment of WT and mutant RTTN protein with predicted hydrophobicity and isoelectric point. Replacement of arginine with tryptophan at position 1812 changes alpha helix to beta strand at position 1816. Alpha helices (pink), coils (gray line), turns (blue arrows) and beta strands (yellow arrows).
Figure 4.5. Expression of *PACRG* and *RTTN*.

Expression of PACRG (A, B) and RTTN (C, D) in asthmatic and non-asthmatic horses in counts-per-million (CPM; y-axis) pre- and post-challenge. PACRG expression varied from ~30.7 to 66.3 CPM in asthmatic horses (A) and ~25.1 to 65.6 CPM on non-asthmatic horses (B), while RTTN expression varied from ~11.5 to 24.9 CPM and ~13.1 to 29.9 CPM in asthmatic (C) and non-asthmatic (D) horses, respectively.
Figure 4.6. GATK variant calls and Sanger sequencing results for *PACRG*.

Comparison of GATK variant calls and Sanger sequencing results for *PACRG* in asthmatic (A) and non-asthmatic (B) horses. For both groups, the bar graph indicates the IGV count for each horse with GATK and Sanger variant calls, and the agreement between the two methods is indicated.
allele (A-red, G-yellow), horse and condition. Below the bar graph is the GATK variant call, the electropherogram of the Sanger sequence, and agreement. (A) Four asthmatic horses (1, 2, 5, 6) had heterozygous alleles [A/G] and two (3 and 4) were homozygous for the mutant allele [A/A]. (B) In non-asthmatic horses, one horse (6) had heterozygous alleles [A/G], three horses (1, 2 and 4) were homozygous for the wild-type allele [G/G] and three horses (3, 5 and 7) were homozygous for the mutant allele [A/A]. All genotypes were consistent across horses and methods. DNA was not available for non-asthmatic horse 1.
Figure 4.7. GATK variant calls and Sanger sequencing results for RTTN.

Comparison of GATK variant calls and Sanger sequencing results for RTTN in asthmatic (A) and non-asthmatic (B) horses. Details as in Fig. 4.6. (A) Four asthmatic horses (3-6) had heterozygous alleles [A/T] and two (1 and 2) were homozygous for the mutant allele [A/A].
Genotypes were consistent across horses and methods. (B) In non-asthmatic horses, one (5) had heterozygous [A/T] alleles, four horses had homozygous wild type [T/T] alleles, and two horses (1 and 4) were inconsistently identified as homozygous wild type and heterozygous in different samples. Sanger sequencing confirmed the genotype of horse 4 as heterozygous. DNA was not available for non-asthmatic horse 1.
Figure 4.8. **PACRG and RTTN sequence alignment.**

Alignment of PACRG (A) and RTTN (B) Sanger sequences for 10 asthmatic and 14 non-asthmatic horses with the reference genome. (A) For PACRG, 8 asthmatic horses (80%) were heterozygous [A/G], 2 (20%) were homozygous mutant [A/A] and none was homozygous wild-type [G/G]. Six non-asthmatic horses (43%) were heterozygous [A/G], 5 (36%) were homozygous mutant [A/A] and 3 (21%) were homozygous wild type [G/G]. (B) For RTTN, in the asthmatic group, there were 5 (50%) heterozygous [A/T], 2 (20%) homozygous mutant [A/A] and 3 (30%) homozygous wild type [T/T] genotypes. In the non-asthmatic group, 4 (29%) were heterozygous [A/G], 10 (71%) were homozygous wild type [T/T], and none had the homozygous mutant [A/A] genotype.
Supplementary figure 4.1. Variants detected in asthmatic and non-asthmatic horses with Genome Analysis Toolkit.

Variants detected in asthmatic and non-asthmatic horses with Genome Analysis Toolkit (GATK). The Venn diagram was generated with SeqMule to identify variants present in all asthmatics before (red) and after challenge (green), and in non-asthmatics before (turquoise) and after challenge (purple).
Supplementary figure 4.2. Predicted effect of variants detected in asthmatics.

