EXHALED BREATH CONDENSATE PH, HYDROGEN PEROXIDE AND LEUKOTRIENE B4 IN HORSES: MEASUREMENT CONSIDERATIONS AND APPLICATION FOR THE INVESTIGATION OF LOWER AIRWAY DISEASE

by

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A thesis submitted for the degree of Doctor of Veterinary Studies, Charles Sturt University

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I would like to dedicate this thesis to my loving wife.
You have been my rock.
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Certificate of Authorship

"I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma at Charles Sturt University or any other educational institution, except where due acknowledgment is made in the thesis. Any contribution made to the research by colleagues with whom I have worked at Charles Sturt University or elsewhere during my candidature is fully acknowledged.

I agree that this thesis be accessible for the purpose of study and research in accordance with the normal conditions established by the Executive Director, Division of Library Services or nominee, for the care, loan and reproduction of theses."

Surita du Preez

07/05/2018
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Animal Care and Ethics Approval

Animal care and ethics approval was attained prior to sample collection. The Animal Care and Ethics Committee, Charles Sturt University approved the study protocol.

The protocol number issued with respect to this project was 14/082

Publications

Part of this thesis has been accepted for publication elsewhere:


Part of this thesis has been presented elsewhere:

du Preez, S., Raidal, S.L., Doran, G.S., Nielsen, S.G. and Hughes, K.J. The variability and influence of animal and environmental factors on exhaled breath condensate hydrogen peroxide and leukotriene B₄ in horses. Presented at the Australian and New Zealand College of Veterinary Scientists, Science Week Conference, Gold Coast, Australia, 7-9 July 2016.
Abstract

The measurement of biomarkers in exhaled breath condensate (EBC) is an expanding field of non-invasive respiratory assessment. In humans, several biomarkers, including hydrogen peroxide (H$_2$O$_2$), pH and leukotriene B$_4$ (LTB$_4$) have proven useful for the detection and monitoring of inflammatory lung diseases, including asthma and chronic obstructive pulmonary disease. While EBC analysis could offer an alternative to conventional invasive methods for the diagnosis of equine lower airway inflammation (LAI), little information is available regarding EBC biomarkers in horses. The aims of this study were to (1) determine the intra- and inter-day consistency of EBC pH, H$_2$O$_2$ and LTB$_4$ measurements in horses, (2) determine whether these biomarkers are influenced by environmental, animal or methodological factors, (3) identify associations between these biomarkers and cytological and endoscopic definitions of LAI and (4) develop a superior tandem mass-spectrometry (MS/MS) method for H$_2$O$_2$ measurement.

Biomarker measurement consistency was assessed using EBC samples collected from 10 horses on three occasions (day 1), and at midday on days 2 and 3. Influences of environmental, animal and methodological factors on biomarkers were determined using EBC samples collected from 20 horses (pH; LTB$_4$) and 10 horses (H$_2$O$_2$). Clinical, endoscopic and airway cytological findings from 47 horses were compared with EBC pH, H$_2$O$_2$ and LTB$_4$ measurements by univariate and multivariable analyses. Dichotomous (presence/absence of LAI) and continuous (respiratory fluid differential cell counts) outcome variables were evaluated and environmental and methodological factors were included. Standard H$_2$O$_2$ solutions were used to compare flow injection analysis and liquid chromatography (LC) using either fluorescence (FLD) or MS/MS detection with instrument grade water or pooled EBC as matrix for standard preparation. The influence of sample matrix was evaluated by comparison of peak areas and gradients of standard curves.

Intra- and inter-day consistency for both H$_2$O$_2$ and pH were adequate (intraclass correlation coefficients ≥0.8). The intra- and inter-day consistency for LTB$_4$ was poor. H$_2$O$_2$ was influenced by ambient temperature (T$_a$, P=0.004), humidity (P=0.009), time
of day (P=0.009) and collection location (P=0.007), while pH was influenced by respiratory rate during EBC collection (P<0.001) and T_A (P<0.001). H_2O_2 concentrations and pH were higher in horses with LAI and were positively associated with bronchoalveolar lavage fluid (BALF) neutrophil percentage (P=0.033 and P=0.01, respectively). BALF mast cell percentage was negatively associated with EBC pH (P=0.041) and BALF eosinophil percentage was positively associated with EBC LTB_4 (P=0.035). The LC-MS/MS method was more sensitive (limit of detection 0.038 μM) than previously published FLD methods. The intra- and inter-day assay coefficients of variations were 1.0-3.2% and 1.7-2.3%, respectively. A significant matrix effect was identified (P<0.001).

