3. General introduction

Equine asthma (EA) syndrome is the most prevalent natural occurring chronic lung disease in adult horses. Until now, the pathophysiology remains partially understood and diagnostic biomarkers or tailored treatment options are lacking (Couëtil et al., 2020; Leduc, Leclère, & Lavoie, 2024). The disease complex incorporates pathological and clinical features of human chronic obstructive pulmonary disease (COPD) and human neutrophilic asthma. Clinical signs are typically induced and worsened by exposure to environmental allergens and include chronic cough, exercise intolerance, nasal discharge or dyspnoea. Two EA phenotypes are distinguished, namely mild-moderate EA and severe EA (Couëtil et al., 2016). However, clinical severity varies within the phenotypes and strict subcategorisation of individual horses, or diagnosing of subclinical horses, remains challenging. Affected horses may present with varying degrees of clinical signs dependent on for example (e.g.), season, management, or if the horse is in disease exacerbation or in clinical remission (Robins et al., 2023).

Chronic airway inflammation (AI) is a hallmark of EA. The main effector cell of AI with increasing disease severity is the neutrophil granulocyte (Uberti & Morán, 2018). Neutrophil granulocytes represent a fundamental component of the first line defence of the innate immune system. They are indispensable cells in host defence and inflammatory processes with several effector and defence functions. The most recently discovered defence mechanism is the formation of extracellular DNA traps, called neutrophil extracellular traps (NETs). NETs were first described in 2004 by Brinkmann et al., who found that these scaffold-like structures are capable of trapping and killing bacteria (Brinkmann et al., 2004). Within the years, beneficial functions of NETs against many pathogens were identified. However, it also became evident that NETs can have detrimental effects on the host, if not strictly regulated (Brinkmann, 2018). NET structures have the capacity to induce autoimmunity, and are associated with disease severity, AI and lung tissue destruction in various lung diseases, including human neutrophilic asthma and human COPD (Cheng & Palaniyar, 2013). NETs were also discovered in bronchoalveolar lavage fluid (BALF) of horses with sEA correlating with disease severity (Janssen et al., 2022; Vargas et al., 2017).

The aim of this study was to investigate the occurrence of NETs and NET modulating factors in blood and BALF of horses with different EA severities.

3.1 Definition of the terms “pheno-” and “endotype”

Pheno- and endotype characterisations aim at further defining subgroups of patients within one larger disease spectrum.

The word phenotype originates from the Greek phaínô (“to shine”, “to show”, “to appear”) and tupos (“mark”, “type”). It is used to describe physical or behavioural characteristics of individuals that result from genetic and environmental influences (Kuruvilla et al., 2019b). However, there are only loose definitions of the term and a phenotype characterisation can be made on different levels of biological organisations or characteristics. This can be, e.g., according to factors such as clinical presentation, disease severity, level of treatment required, or duration of the disease (Agache et al., 2012; Richesson et al., 2013). In human asthma, phenotype definitions are widely utilised. Correct phenotype definitions are e.g., “early-onset asthma”, “late-onset asthma” or “severe asthma of current or ex-smoker” (Wenzel, 2012).

The word endotype originates from the Greek endon (“within”, “in”) and tupos (“mark”, “type”) and is used to describe a specific pathogenesis or pathomechanisms underlying a phenotype. A differentiation of endotypes is beneficial since different pathomechanisms, e.g., molecular or immunological pathways, could lead to the same phenotype, but would respond to dissimilar treatment protocols (Agache et al., 2012). Endotypes used in human asthma are e.g., “T-helper type 2 (Th2)-high asthma” or Th2-low asthma” (Svenningsen & Nair, 2017).

Phenotype descriptions are often elaborated before knowledge about the underlying endotypes. Therefore, it would be beneficial to re-evaluate and revise published phenotypes periodically according to current knowledge.

3.2 Equine asthma

Specialists have long tried to sufficiently describe and correctly name chronic obstructive lung disease of the adult horse. The terminology has changed over the decades, leading to different terms used interchangeable in literature.