Predicted effect of variants in all (left) and coding (right) regions for asthmatic horses before (upper) and after (lower) challenge. Analysis was done using Ensembl Variant Effect Predictor (VEP) tools.
Supplementary figure 4.3. Predicted effect of variants detected in non-asthmatics.

Predicted effect of variants from all (left) and coding (right) regions for asthmatic horses before (upper) and after (lower) challenge. Analysis was done using Ensembl Variant Effect Predictor (VEP) tools.
Supplementary figure 4.4. Polyphen2 results for PACRG and RTTN.
Results of analysis of PACRG (A) and RTTN (B) mutations PolyPhen2 software. Mutations were predicted as probably damaging in PACRG and RTTN proteins with confidence scores of 0.993 and 0.979, respectively.
Table 4.1. SNAP2 predicted effect of amino acid substitution at position 182 for PACRG.

<table>
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<th>Outcome</th>
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Table 4.2. SNAP2 predicted effect of amino acid substitution at position 1807 for RTTN.

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CHAPTER 5

Morphological abnormalities in the bronchial and bronchiolar epithelium of asthmatics

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Short running head: Ciliary and microvillar ultrastructure in asthma

At a Glance Commentary: This study shows that asthmatic horses have morphologically abnormal cilia and microvilli that are likely caused by chronic inflammation and contribute to mucus accumulation and the clinical features of asthma.

We thank Cameron Ackerley, Department of Physiology and Experimental Medicine, Peter Gilgan Centre for Research and Learning, Hospital for Sick Children, 686 Bay St, Toronto, Ontario M5G 0A4, Canada, for provision of electron microscopic images.
Abstract
Severe asthma is a chronic inflammatory disease of the lung in horses similar to non-eosinophilic late-onset asthma in humans. Clinical signs of severe equine asthma include mucus hypersecretion, cough, bronchial hyperreactivity, bronchospasm and neutrophilic airway inflammation. Alterations in composition and maturity of bronchial epithelial cells have been observed in asthmatic horses, but ciliary abnormalities have only been described in human asthmatics. The objective of this study was to assess epithelial cell and ciliary morphology of asthmatic and non-asthmatic horses. Tissues were collected from the bronchi and bronchioles of asthmatic and non-asthmatic horses, fixed and assessed by scanning and transmission electron microscopy. Abnormal ciliary and microvillar morphology was observed in asthmatic but not in non-asthmatic horses, and findings were concordant between scanning and transmission electron microscopy. Cilia had different length, shape and orientation and included compound cilia. Some cilia had marked asymmetric cytoplasmic expansions at the tip or core. Branching or deformed microvilli were also present in all samples. Abnormal cilia and microvilli likely result from pre-existing chronic inflammation in the bronchi and bronchioles of asthmatics. We however suspect these abnormalities to impair proper cilia beating and impact microvilli signaling, therefore contributing to inflammation chronicity and asthma clinical signs worsening.