The study demonstrated adequate consistency of H_2O_2 and pH, and that both biomarkers were altered by inflammation, suggesting that use of these biomarkers in the diagnosis and monitoring of LAI, may be warranted. However, environmental, animal and methodological factors can influence these biomarkers and should be considered in the interpretation of results. The LC-MS/MS method was 3.7 times more sensitive with superior selectivity and repeatability compared with existing FLD methods. However, the effect of matrix-solution should be considered prior to sample analysis.
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Glossary of abbreviations

BAL  Bronchoalveolar lavage
BALF Bronchoalveolar lavage fluid
CV  Coefficient of variation
CI  Confidence intervals
DHPA 2,2’-dihydroxybiphenyl-5,5’-diacetate
EB  Exhaled breath
EBC Exhaled breath condensate
EBTV Mean total volume of exhaled breath
EIA Enzyme immunoassay
EIPH Exercise-induced pulmonary haemorrhage
ESI Electrospray ionisation
FA Formic acid
FIA Flow injection analysis
FLD Fluorescence detection
FOM Forced oscillatory mechanics
FP Flowmetric plethysmography
FR Respiratory frequency
H₂O₂ Hydrogen peroxide
HPAA 4-hydroxyphenylacetic acid
HR Heart rate
HRP Horseradish peroxidase
IAD Inflammatory airway disease
ICC Intra-class correlation coefficient
LAI Lower airway inflammation
LAI-1 IAD or EIPH or STI or any combination
LAI-2 IAD or EIPH or any combination
LC-MS/MS Liquid chromatography-tandem mass-spectrometry
LLoQ Lower limit of quantitation
LoB Limit of blank
LoD Limit of detection
LTB₄ Leukotriene B₄
LTB₄-d₄ Deuterated leukotriene b₄
PFT Pulmonary function testing
PLH Pharyngeal lymphoid hyperplasia
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>RAO</td>
<td>Recurrent airway obstruction</td>
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<tr>
<td>RH</td>
<td>Relative humidity</td>
</tr>
<tr>
<td>RL</td>
<td>Lung resistance</td>
</tr>
<tr>
<td>RR</td>
<td>Respiratory rate</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SPA-RAO</td>
<td>Summer pasture associated - recurrent airway obstruction</td>
</tr>
<tr>
<td>STI</td>
<td>Syndrome of tracheal inflammation</td>
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<tr>
<td>TA</td>
<td>Ambient temperature</td>
</tr>
<tr>
<td>TA</td>
<td>Tracheal aspirate</td>
</tr>
<tr>
<td>THS</td>
<td>Total haemosiderin score</td>
</tr>
<tr>
<td>Ti</td>
<td>Inspiratory time</td>
</tr>
<tr>
<td>TIS</td>
<td>Tracheal inflammation score</td>
</tr>
<tr>
<td>trIAD</td>
<td>Tracheal inflammatory airway disease</td>
</tr>
<tr>
<td>V̇E</td>
<td>Pulmonary ventilation</td>
</tr>
<tr>
<td>V̇T</td>
<td>Tidal volume</td>
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Introduction

Non-infectious lower airway inflammatory (LAI) conditions are common causes of poor performance and wastage in horses [1]. These conditions include the heterogeneous syndrome of mild equine asthma (previously termed inflammatory airway disease, IAD), the syndrome of tracheal inflammation (STI) and exercise-induced pulmonary haemorrhage (EIPH). Mild equine asthma is common in athletic horses [2] and has been associated with poor performance, exercise intolerance, coughing, excess tracheal mucus and abnormal pulmonary function [3]. The clinical presentation of LAI conditions is variable and has been related to differing inflammatory cell populations and inflammatory mediators in bronchoalveolar lavage fluid (BALF) [4,5]. Exercise-induced pulmonary haemorrhage is ubiquitous among racehorses and a significant negative association between EIPH and race performance has been demonstrated [6,7]. Increased risk of EIPH has been associated with lower airway inflammation [8] and instillation of autologous blood into the airways elicits an inflammatory response [9]. Although EIPH and IAD commonly coexist, the aetiopathological relationship between the two conditions remains controversial [3]. Diagnosis of LAI conditions are currently based on tracheobronchoscopic examination, pulmonary function testing and/or BALF cytology. These conventional diagnostic modalities are invasive, often require sedation, are not without sequelae and do not provide information about the level of active inflammation.