Since first introduced in 1971, equine chronic airway disease was named “equine chronic obstructive pulmonary disease (eCOPD)” (Sasse, 1971). The term eCOPD was inspired by the human counterpart, the human COPD. With growing knowledge, discrepancies between COPD and eCOPD became evident. In 2000, a workshop with equine specialists was held on eCOPD and agreement on renaming eCOPD was reached (Robinson, 2001). The terms “inflammatory airway disease (IAD)” and “recurrent airway obstruction (RAO)” were elaborated. With this, an attempt on a clinical description of a milder and a more severe form of equine chronic lung disease was made. For a pasture season associated form of RAO the term “summer pasture-associated RAO (SPA-RAO)” was used. The terms were then internationally recommended in the 2007 published Consensus Statement of the American College of Veterinary Internal Medicine (ACVIM) (Couëtil et al., 2007). Finally, in 2016, the term “EA syndrome” was established in the revised ACVIM Consensus Statement (Couëtil et al., 2016). This term is supposed to emphasise similarities to human asthma, such as a pronounced airway hyperresponsiveness to aeroallergens and a wide variability of clinical presentations of diseased individuals (Bond et al., 2018; Bullone & Lavoie, 2015). However, the authors of the Consensus Statement highlight that although the new terminology suggests the disease to be a continuum, clear evidence for mEA (IAD) progressing to sEA (RAO) is lacking.

3.2.1 Aetiology

The aetiology of EA is multifactorial. A complex interaction of environmental factors in genetically heterogenous and susceptible individuals, alongside differences in the immunological status are discussed to contribute to the development of EA (Hotchkiss et al., 2007b; Thomas et al., 2021).

All breeds are susceptible to develop EA. However, a genetic familiar predisposition for eCOPD was long assumed (Marti et al., 1991; Marti & Harwood, 2002). In affected families, the prevalence can reach up to 30% (Ramseyer et al., 2007). Regions on equine chromosome 13 (ECA13, autosomal recessive) and ECA15 (autosomal dominant) were identified to be

associated with sEA in two half-sibling warmblood families. Regions of interest encode, e.g., for genes such as the Interleukin-4-receptor (IL-4R)-α chain gene or the Suppressor of Cytokine Signaling 5 (SOCS5)-gene (Gerber et al., 2015; Swinburne et al., 2009). However, a causal gene was not yet identified in the overall population. Gene differences between families suggest that there is genetic heterogeneity within the disease spectrum.

Environmental risk factors, such as non-infectious organic and inorganic aeroallergens, play a major role in EA development. Stabling and high concentrations of aeroallergens in the environment have been identified as great risks for horses to develop EA (Hansen et al., 2019; Hotchkiss et al., 2007a). Organic dust, which is a term used for dust from animal, bacteria or plant origins, represents the major component of the airborne allergen concentration in a stabled horse (Mańkowska & Witkowska, 2024). Thereby, dry hay feeding generates the majority of organic dust in the stabling environment (Moore-Colyer et al., 2016). Allergens can be cell wall components of fungi, plants or microbiota, such as e.g., β-glucans, which are polysaccharides of D-glucose monomers, or e.g., endotoxins, which are cell wall components of gram-negative bacteria consisting of lipopolysaccharides (LPS) (Barbazanges et al., 2024; Dauvillier et al., 2019). Noxious gases such as ammonia and carbon or nitrogen dioxide may also function as strong allergens (Kwiatkowska-Stenzel et al., 2014). In addition to the concentration and quality of airborne particles, factors such as humidity, ventilation and temperature directly impact the horses' lungs and can influence the concentration and distribution of inhalable organic dust throughout the seasons (Bullone & Lavoie, 2016). Seasonal changes can additionally be triggered by individual pasture-allergen sensitivity in some horses. These horses exhibit equine pasture asthma in the summer months upon contact to grass and tree pollen, rather than developing clinical disease in the stabling environment in the winter (Rodrigues Costa et al., 2000).

The potential role of different infectious agents of the upper or lower airways have long been discussed. Some studies found associations between the occurrence of equine herpes viruses 2 and 5, or equine rhinitis viruses, and clinical EA (Couëtil et al., 2021; Doubli-Bounoua et al., 2016; Houtsma et al., 2015). However, longitudinal studies have not identified a causal relationship between viral load of these viruses and clinical signs (Back, Penell, et al., 2015; Back, Ullman, et al., 2015). Other studies have identified differences of the lower respiratory tract microbiota between healthy horses and horses with EA (Bond et al., 2017; Fillion-Bertrand et al., 2019; Manguin et al., 2020). However, a causal relationship remains unproven as well.

Nevertheless, it cannot be excluded that subclinical viral or bacterial infections contribute to a subclinical AI and subsequently facilitate the development of EA (Couëtil et al., 2020).