Keywords
Asthma, bronchus, electron microscopy, cilia, microvilli, horse

Background
Severe equine asthma (recurrent airway obstruction or “heaves”) is a naturally occurring chronic lung disease affecting horses chronically exposed to environmental dust and microbial components [1]. Clinical signs of the disease during exacerbation include mucus hypersecretion, cough, bronchial hyperreactivity, bronchospasm and neutrophilic airway inflammation. Equine asthma is a multifactorial disease influenced by multiple genes and factors such as sex, age and exposure to noxious environments [2-4]. Severe equine asthma is most similar to non-eosinophilic late-onset asthma in humans [5,6]. The specific mechanisms leading to the development and chronicity of the disease remain elusive, recent evidence suggests cell cycle dysregulation and involvement of hedgehog (HH) signaling [7,8].
Abnormal ciliary function has been identified in humans with severe asthma [9]. Mucociliary clearance is an innate immune defense mechanism crucial for lung health [10,11] and includes three components: the motile cilia, the periciliary liquid (PCL), and the mucus itself. The role of motile cilia is to move the mucus layer and entrap airborne particles in a cranial direction into the oropharynx through a “metachronal wave” motion [12-14]. The desynchronized wave motion creates flow allowing efficient movement of the mucus layer. Structural or functional abnormalities of motile cilia can result in reduced mucus clearance and lead to lung disease [15]. The PCL is located between the mucus layer and the epithelial cells proper, and provides a low-viscosity environment for unrestricted ciliary beating and a lubricated surface for the movement of mucus [16,17]. Viscosity, pH and height of the PCL layer depend on its ionic composition [17], and therefore proper regulation of ion transport through the apical epithelial membrane is an essential factor for ciliary function. Changes in voltage-gated and store-operated ion channels were identified in experimental models of ovalbumin-induced asthma and were associated with altered ciliary beat frequency [18]. Oxidative injury and inflammation, such as in asthma and other lung conditions, can affect cellular calcium (Ca\(^{2+}\)) homeostasis, which in turn can alter epithelial chloride and potassium channel activity and the ionic composition of the PCL [19]. Whether the ionic composition of the PCL in horses with asthma is abnormal or not is unknown, but multiple ion exchange transporters were differentially expressed in the bronchial epithelium of affected relative to control horses. These included upregulated solute carriers *SLC46A2, SLC7A11, SLC7A5,* and *SLC4A11,* and downregulated *SLC4A5, SLC16A10, SLC2A4RG* [8]. While the specific function and localization of these ion channels is unknown, there is strong evidence of differential regulation of epithelial ion transport in asthma.

The composition of the bronchial microenvironment can be influenced by signaling from motile cilia [20], primary cilia [14] and microvilli [21]. Crucial for lung homeostasis, the primary cilium is present on most terminally differentiated cells and is involved in cell signaling and environmental sensing [14]. Primary cilia also regulate smooth muscle cell (SMC) remodelling through calcium and hedgehog (HH) signaling [22]. Microvilli are also numerous on the surface of ciliated and other differentiated cells [23] and are involved in multiple cell functions such as ion transport through a variety of ion channels including those for Na\(^{+}\), K\(^{+}\), Cl\(^{-}\), Ca\(^{2+}\) and other large anions [21]. Current knowledge suggests that the main core of each microvillus is
composed of F-actin filament bundles, an important calcium reservoir, linked by fimrin, villin and epsin and attached to the plasma membrane by myosin and calmodulin [24,25]. However, there are tissue-specific microvillar characteristics, and airway microvilli have not been thoroughly described. Microvilli have not been directly linked to asthma, but their important role as calcium reservoir suggests they may contribute to impaired calcium regulation in the lung of asthmatics [26].

The aim of this study was to visualize the bronchial and bronchiolar epithelium of asthmatic and non-asthmatic horses to determine whether ultrastructural abnormalities are a feature of severe equine asthma. Transmission and scanning electron microscopy was performed on tissue from the bronchi and bronchioles of asthmatic and non-asthmatic horses. We found major morphologic changes in cilia and microvilli in horses chronically affected by asthma compared to those without asthma. The degree of morphologic change is more profound that identified previously in either experimentally-induced or naturally-occurring asthma.

Methods

Animal procedures and sample collection

Three asthmatic horses in remission were exposed to dusty hay until respiratory impairment was apparent [8]. Physical examinations were performed daily, and horses were euthanized once clinical signs of exacerbated asthma were apparent. Two horses free of respiratory disease and euthanized due to untreatable orthopedic conditions served as controls. Samples of the respiratory tract from trachea to bronchioles were obtained post mortem. All procedures were conducted in accordance with protocols approved by the Institutional Animal Care Committee of the University of Guelph (R10-031) and conducted in compliance with Canadian Council on Animal Care guidelines.