Analysis of biomarkers in exhaled breath condensate (EBC) is a non-invasive, widely used method for direct assessment of the lower respiratory tract in human medical research. EBC is collected by cooling of expired breath to a liquid or frozen state [10]. EBC constituents are likely present in concentrations that reflect those within the epithelial lining fluid (ELF) of the lower airways [11] and contain many non-volatile and water-soluble volatile molecules. However, biomarker concentrations may be influenced by methodological, physiological and environmental factors [12-14].

EBC acidification has been reported in humans with asthma, chronic obstructive pulmonary disease (COPD) and bronchiectasis [15], suggesting that pH may be a useful biomarker for detection of acid-base disturbances caused by inflammation of
pulmonary tissues. Similarly, LAI has been associated with EBC acidification in calves [16]. However, EBC pH may be affected by collection and measurement temperatures [17,18] and ambient temperature (TA), though other studies reported EBC pH to be independent of TA [19].

The concentration of hydrogen peroxide (H₂O₂) in human EBC has been reported to be correlated with the severity and treatment response of several diseases, including asthma [20] and is considered to be a marker of pulmonary inflammation and oxidative stress. Associations between EBC H₂O₂ and LAI in horses with recurrent airway obstruction (RAO) [21], in foals with *Rhodococcus equi* pneumonia [22] and in *Ascaris suum* (AS)-sensitised cats [23] have been reported, while in other studies, no association between H₂O₂ and airway inflammation in horses were found [24,25]. H₂O₂ concentrations may be affected by diurnal variation [12], age [26], respiratory rate (RR) [14] and methods used for EBC collection and quantification of H₂O₂ [27].

Leukotriene B₄ (LTB₄), an arachidonic acid metabolite, is a non-specific pro-inflammatory mediator [28]. Increased concentrations of LTB₄ in EBC from humans with COPD and asthma have been reported [29]. LTB₄ has been associated with airway inflammation in calves with bacterial and viral pneumonia [30], in pigs with *Chlamydia suis* pneumonia [31] and horses with RAO [32]. The influences of environmental factors on EBC LTB₄ have not been evaluated previously.

Measurement of specific EBC biomarkers could offer a non-invasive alternative to conventional diagnostic methods and allow more frequent monitoring in horses with LAI. To date, reports evaluating EBC biomarkers in horses with LAI have been scarce and some results are conflicting, as such further investigation of EBC analysis in this species is warranted.
The research questions for this study were

1. What is the consistency of EBC pH measurements and H$_2$O$_2$ and LTB$_4$ concentrations in horses and do animal, environmental and analysis factors influence these biomarkers?

2. Is EBC useful for the diagnosis of lower airway inflammation in horses?

3. Can a superior method be developed for the determination of H$_2$O$_2$ concentration in EBC?

**Thesis structure**

This thesis is compiled in accordance with Charles Sturt University recommendations for a professional doctorate by publication. Chapter 2 includes a comprehensive literature review of mild, non-infectious lower airway inflammatory conditions in horses, an overview of conventional diagnostic modalities used to assess the lower airways in horses, a review of published research findings of the measurement and interpretation of EBC pH, H$_2$O$_2$ and LTB$_4$ in humans and veterinary species and the study research objectives and hypotheses.

Chapter 3 consists of a manuscript accepted for publication in The Veterinary Journal as a short communication (in press), entitled: “The consistency and influence of environmental and animal factors on exhaled breath condensate hydrogen peroxide, pH and leukotriene B$_4$ in horses” and the accompanying supplementary material for online publication. This chapter presents and discusses the study designed to evaluate the consistency of EBC pH measurements and H$_2$O$_2$ and LTB$_4$ concentrations in equine EBC and to determine if animal, environmental and analysis factors influence these EBC biomarkers in the population of horses used (research question 1). The originally prepared full-length manuscript is also included in Appendix 1, as it provides additional information that could not be presented within the constraints of a short communication. The EBC collection method used throughout the study is discussed and illustrated in full in this chapter.

Chapter 4 consists of the manuscript submitted for publication to the Equine Veterinary Journal (under review), entitled: “Exhaled breath condensate hydrogen
peroxide, pH and leukotriene B₄ are associated with lower airway inflammation and airway cytology in the horse” and the accompanying supplementary material for online publication. This chapter reports the study designed to compare EBC pH measurements and H₂O₂ and LTB₄ concentrations in horses with and without various categories of LAI and to determine if correlations between EBC biomarkers and cytological and endoscopic definitions of LAI exist (research question 2).

Chapter 5 consists of the manuscript and supplementary material for online publication prepared in preparation for submission to the Journal of Chromatography B, entitled: “Sensitive and selective analysis of H₂O₂ in exhaled breath condensate using LC-MS/MS”. This chapter reports the development of a novel, sensitive and more selective liquid chromatography tandem mass-spectrometry (LC-MS/MS) method for determination of H₂O₂ concentrations in EBC, with results compared directly with FLD (research question 3).