3.2.2 Pathogenesis

Contact to aeroallergens triggers AI and airway hyperresponsiveness (AHR). AI is defined by the quantity and quality of inflammatory cells within the lungs. Inflammatory cells recognized in lungs of horses with EA are neutrophil granulocytes (neutrophils), mast cells and, to a smaller extent, eosinophil granulocytes (Couëtil et al., 2016). Airway neutrophilia is associated with disease severity and the neutrophil the main effector cell in severe EA. Whereas a mixed inflammatory response is more likely to be found in mild-moderate affected horses (Amstrup et al., 2023). The local inflammation consequently causes further inflammatory cell recruitment, leads to mucosal swelling and epithelial destruction, lung tissue degradation and mucus hypersecretion (Debrue et al., 2005; Saffarzadeh et al., 2012). Furthermore, BALF and blood neutrophils undergo delayed apoptosis and show a greater viability compared with controls, which can lead to unresolved AI and chronicity of the disease (Bureau, Bonizzi, et al., 2000; Bureau, Delhalle, et al., 2000; Mainguy-Seers et al., 2018).

Following, bronchospasm, mucus accumulation and AI result in narrowing of the airways. An increase of pulmonary resistance and pleural pressure can be observed (Jackson et al., 2000). This initial acute airway narrowing is typically reversible with allergen avoidance. In remission and under environmental control, affected horses might not show any clinical signs and are therefore clinically not distinguishable from healthy horses. However, AHR can remain elevated in these individuals (Hunter et al., 2020).

In disease progression, remodelling of the airways can be investigated, with an increase of smooth musculature (Bullone et al., 2017; Bullone & Lavoie, 2020), extracellular matrix fibrosis (Ferrari et al., 2018; Herszberg et al., 2006; Höglund et al., 2024), bronchial angiogenesis (Millares-Ramirez & Lavoie, 2021), increased airway innervation (Leduc, Leclère, Gauthier, et al., 2024) and thickening of pulmonary arteries (Ceriotti et al., 2020). Airway remodelling includes peripheral and central airways and is only partly reversible (Bullone, 2017; Leclere et al., 2011). With the course of the disease, airway neutrophilia observed in sEA can be persistent and insensitive to treatment (Bullone et al., 2017; Leclere et al., 2012; Léguillette et al., 2017). Many detrimental effects that contribute to bronchoconstriction and pulmonary remodelling are attributed to neutrophil-associated

cytokines or functions (Uberti & Morán, 2018). In horses with structural alterations the airway resistance can remain high in remission, although no bronchospasm is present (Stucchi et al., 2021). The structural changes can lead to a severely decreased lung function, which may be visible as persisting clinical dyspnoea at rest. Furthermore, AI and remodelling can lead to hypoxemia (Simões & Tilley, 2023).

Recurring disease exacerbations can be triggered by further allergen exposure at any time (Bullone et al., 2018). The different disease states of exacerbation and remission can lead to a divergent clinical appearance in the same horse.

3.2.3 Immunological pathways

The immunological processes in EA are complex and only partly understood. In humans, distinct cytokine profiles allow the differentiation of two major asthma endotypes:

Human allergic asthma is characterised by a T-helper cell type (Th) 2 pathway involving high levels of interleukin (IL) -4, IL-5, IL-9, IL-13 and granulocyte colony stimulating factor (G-CSF). This pathway consequently results in excessive airway eosinophilia and high concentrations of immunoglobulin (Ig) E. These characteristics are typically not evident in EA.

Human non-allergic asthma ("Th2-low"), on the contrary, shares some similarities with EA. Typical Th2 markers are lacking and various cell populations and innate and acquired immune processes appear evident. Links can be drawn to an activation of Th1 and Th17 cells or an imbalance between Th17 and T-regulatory cells. Key cytokines of Th17 are IL-17A, IL-17F and IL-22, which can further induce IL-8 secretion. These cytokines are associated with neutrophilic recruitment and inflammation (Bullens et al., 2006). The primary effector cell of AI is indeed the neutrophil, however, a pauci-granulocytic (e.g., eosinophils and neutrophils) AI is possible (Kuruvilla et al., 2019a). Response to corticoid treatment is typically low, while severity and morbidity are high (Klain et al., 2022; Panettieri, 2016).

A clear differentiation of distinct immunological pathways has not been successful in EA. The wide variety of clinical presentations and the research based on the more homogenous sEA phenotype aggravates a comprehensive understanding of the underlying mechanisms (Simões et al., 2022). Since airway neutrophilia is a hallmark of sEA, a Th17 involvement has been hypothesised in some studies (Debrue et al., 2005; Hansen et al., 2020; Murcia et al., 2016). Horses with mEA present with a mastocytic, neutrophilic, eosinophilic or mixed inflammatory

cell involvement, which could be explained by a Th2-high response. However, approaches to unravel one underlying T-helper cell pathway have been unsuccessful and different cytokine profiles were found in a variety of equine studies (Simões et al., 2022).