Tissue preparation

Tissues were collected and prepared for transmission (TEM) and scanning electron microscopy (SEM). Tissues were fixed in 4% paraformaldehyde and 1% glutaraldehyde, post-fixed in 1% OsO₄ and dehydrated in an ascending series of acetone. For SEM, samples were critical point dried and mounted on aluminum stubs with carbon dag and rendered conductive with a thin film of sputtered gold palladium. Photographs were taken in a scanning electron microscope (JEOL
JSM 820, Peabody, MA). For TEM, samples were infiltrated with an Araldite-Epon mixture and embedded in BEEM capsules prior to polymerization at 60 °C overnight. Areas of interest were selected from 0.5-mm sections stained with toluidine blue and cut using an ultramicrotome. Blocks were trimmed to the area of interest. Ultrathin sections were then prepared and mounted on grids, and stained with uranyl acetate and lead citrate prior to examination in the transmission electron microscope (JEM 1230, JEOL, Peabody, MA). Images were acquired with a charge-coupled device (CCD) camera (AMT, Danvers, MA).

Results

Ciliary structure
Cilia were abundant and readily recognized. On cross-section, the structure of the inner and outer ciliary doublet was normal in asthmatic (Figure 5.1) and non-asthmatic horses (data not shown). Bronchi/bronchioles of asthmatic horses had denser clusters of heterogeneous cilia viewed with TEM and SEM (Figure 5.2A) than those of non-asthmatic horses (Figure 5.2B). On highly magnified SEM images marked heterogeneity in length and shape of cilia and a relatively greater proportion of surface area covered by microvilli was apparent in asthmatics (Figure 5.3A) compared to non-asthmatics (Figure 5.3B).

Groups of entangled cilia of unusual shapes, lengths and orientation were present only in asthmatics (Figure 5.4). In these cases cilia with distal loops, abnormal curves, lateral or angular rather than perpendicular protrusion (Figure 5.5A), corkscrew shapes (Figure 5.5B) and narrow bent tips (Figure 5.6) were observed.

Compound cilia were only present in asthmatics, and identified by TEM (Figure 5.7A) and SEM (Figure 5.7B). Additional ciliary abnormalities consisted of cilia growing within the epithelium or as epithelial outgrowths (Figure 5.8). In some cilia abnormally narrow bases in the region of the transition zone were apparent (Figure 5.9).

Finally, cilia with distended club-like tips were observed in the bronchi/bronchioles of asthmatic but not in non-asthmatic horses (Figure 10A). This highly abnormal appearance on SEM was also present on TEM, which, in addition to tip protrusions, also revealed accumulation of particles in the tip cytoplasm (Figure 10B).
Microvillar abnormalities

Asthmatic horses had numerous abnormal microvilli. The initial observations on TEM were of large epithelial outgrowths with branching microvilli at the tip (Figure 5.11A, in concordance with findings on SEM (Figure 5.11B). However, at the base of the outgrowths F-actin filament bundles were noted (Figure 5.11A), which suggests the entire structures are abnormal microvilli.

Discussion

Analysis of genes and pathways differentially expressed in the epithelium of asthmatic and non-asthmatic horses yielded many indicators of ciliary dysfunction [7,8]. Furthermore, in-depth assessment of gene variants that alter amino acid composition and predicted protein function found mutations in parkin co-regulated (PACRG) and rotatin (RTTN) [27], both of which are proteins essential for ciliary function. Hence, the goal of this study was to examine whether the ultrastructural appearance of cilia is different in asthmatic than non-asthmatic horses. While the findings have to be considered preliminary since tissues from only a small number of individuals were assessed, and findings remain to be confirmed with ancillary assays, we nevertheless observed pronounced and diverse ciliary changes only present in asthmatic horses. We also report for the first time the presence of abnormal microvilli in asthmatics, including branching and abnormally long and swollen microvilli. Such changes have not yet been reported in lung diseases, but they are a feature of other chronic inflammatory diseases such as those affecting the intestinal epithelium [28].

Morphologically abnormal cilia have previously been reported human severe asthma [9], and included ciliary disorientation and a reduced number of cilia per cell. In addition there were changes in location of ciliated epithelial cells, presence of cytoplasmic blebs and mitochondrial injury [8]. However, the extent of morphological changes observed in cilia of asthmatic horses is unprecedented. We first observed that the inner and outer doublet structure of cilia in asthmatic horses was normal, which suggested that abnormal morphologic features are not the result of a genetically inherited ciliopathy but is rather a consequence of inflammation. Asthmatic horses had denser clusters of cilia with heterogeneous lengths and numerous shape changes compared to non-asthmatic horses. Asthmatic horses also had a larger surface area occupied by microvilli surrounding cilia compared to non-asthmatics. These findings strongly suggest impaired maintenance of epithelial integrity and regeneration.
A large number of cilia had abnormal morphology consisting of variable length, shape and orientation, deformations such as abnormal curvatures and distal protrusions or narrowing. Impaired orientation and position of the basal body will result in abnormal ciliary orientation and hence abnormal beating and mucociliary clearance [29].