The studies designed to answer each specific research question resulted in the identification of multiple significant associations with EBC pH measurements and H₂O₂ and LTB₄ concentrations. Chapter 6 is a general discussion in the form of an exegesis that summarises the findings of this thesis and discusses issues that were identified in the studies. Issues that are relevant to all three EBC biomarkers evaluated in this thesis are discussed first, followed by findings and issues identified for each individual EBC biomarker.
References


1. Introduction

Respiratory tract disorders are second in importance only to musculoskeletal disorders in limiting the athletic performance of the horse [1,2]. To limit economic losses and premature retirement of the athletic horse, early detection and treatment of respiratory disorders is essential [3]. Respiratory disorders of the horse can broadly be classified into disorders of the upper or lower respiratory tract. Lower airway disorders are common in horses. Presenting signs vary with these disorders and may include nasal discharge, cough, exercise intolerance, poor performance, fever, respiratory distress, increased respiratory rate or effort or lethargy, inappetence and weight loss. These disorders may be infectious or non-infectious in origin (Table 2.1) and careful clinical examination and ancillary diagnostic testing are usually required to confirm a suspected diagnosis [3]. This study furthers the understanding of non-invasive diagnostic methods for the investigation of non-infectious equine lower airway inflammation (LAI), specifically mild equine asthma, formerly inflammatory airway disease (IAD), exercise-induced pulmonary haemorrhage (EIPH) and the syndrome of tracheal inflammation (STI) in adult horses.
### Table 2.1
Common non-infectious disorders of the lower respiratory tract in horses [3]

<table>
<thead>
<tr>
<th>Adult horses</th>
<th></th>
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</thead>
<tbody>
<tr>
<td><strong>Severe equine asthma</strong></td>
<td><strong>Recurrent airway obstruction (RAO)</strong></td>
</tr>
<tr>
<td>Affects mature horses, housed in stables. Postulated to be caused by:</td>
<td>Specific hypersensitivity to fungal aeroallergens [4] as well as non-antigen specific inflammatory response to endotoxins or B-glucans [5].</td>
</tr>
<tr>
<td>Summer pasture associated recurrent airway obstruction (SPA-RAO)</td>
<td>Develops in mature horses maintained at pasture. Unknown aetiology. Clinically indistinguishable from RAO</td>
</tr>
<tr>
<td><strong>Mild to moderate equine asthma</strong></td>
<td><strong>Inflammatory airway disease (IAD) [6]</strong></td>
</tr>
<tr>
<td>Most commonly in young racehorses, but affects horses of any age or breed</td>
<td>Cause is poorly defined, but respirable irritants, particles and antigens may be involved</td>
</tr>
<tr>
<td><strong>Exercise-induced pulmonary haemorrhage (EIPH)</strong></td>
<td></td>
</tr>
<tr>
<td>Numerous mechanisms have been suggested to cause EIPH. The most widely accepted as postulated by Derksen et al. [7] is; regional pulmonary capillary hypertension with capillary stress failure and rupture, bleeding, haemosiderin accumulation, localised inflammation, bronchial circulation angiogenesis and lung fibrosis.</td>
<td></td>
</tr>
<tr>
<td><strong>Syndrome of tracheal inflammation (STI)</strong></td>
<td></td>
</tr>
<tr>
<td>The definition of STI remains variable. It is generally accepted to represent a syndrome of lower airway inflammation characterised by a combination of increased mucus accumulation within the tracheal lumen and neutrophilic inflammation identified by TA cytology with or without bacterial colonisation [8-10]. The term is used interchangeably with tracheal IAD (triAD) in the literature. The pathogenesis remains to be defined however is likely similar to that of IAD.</td>
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</table>
2. Conventional diagnostic methods to investigate LAI in horses

2.1. Physical examination
Examination of a horse with clinical signs suggestive of pulmonary disease includes collection of a complete history and physical examination, including thoracic auscultation during eupneic and hyperpnoeic breathing. To induce hyperpnoeic breathing, a rebreathing bag technique is used to induce hypercapnoea. This method facilitates detection of abnormal breath sounds [3,11]. Thoracic percussion may be performed: aerated tissues produce a resonant sound whereas fluid filled structures, such as bowel, heart, abscesses or consolidated lung, produce a dull sound [12,13]. These preliminary examination techniques direct the selection of further diagnostic tests, if required. Common ancillary diagnostic tests of the lower respiratory tract include endoscopy, tracheal aspiration (TA), bronchoalveolar lavage (BAL), thoracic radiography and ultrasonography, arterial blood gas analysis, complete blood count (CBC) and serum biochemical analysis.