In sEA, there is evidence for a maturation towards a Th2- or Th17- pathway, as well as mixed Th1- and Th2-, Th1- and Th17-, or Th2- and Th17- pathways. For example, microRNAs supporting a mixed Th2/Th17 cell differentiation were found in one study (Pacholewska et al., 2017), whereas another study found a Th2 type mRNA expression with mRNA upregulation for IL-4 and IL-5, and a downregulation for interferon-γ in affected horses (Lavoie et al., 2001). Further studies, on the contrary, postulated a downregulation of cytokines consistent with Th1 and Th2 (Kleiber et al., 2005) or found an increase of IL-17 mRNA in horses with chronic disease exacerbation (Debrue et al., 2005). One study further attempted to differentiate between EA phenotypes and found BALF mRNA expressions of cytokines from a Th2 pathway for the mastocytic EA phenotype and a Th1/Th17 pathway in sEA (Hansen et al., 2020).

Despite the uncertainties of the underlying immunological pathway, it is undisputed that the neutrophil is the key effector immune cell of non-infectious AI in severe cases of EA (Uberti & Morán, 2018). It is known that blood neutrophils of asthmatic horses exhibit an IL-17 associated glucocorticoid insensitivity and an enhanced IL-8 production despite glucocorticoid treatment, which are both cytokines that are associated with neutrophil recruitment to the lungs (Murcia et al., 2016). However, the mechanisms driving airway neutrophilia and inflammation are only partly understood. Identifying the underlying cause of the persistence of the chronic neutrophilic AI is objective of ongoing research. A possible driving factor might be an imbalance in the pro-inflammatory and anti-inflammatory pathways; e.g., increased NET formation (Cheng & Palaniyar, 2013). Therefore, research focussing on neutrophils and neutrophil functions remains promising.

3.2.4 Classification of phenotypes and endotypes in EA

The internationally accepted ACVIM phenotype definitions underly a wide range of parameters including medical history, clinical presentation, BALF cytology and factors regarding the pathogenesis. Classifying treatment responses is not part of the phenotype definitions. With-in the EA spectrum, two phenotypes are differentiated: mild-moderate EA (mEA) and severe EA (sEA) (Couëtil et al., 2016). A further subdivision was made in sEA, describing the summer-

pasture associated form of sEA as severe equine pasture asthma (sEPA) (Bullone & Lavoie, 2016).

The mEA phenotype is defined as present mostly in younger horses (<7 years of age) with no or mild to moderate clinical signs, lacking dyspnoea at rest. Clinical signs may vary from minor exercise intolerance to occasional cough. AI and clinical signs can resolve spontaneously and the recurrence rate in mEA is low. The stated prevalence varies between studies with values up to 80% of the horse population. However, this phenotype comprises also subclinical diseased horses diagnosed solely according to BALF cytology (Ivester et al., 2018). International consensus suggests that cut-off values of >10% neutrophils, >5% mast cells and >5% eosinophils are consistent with mEA (Couëtil et al., 2016). Airway endoscopy can be inconspicuous or with slight abnormalities such as accumulation of minor amounts of tracheal mucus (Couëtil, 2002). Horses with mEA show no to slight abnormal lung function (e.g., tested with oscillometry or flowmetric plethysmography). AHR is more likely confirmed in horses showing higher mast cell counts after bronchoprovocation challenge; however, AHR can also be absent in mEA (Cullimore et al., 2018; Wichtel et al., 2016). A suggestion of distinguishing a mild (= subclinical) and a moderate (= clinical) EA phenotype was made by some authors but is limited by the absence of non-invasive screening methods or biomarkers for subclinical horses.

The sEA phenotype typically includes horses older than 7 years and has a prevalence of 14% in the northern hemisphere (Hotchkiss et al., 2007b). Horses show chronic clinical signs such as frequent cough, exercise intolerance, nasal discharge and possibly expiratory dyspnoea at rest (Pirie, 2017). Airway endoscopy typically reveals pathological findings such as tracheal mucus accumulation and mucosal thickening (Gerber et al., 2000, 2004). Severe airway neutrophilia (BALF neutrophils >25%) is a hallmark of sEA. However, BALF cytology does not always correlate with clinical severity (Bullone & Lavoie, 2017; Couëtil et al., 2005). Recurrence rates of exacerbations are high and clinical signs can vary seasonally. Treatment failure in sEA is common.