Compound cilia and cilia extending on the surface of or within apparent epithelial outgrowths were common. Compound cilia are thought to stem from cytoplasmic membrane fusion of multiple cilia, and have been observed in humans following exposure to cigarette smoke [30]. These findings further suggest that the ciliary abnormalities in asthmatic horses result from chronic exposure to inhaled dust and microbes, and the ensuing inflammation, rather than a genetic defect.

Interestingly, we observed number of cilia with abnormally narrow transition zones. The transition zone is a crucial component of the cilium thought to play a central role in regulating intra-ciliary composition [14,15]. Cilia with bulging, club-shaped tips were also observed in asthmatic horses. This abnormality is likely the result of impaired intraflagellar transport (IFT) and inappropriate accumulation of proteins at the tip of the cilium. Anterograde and retrograde IFT occur simultaneously, but can be dissociated in vitro [29]. We suggest that these ciliary tip distensions result from uncoupling of anterograde and retrograde IFT leading to hyperactive anterograde IFT or impaired or inefficient retrograde IFT. Considering that ciliary tip bulges were noted in the single-celled algae *Chlamydomonas reinhardtii* with mutations in the genes coding for IFT complex A [31], suggests that impaired or inefficient retrograde IFT is the most likely cause. Another hypothesis may be impaired function of BBS proteins since BBS1-knockout mice may have swollen ciliary tips [32].

Lastly, we observed a large number of what appeared to be branching microvilli of heterogeneous size and length. Ultrastructurally, presence of F-actin bundles at the base of the branching structures indicates their microvillar rather than ciliary identity. Furthermore, there was no evidence of internal microdoublet structures, which would have indicated ciliary differentiation. Disassembly or loss of axonemal microtubules is unlikely considering that these are highly stable organelles [29], therefore the branching structures are unlikely cilia lacking microtubules. Branching microvilli have been observed in nasal polyps in humans [33] and malignant mesothelioma [34], and invasive forms of the latter included cells with abnormally long and slender microvilli. In addition, microvilli on hepatocytes exhibited change in shape
following activation of calcium signaling or insulin-induced activation [35,36]. Similar changes have not yet been reported in the lung. We suggest that these unusual structures may be activated microvilli, although their specific composition and role in disease remains to be determined.

Conclusions
We have identified multiple and striking ultrastructural abnormalities of cilia and microvilli in asthmatic but not non-asthmatic horses. Correlation of these abnormalities with severity of clinical illness and irreversibility remains to be determined, but the changes are likely pivotal in the pathogenesis of severe asthma. We suggest that abnormal cilia and microvilli result from chronic inflammation and dramatically impair mucociliary clearance. Further characterization of microvillar and ciliary internal structure by immunochemical detection of F-actin and tubulin, respectively, and in situ analysis of ion channel expression, are warranted.

References


27. Tessier L, Côté O, Dorothee B. Variant analysis of RNA sequences in severe equine asthma. Under review, PeerJ.


Figure 5.1. Cilia in the airway epithelium have normal doublet structure in asthmatic horses.
Figure 5.2. Ultrastructure of the airway epithelium in asthmatic and non-asthmatic horses.
A. Bronchi/bronchioles in asthmatic (A) and non-asthmatic horses (B) viewed in transmission electron microscopy (upper) and scanning electron microscopy (lower). In asthmatic horses (A), patches of cilia and microvilli appear to be more dense (greater number of cilia per cells) and occupy a greater surface. In non-asthmatic horses (B), cilia are more scattered.
Figure 5.3. Ultrastructure of the airway epithelium in asthmatic and non-asthmatic horses. Abnormal microvillar and ciliary morphology in the bronchial/bronchiolar epithelium of asthmatic (A) compared to non-asthmatic horses (B) on scanning electron microscopy (SEM). In asthmatic horses (A), microvilli tend to occupy a larger proportion of the surface around patches of cilia, and cilia are of variable lengths. In non-asthmatic horses (B), patches of cilia are more homogeneous in length.
Figure 5.4. Electron microscopic abnormalities of cilia in the bronchial and bronchiolar epithelium of asthmatic horses.