2.2. Respiratory tract endoscopy
Endoscopy of the respiratory tract allows direct visualisation of the upper and lower airways and any pathologic changes. Disorders of the upper respiratory tract, including recurrent laryngeal neuropathy, dorsal displacement of the soft palate, abnormalities of the palatopharyngeal arch, aryepiglottic folds, pharynx (e.g. pharyngeal lymphoid hyperplasia [PLH]) and epiglottis may be identified during endoscopic examination [3,14-16]. PLH is associated with antigenic stimulation of the mucosal lymphoid tissue of the pharynx, most often in younger horses [17,18]. PLH may be associated with reduced performance in Thoroughbred racehorses [18], increased tracheal mucus accumulation score [17] and palatal instability [19], suggesting this condition can influence respiratory function.

Neutrophilic inflammation may cause mucus accumulation in equine airways through increased production and secretion of mucins [20] and/or by altering physical properties and clearance of mucus [21,22]. Gerber et al. [23] investigated the validity
of endoscopic scores used to assess mucus accumulation, viscosity, localization and colour. The authors reported that the mucus accumulation score (Fig. 2.1) was the most reliable score: it was highly reproducible within and between observers, had moderate variation over time within horses and was correlated with measured volumes of mucus, TA neutrophil percentage, bronchoalveolar lavage fluid (BALF) absolute neutrophil numbers and BALF neutrophil percentage [23]. These findings indicate that tracheal mucus accumulation scoring is a reliable clinical and research tool.

![Figure 2.1](image)

Tracheal mucus accumulation score (from Gerber et al. [23])

### 2.3. Tracheal aspirate and bronchoalveolar lavage

Aspiration of tracheobronchial secretions using an endoscopically guided catheter is easy to perform and represents a collective sample from all areas of the lung that can be used for cytological examination [24]. When obtained via a plugged triple lumen guarded catheter, samples are also suitable for microbiological examinations [25].

The use of TA has been challenged for the investigation of chronic inflammatory disorders, due to the lack of correlations between TA and BALF cytology and pulmonary histopathology [24,26,27]. However, other authors argue that TA samples are suitable for investigation of LAI (e.g. mild equine asthma) as TA neutrophil percentage has been associated with both inspiratory and expiratory indices of pulmonary function using spirometry [28] and coughing [29]. According to the conclusions of the Havemeyer Workshop of 2003, TA cytology of a young healthy racehorse should have <20% neutrophils and <1% eosinophils [30] and abnormal TA cytology may reflect a response within the trachea or more distal airways.

Bronchoalveolar lavage allows recovery of respiratory secretions from the bronchioles
and alveoli. Specific indications for BAL are investigation and diagnosis of equine asthma, exercise-induced pulmonary haemorrhage (EIPH) and interstitial pneumonias [6,31,32]. There is good correlation between BAL differential cell counts and exercise-induced hypoxaemia or lactic acidosis [33,34], airway obstruction [35], airway responsiveness [36] and pulmonary histopathology findings [37]; illustrating the link between BALF cytology and the lower airway structure and function. BALF may also be used for PCR and proteomic detection of pathogens and inflammatory responses [38,39].

The influence of lung site, lavage volume, prior BAL, exercise and cold weather exercise on BAL reproducibility has been assessed. No significant difference in BALF cytology between different sampling sites was found in earlier studies [32,40-42]. However, more recently, a significant difference in BALF neutrophil percentage and haemosiderophage to macrophage ratio between the left and right lungs of 138 French trotters in race training [43] was detected. This suggests that horses can be incorrectly classified as free of IAD if only one side of the lung is evaluated.

Large volume lavage (250-500mL) with sterile saline is preferred for BAL. Cytological findings did not differ significantly when three sequential aliquots of 100ml saline or one pooled infusion of 300ml was used [42,44]. While in a recent study, the BALF cytological profiles were significantly different when 250mL or 500mL during lavage was used [45], indicating that a consistent BAL protocol is needed to achieve comparable results. The BAL procedure can affect subsequent BALF cytology with a transient increase in neutrophil percentage for 48-72 hours [45,46]. Further, exercise can result in a mild, transient increase in neutrophil percentage [33], particularly during cold weather (-5°C) [47]. There is no evidence that BAL affects lung function [48] and prior pulmonary function testing, including histamine bronchoprovocation, does not alter BALF cytology or cytokine levels [49].