One approach to further subcategorise EA phenotypes for targeted therapy was previously made. The authors suggested the division of phenotypes strictly based on severity (mild, moderate and sEA), or BALF cytology characteristics (e.g., neutrophilic, mastocytic, eosinophilic, pan-granulocytic, pauci-granulocytic), or trigger factors (sEA, sEPA), or the progression of airway remodelling (Leduc, Leclère, & Lavoie, 2024).

To the best of current knowledge, there are no internationally accepted definitions for endotypes in EA.

3.2.5 Treatment

Treatment of EA aims at resolving clinical signs by reducing AHR and AI, and reversing airway obstruction (Pirie, 2017). Per phenotype definition, sEA cannot be cured, while mEA may resolve with treatment or spontaneously. Therefore, the aim of treating sEA is to relieve symptoms, to prevent further exacerbation and to slow disease progression. Consequently, the reduction of allergen exposure is considered to be the fundamental principle that forms the basis of any comprehensive therapeutic approach (Ivester & Couëtil, 2014; Simões et al., 2020). Furthermore, medical treatment is symptomatic and aims at reducing bronchospasm (e.g., through bronchodilators) and controlling AI (e.g., through glucocorticoids). Targeted therapy of underlying pathomechanisms and tailored treatment options are currently not available for horses (Leduc, Leclère, & Lavoie, 2024).

3.3 Neutrophils

Neutrophil granulocytes, otherwise referred to as “neutrophils”, represent the most prevalent blood leucocytes in horses, a characteristic that is analogous to that observed in humans (Fingerhut et al., 2020). The neutrophil is one of the first cells of the innate immune system recruited in response to an infection and essential in yielding the first line defence against pathogens. Physiologically, neutrophils exhibit antimicrobial and phagocytic functions and are well recognised and balanced as key effector cells of infectious or non-infectious inflammatory processes (Witko-Sarsat et al., 2000). Furthermore, they can modulate immune responses through the release of immunomodulatory cytokines, chemokines and prostaglandins, and through the direct interaction with immune cells such as dendritic cells, macrophages or natural killer cells (Bliss et al., 2000).

3.3.1 Granulopoiesis and homeostasis

Granulocytes are originating from myeloid progenitor stem cells which are differentiating from multipotent haematopoietic stem cells in the bone marrow (Borregaard, 2010). This overall process is called granulopoieses. In the process of neutrophil maturation, the cell has to go through several progenitor cell stages to become a mature polymorphonuclear neutrophil (myeloblasts, promyelocytes, myelocytes, metamyelocytes and band cells; respectively). Homeostasis of granulopoieses is controlled by growths factors, such as the G-CSF (Liu et al., 1996). Elevated levels of G-CSF enhance the production of neutrophils upon higher needs during infections.

In horses, physiological values of circulating, peripheral blood leucocytes range from 3.5 - 12.1 x 10^9/L, of which 45 - 70% are presented by neutrophils (1.58 - 8.47 x 10^9/L) (Fingerhut et al., 2020). These numbers are influenced by a variety of internal and external factors, e.g., an increase in case of systemic inflammation or infection, or a decrease in e.g., endotoxemia or viral infections (Lilliehöök et al., 2016; Törner & Kaufhold, 2019). Under physiological circumstances, the majority of the neutrophils is not circulating within the blood but is stored in small vessels or relocated in bone marrow, spleen, liver and lung, which can be seen as reservoirs until activation (Peters, 1998; Summers et al., 2010; Suratt et al., 2001).

Neutrophils undergo apoptosis at the end of their life-span or antimicrobial activity and are subsequently phagocytosed by macrophages and dendritic cells (Greenlee-Wacker, 2016). This process is essential in maintaining granulocyte homeostasis (Stark et al., 2005), resolving inflammation (Fadok et al., 1998; Voll et al., 1997) and preventing lysis of the neutrophils and uncontrolled release of active components with detrimental effects on the host (Epstein & Weiss, 1989). However, there is evidence that circulating neutrophils are able to remigrate into the bone marrow under certain circumstances, e.g., after excessive trauma (Teuben et al., 2022). Furthermore, the overall life-span can be modulated by neutrophil activation which ensures the cells viability in case of inflammation (Colotta et al., 1992).

3.3.2 Morphology

The mature, circulating equine neutrophil has an average diameter of 10 - 12 µm (Herteman et al., 2017). It is easily recognised by its segmented, multilobed nucleus. The segmentation of the nucleus is particularly pronounced in the horse, compared to other mammals (Tvedten &