Cilia in asthmatic horses have irregular shapes, uneven lengths and club-like distal deformities, and the surface membrane of cilia is not smooth. (A) and (B) are images from two separate areas, and arrows and arrowheads in the images on the left indicate the site of higher magnification on the right.
Figure 5.5. Electron microscopic abnormalities of cilia in the bronchial and bronchiolar epithelium of asthmatics.

Cilia with abnormal shape are frequently non-erect and at times form loops (A, arrowhead) or abnormal curves (A, arrow). Cilia with extensive curvatures and corkscrew twists were also observed (B).
Figure 5.6. Electron microscopic abnormalities of cilia in the bronchial and bronchiolar epithelium of asthmatics.

Cilia have narrowed and bent tips or abnormal distal protrusions. Transmission electron microscopy (left) and scanning electron microscopy (right).
Figure 5.7. Electron microscopic abnormalities of cilia in the bronchial and bronchiolar epithelium of asthmatics.

Multiple cilia cores were found within single plasma membrane forming compound cilia (A) seen as excessively large cilia (B). Transmission electron microscopy (A) and scanning electron microscopy (B).
Figure 5.8. Electron microscopic abnormalities of cilia in the bronchial and bronchiolar epithelium of asthmatics.

Cilia are growing within the epithelium or as epithelial outgrowths. Transmission electron microscopy (A) and scanning electron microscopy (B).
Figure 5.9. Electron microscopic abnormalities of transition zone in cilia of the bronchial and bronchiolar epithelium of asthmatics.

Cilia with narrow base were prevalent. Original picture can be seen on the left and zoomed-in designated area on the right. Arrows in the images on the left indicate the site of higher magnification on the right. Transmission electron microscopy (A) and scanning electron microscopy (B).
Figure 5.10. Electron microscopic abnormalities of cilia in the bronchial and bronchiolar epithelium of asthmatics.

Patches of cilia with swollen tip were viewed by scanning electron microscopy (A). Cilia with slightly swollen tip had cytoplasmic accumulation of proteins, as view with transmission electron microscopy (B).
Figure 5.11. Electron microscopic abnormalities of microvilli in the bronchial and bronchiolar epithelium of asthmatics.
Microvilli had an apparent F-actin bundle inner core and formed branching structures. Arrows in the images on the left indicate the site of higher magnification on the right. Transmission electron microscopy (A) and scanning electron microscopy (B).
GENERAL DISCUSSION

Severe equine asthma (recurrent airway obstruction, heaves) is a lung inflammatory disease, affecting horses chronically exposed to airborne microbial components [1]. Sensitized horses display progressive airway obstruction caused accumulation of mucus and neutrophils in the airway lumen, bronchospasm and airway hyperreactivity. Asthmatic horses also have chronic inflammation characterized by smooth muscle and mucous cell hyperplasia, mucous cell metaplasia, and fibrosis [2-5]. Interactions between environmental and genetic factors contribute to the development of asthma in horses [6]. Evidence suggests that asthma has a complex mode of inheritance [7], however no consistent genetic markers have been identified in genetically heterogeneous horses. Specific mechanisms leading to the development of the disease remain elusive.