Reference values for BALF differential cell counts from healthy horses are: macrophages 50-70%, lymphocytes 30-50%, neutrophils ≤5%, mast or metachromatic cells ≤2% and eosinophils ≤1%. Epithelial cells are generally rare although a few non-ciliated bronchial epithelial or goblet cells may be observed [6,50]. Malikides et al. [24]
demonstrated that 37% of paired BAL and TA samples had differences in diagnostic interpretation and recommended that both procedures be performed to assess the lower airways cytologically.

2.4. Thoracic radiography and ultrasonography
Thoracic radiography is useful for detection of diffuse parenchymal diseases such as interstitial pneumonia, pulmonary oedema, chronic airway disorders, neoplasia and deep parenchymal abscesses [51,52]. However, radiography of adult horses requires multiple exposures and is often limited to referral institutes [53]. Radiographic changes can be non-specific and several pulmonary disorders (e.g. IAD, EIPH, RAO and lungworm infection) may not have detectable radiographic abnormalities [3,54].

Thoracic ultrasonography is widely available in equine practice and is more sensitive than thoracic radiography for detection of pleural effusion, peripheral lung changes and lesions overlying the heart or liver. Ultrasonography should be performed when clinical examination reveals abnormal lung sounds or areas of dullness within the thorax are detected during thoracic percussion [51,55]. Disadvantages of ultrasonography is the inability to evaluate lesions deep to aerated lung [51,55] and the frequent lack of specific abnormalities in horses with non-infectious pulmonary disorders (e.g. equine asthma and EIPH) [55].

2.5. Ancillary diagnostic tests
A complete blood count and serum biochemical analysis is often indicated when investigating lower respiratory tract disorders of suspected infectious or neoplastic origin, however non-infectious disorders of the lower respiratory tract are infrequently associated with alterations in the leukogram or acute phase protein concentrations [3]. Arterial blood gas analysis can be used to assess the effectiveness of alveolar gas exchange. However, it requires the collection of an arterial blood sample and sophisticated equipment, which is gradually becoming more readily available in practice, though mostly limited to referral practices. Additionally, alterations in blood gas parameters are often only detected when collected during maximal exercise in horses with non-infectious lower airway inflammatory conditions [33].
3. Equine asthma

The term “equine asthma” incorporates both horses with mild to moderate (previously inflammatory airway disease – IAD) and severe (previously recurrent airway obstruction - RAO) non-infectious LAI [6]. IAD is a disease of adult horses of any age but more commonly in young horses. IAD is defined by poor performance with or without chronic, intermittent coughing of more than 3 weeks duration, excess tracheal mucus accumulation as well as mild airway inflammation in BALF, without systemic signs of infection or increased respiratory rate and effort at rest. Thoracic auscultation does not usually reveal any abnormalities, although some horses may exhibit subtle wheezes with rebreathing procedures. The absence of overt clinical signs does not rule out IAD [6,56]. Further confirmation of IAD can be achieved by demonstrating abnormal pulmonary function test results [6].

Lower respiratory tract disorders are important causes of poor performance and racing interruptions in athletic horses [10,57] and are of economic concern to horse racing industries, worldwide [2,58]. The reported prevalence of IAD has ranged from 13.8% in the UK [59] to 33% in Australian racing Thoroughbred populations [60]. In other studies, a prevalence of IAD up to 70% in horses presented for poor performance were reported [61]. However, differing definitions of IAD were used in these studies, and further epidemiological studies are required, using accepted, current case definitions [6].