Our studies aimed to identify pathways, networks and genetic markers involved in severe equine asthma, and to define phenotypic consequences associated with these mechanisms. We assessed the differential genome-wide gene expression response of the bronchial epithelium in asthmatic and non-asthmatic horses to an asthmatic challenge. Our results suggest that following challenge, asthmatic horses have significant upregulation of genes involved in neutrophil migration and chemotaxis, immune and inflammatory response, coagulation, apoptosis, and secretion, and downregulation of genes involved in rhythmic processes. MMP1, MMP8, MMP9 and IL8 centrally linked different proteins of the network, suggesting a pleiotropic effect in the pathogenesis. Upregulation of neutrophil-, inflammation- and immune response-linked genes is consistent with the changes observed clinically and endoscopically during exacerbation, a feature of severe equine asthma. Increased coagulation and systemic inflammation were reported in horses with asthma [8], along with dysregulated apoptosis in bronchoalveolar lavage (BAL) fluid but not peripheral blood leukocytes [9,10]. Five genes involved in circadian rhythm regulation were differentially expressed in asthmatic horses, a finding reported first here. In mice, club cells contribute to regulating circadian rhythm in the lung, and imbalance in circadian rhythm may lead to impaired neutrophil regulation [11]. Secretoglobin 1A1 (SCGB1A1) is significantly downregulated in asthmatic compared to non-asthmatic horses [12], suggesting a decrease in the number of club cells. Our results are consistent with an aberrant pulmonary circadian rhythm that may be caused by impaired
epithelial precursor recruitment and differentiation, and contributes to chronic inflammation in asthmatic horses.

Further analysis of gene expression in asthmatic and non-asthmatic horses separately identified 2341 and 120 differentially expressed genes in asthmatic and non-asthmatic horses following the asthmatic challenge. In asthmatic horses, upregulated gene sets were involved in cell cycle, neutrophil migration and chemotaxis, wound healing, hemostasis, coagulation, regulation of body fluid levels and the hedgehog pathway. A large number of cell cycle-related genes were significantly enriched in asthmatics. Additionally, also among significantly upregulated genes were many that have binding sites for the E2F family of transcription factors that are central cell cycle regulators [13]. In asthmatic horses, three members of the E2F family (E2F2, E2F3 and E2F8) were significantly upregulated. A dysregulated cell cycle was also reported in PBMC of asthmatic horses [14]. AP-1 and NF-κB, from bronchial brush and BAL leukocytes respectively, were associated with disease activity [15-17] and serum response factor (SRF) and myocardin (MYOCD) were increased in airway smooth muscle (ASM) cells of peripheral airways in asthmatic horses [18].

Our data also suggests a direct link between E2F transcription and hedgehog (HH) signaling. FOXM1 was significantly upregulated in asthmatic horses [19] and is known to regulate CDC25A transcription by binding directly to the gene promoter, or through E2F transcription [20]. HH signaling was perpetuated through aberrant FOXM1 expression and linked to cell growth and proliferation in intestinal epithelial cells [21], and significant enrichment of HH-linked gene sets may indicate a link to cell cycle dysregulation. Furthermore, altered HH signaling may be implicated in nefarious ciliary signaling in equine asthma. PTCH1, a HH receptor expressed on primary cilia [22], was differentially expressed between asthmatic and non-asthmatic horses after challenge [23] suggesting differential regulation of this pathway. Primary cilia play a crucial role in HH signaling [24]. We put forth the possibility that impaired homeostasis in the bronchial epithelium may be perpetuated by dysregulated cell cycle and progenitor cell differentiation, and driven by E2F and CDC25A transcription through FOXM1 action, eventually leading to impaired HH signaling. The origin of this signaling impairment is unknown, but gene variants causing loss of function affecting cilia is a possible cause.

We further aimed to better understand the cause of gene expression differences between asthmatic and non-asthmatic horses. Analysis of genetic variations with RNA-Seq was
performed to identify factors that predispose or promote the development of severe asthma in horses. Prior to our study, no consistent genetic markers had been identified in genetically heterogeneous horses. Analysis of novel gene mutations in coding regions, prevalent in asthmatic horses and predicted to cause loss of protein function, yielded single mutations in *PACRG* and *RTTN*. For *PACRG*, a G/A mutation resulting in a V->M substitution in the protein in at least one allele was observed in all asthmatics compared to 79% of non-asthmatics; and 80% of asthmatics were heterozygous compared to 43% of non-asthmatics. For *RTTN*, an A/T mutation associated with a R->W substitution in the protein in at least one allele was observed in 70% of asthmatics compared to 29% of non-asthmatics. *RTTN* is a centrosome-associated protein [25] while *PACRG* is involved in ciliary morphogenesis, function and beating [26,27]. Although the effect of the mutation on protein function remains to be fully characterized, higher prevalence in asthmatics and a crucial role of both proteins in ciliary integrity warrant further investigations. These findings are also consistent with prior evidence of dysregulated HH signaling. Mutations in cilia-linked proteins have not previously been reported in horses but are present in a variety of pulmonary diseases in humans [28].

In our final study, we investigated whether abnormal morphology in ciliated or other epithelial cells was observed, consistent with gene expression and gene variants detected in asthmatics. TEM and SEM images showed major morphological abnormalities in cilia and microvilli. Crooked, swollen and compound forms of cilia were observed along with entangled groups of cilia. Abnormal cilia have been reported in human asthmatics [29], but the degree of abnormality observed here is unprecedented in equine or human asthma. Normal inner and outer doublet structure suggests that the abnormalities stem from exposure to an inflammatory environment. Furthermore, these cilia are unlikely to be able to beat properly, and thereby would preclude appropriate mucociliary clearance and perpetuate chronic inflammation. Both primary and motile cilia are chemosensory and contribute to calcium signaling [22,30,31]. Together with impaired HH signaling, these abnormalities may also affect ionic imbalance in the airway epithelium.

Microvilli of abnormal length and shape, as well as branching microvilli, were also observed in asthmatics. Microvilli are an important calcium reservoir [22,32] and involved in ion balance in the airway. Calcium signaling is impaired in human asthmatics [33], and although the cause is unknown, we suggest that abnormal microvillar and ciliary signaling may be the cause.
In conclusion, our studies suggest that dysregulated cell cycle and progenitor cell differentiation in the bronchial epithelium of asthatics may be a primary cause of chronic inflammation due to the inability of the epithelium to re-establish homeostasis. Activation of E2F transcription factors and HH signaling would then drive epithelial remodeling in asthatics. This chronic state of inflammation may lead to the formation of abnormal cilia and microvilli in predisposed individuals, in turn impairing mucociliary clearance and ionic balance. This vicious cycle of chronic inflammation and airway remodeling is hypothesized to drive the pathogenesis of severe equine asthma, and we suggest it is underpinned by lack of homeostasis in the absence of mature, differentiated and fully functional epithelial cells.
FUTURE DIRECTIONS

The work presented in this thesis provides a clearer picture of the gene expression landscape defining the asthmatic bronchial epithelial response to environmental challenge. Furthermore, we report genetic variations that correlated with the development of severe asthma in horses and ensuing morphological abnormalities of the epithelium.

Specifically, we report potential nefarious expression of HH pathways. The exact role of HH signaling and the effect on ciliary and microvillar morphology is unknown. Future experiments could investigate the expression of HH receptors and transcription factors at multiple timepoints before and after exposure to an asthmatic challenge. Such experiments might give better understanding of the differences in key signaling events between asthmatic and non-asthmatic horses.

The association of *PACRG* and *RTTN* mutations with asthma susceptibility should be confirmed in a larger number of phenotypically defined horses. It is feasible that the relative contribution of these two genetic variants is small, and combines with other so far undefined variants to constitute genetic predisposition. Apparent limited genetic heritability in asthma might be attributed to epigenetic factors. Comparison of histone mark patterns in immunoprivileged and bronchial epithelial tissues of asthmatic horses could reveal effects of chronic inflammation and identify heritable differences to non-asthmatic horses.

To investigate the significance of abnormal ciliary and microvillar morphology, their temporal association with acute exacerbation and/or remission should be evaluated. Endobronchial biopsies could be collected during remission and at various times after exposure to an asthmatic challenge for electron microscopic analysis. Cytoskeletal abnormalities could be investigated by immunofluorescence to distinguish altered cillum formation from inflammatory effects. Finally, considering the importance of HH receptors and ion channels in cilia and microvilli, evaluating their in situ expression and function may be rewarding avenues for further research.
References