3.1. Putative causes of poor performance in horses with IAD

BALF neutrophilia has been linked with poor racing performance in Thoroughbred racehorses [62]. More recently, it has been shown that horses with IAD had diminished athletic parameters during controlled exercise tests when compared to control horses, including increased heart rate (HR) response, reduced velocity at blood lactate concentrations of 4 mmol/L (V_{La4}) and reduced velocity at HR 200 beats/min (V_{200}) [10]. Similarly, studies have demonstrated that IAD has a negative impact on pulmonary function parameters [63] and physiological responses to exercise [64]. Poor performance in horses with IAD is postulated to result from increased resistance to breathing. Pirrone et al. [65] found that lung resistance (R_{L}) did not progressively decline in horses with IAD as a function of an increased ventilatory level as occurs in
healthy horses [66]. During exercise, healthy horses employ physiological mechanisms, including bronchodilation to increase airway cross-sectional area and decrease lung resistance, to compensate for increased airflow [67]. Horses with IAD may be unable to sustain these alterations because of the mechanical limitation of their narrow, inflamed airways [65]. In addition, horses with IAD had a larger tidal volume (V₁), longer inspiratory time (Tᵢ) and lower respiratory frequency (Fᵣ) during rebreathing manoeuvres at the same ventilatory level as healthy horses [65]. Therefore, at any level of pulmonary ventilation (Vₑ) the Hering-Breuer inspiratory volume threshold ‘off-switch’ was reached later and at a larger Vₑ in IAD horses. Increased Hering-Breuer inspiratory volume threshold is one of the neuronal compensation mechanisms to increased resistance to flow (or Rₑ) [68]. As a consequence of increased Rₑ, horses with IAD have significantly increased work of breathing (Wresp, the work/energy expenditure associated with breathing), greater O₂ requirement by the respiratory musculature and reach the ‘critical point’ of respiration at lower levels of ventilation and exercise intensity than healthy horses, which may restrict athletic performance [65]. The ‘critical point’ of respiration is the point where O₂ requirements by the respiratory machinery would compete with the O₂ demand of the exercising muscles [69]. Airway inflammation may also lead to reduced gas exchange, hypoxaemia and hyperlactataemia at lower velocities and resultant poor performance [33,70]. However the pathophysiological effects of IAD remain unclear as no associations between airway inflammation and parameters of performance, hypoxaemia and airway reactivity were found in other studies [71,72].

3.2. Pathogenesis of IAD
The pathogenesis of IAD is poorly defined, as it is a multifactorial syndrome. A variety of aetiological agents may be involved and contributions to the development of IAD will vary depending on animal age, breed, athletic use and genetics, husbandry practices (including housing, feeding), preventative medicine practices and the distribution of infectious agents [6,56]. IAD may reflect the pulmonary response to one or all of the postulated factors: 1. non-infectious agents (particles and gasses); 2. innate immune system dysfunction; 3. infectious agents; and 4. EIPH [3,6].
Non-infectious agents are likely central in the development of IAD. Horses housed in stables are potentially exposed to aerosolized particles, aeroallergens and gases in a cumulative manner [6]. The introduction of horses to the stable environment is a risk factor for the development of IAD [73-76]. The respirable fraction of stable dust may contain organic and inorganic particles including endotoxin, micro-organisms (bacteria and fungi), fungal wall components, ultra-fine particles (particle size <100 nm in diameter), mite debris, vegetative material, inorganic dusts and noxious gases like ammonia and carbon monoxide [56,77]. Moreover, the concentration of airborne endotoxin in the environment has been shown to be significantly associated with the percentage of neutrophils in airway fluid samples [60]. However, the relative contribution of environmental and stable factors remains unclear. The temperature of inspired air during exercise may also contribute to the development of IAD as cold air (-5°C) resulted in neutrophilia and increased mRNA expression of inflammatory cytokines IL-1, IL-5, IL-6, IL-8 and IL-10 in BALF [47]. However, the role of cold air is likely to be limited [6].

Limited information on the cytokine responses in IAD is available. Metachromatic and eosinophilic forms of IAD have been associated with increased expression of the Th-2 cytokines, IL-4 and IL-5, suggesting a role of aeroallergens and the adaptive immune response [78,79]. In other studies, innate immune and Th-1 mediated responses have been implicated in the pathogenesis of IAD, with increased gene expression for TNF-α, IL-1β, IFN-γ and IL-23 [39,80]. Differences between studies may reflect multiple IAD phenotypes with differing inflammatory pathways involved.

The role of infectious agents in the development of IAD remains uncertain [6]. Earlier studies suggested a role of bacterial agents (Streptococcus equi subsp. zooepidemicus, Streptococcus pneumoniae, Actinobaccillus equuli and Pasteurella spp, Mycoplasma spp.) in the pathogenesis of IAD and speculated that the decreased prevalence of IAD with increasing age of the horse may be attributable to the development of an effective immune response [81-83]. More recently a difference in respiratory tract microbiota between healthy and IAD affected horses was detected, with a larger Streptococcus spp. total number and proportion detected in the IAD horses [84]. However, it remains to be determined if these bacterial species contribute to the
development of IAD or whether their increased presence is as a result of lower airway inflammation and impaired mucociliary action [6]. Several studies concluded that IAD is not associated with viral respiratory tract infections [85,86], while others have demonstrated a decreased total nucleated cell count in BALF and reduced coughing in horses with IAD treated with rest and oral administration of interferon-\(\alpha\), suggesting a viral aetiology [87-89]. However, viral detection was not attempted and the role of viruses remains speculative.

Autologous blood from ruptured pulmonary capillaries during exercise has been suggested as a cause of IAD [90]. The installation of autologous blood causes a neutrophilic pulmonary inflammatory reaction [90]. In another study, inoculation of blood into the bronchial tree induced a subacute inflammatory response, characterised by increased macrophage activity and increased alveolar septal thickness and collagen content in older retired racehorses [91]. In contrast, no alveolar fibrosis was found in younger (2-4 years) horses [92] and this discrepancy may reflect previous lung damage in the older retired racehorses, as a recent study demonstrated that free blood within the airways of horses does not result in a qualitative increase in the amount of interstitial collagen within 8-10 weeks [93]. However, any aetiological associations between EIPH and IAD remain uncertain [6].

3.3. Diagnosis of IAD

3.3.1. Respiratory fluid samples
A presumptive diagnosis of IAD is based on clinical signs and airway endoscopy and is usually confirmed by BALF cytology [6]. While the cytological profile of BALF is influenced by methodology (fluid volume, sampling site, aliquot selection, sample preparation and cell-count method), the following cytological criteria for diagnosis of IAD have been proposed: mild to moderate neutrophilia (>10%) and/or increased mast cells (>5%) and/or eosinophils (>5%) [6]. BALF cell percentages between these and the recommended normal values are equivocal and likely technique dependent [6]. Metachromatic and eosinophilic forms of IAD are common in younger horses [30,36,94,95]. Increased percentage of neutrophils (>20%) in TA samples has been proposed previously as a cytological indicator of IAD [24,96]. More recently, this
cytological phenotype with or without increased tracheal mucus has been termed the syndrome of tracheal inflammation (STI) [97]. Even though tracheal inflammation is highly suggestive of IAD [98], and the association between TA neutrophilia and coughing cannot be refuted [29]; the discordance of TA and BALF cytology and the paucity of data relating tracheal wash inflammation and performance, preclude the link of STI in the aetiology of IAD [6,56]. As such, IAD and STI are now considered separate manifestations of LAI [6,56] and performing both a TA and BAL for investigation of LAI is warranted.

### 3.3.2. Pulmonary function testing

Pulmonary function testing (PFT) (discussed in more detail under the heading, non-invasive diagnostic modalities) can also be used to identify horses with IAD [6]. Standard lung mechanics are usually within reference values in horses with IAD, but airway obstruction can be detected using a rebreathing method [65]. Specialized PFT, including forced expiration and forced oscillation mechanics [35,99], impulse oscillometry [63] and spirometric pulmonary function testing after exercise [28] has also been used to detect IAD. Additionally, airway hyper-responsiveness is common in horses with IAD, especially those with increased eosinophil and/or mast cell percentages [6,100] and can be detected using PFT and bronchoprovocation. Bronchoprovocation is a measure of airway hyper-reactivity, performed by determining dose-dependent alterations in respiratory mechanics after nebulization of histamine or methacholine [35,36,95].

### 3.3.3. Ancillary diagnostic testing

Endoscopic examination of the upper respiratory tract and trachea is useful for detection of concurrent conditions, scoring of mucus accumulation within the tracheal lumen [23] and trans-endoscopic TA sampling for cytology and/or culture [6]. Additionally, horses with IAD may have increased grades of tracheal mucus [6,71], which is associated with coughing [29,101] and poor performance [17,102].

Arterial blood gas analysis may reveal more marked exercise-induced hypoxaemia in horses with IAD with/without EIPH [33,34]. However, exercise-induced hypoxaemia in
IAD horses was not found in a later study [72], although this finding may have been influenced by the athletic ability of the subjects. Nevertheless, usefulness of this method for diagnosis of IAD is uncertain. Thoracic radiography is useful to exclude other diagnoses but is insufficiently sensitive for the diagnosis of IAD [103]. Haematological and serum chemistry profiles are usually within normal limits in horses with IAD but are helpful to rule out infectious respiratory tract conditions.

### 3.4. Suggested subtypes of IAD

Several studies have suggested that multiple subtypes or subcategories of IAD with different aetiologies may be present [72,79,100]. Horses with neutrophilic BALF are more likely to be older, have a cough [100] and have lower exercising arterial oxygen tension [33,34]. Horses with eosinophilia or mastocytosis are often racehorses, younger, examined because of poor performance rather than a cough and have increased baseline respiratory resistance, decreased respiratory reactance and airway hyper-responsiveness (AHR) to histamine bronchoprovocation [36,63,95,100]. Despite these generalisations, the relationship of IAD subtypes and age remains controversial [63,78]. Some horses have both neutrophilic and eosinophilic-metachromatic BALF, termed mixed IAD.

Neutrophilic IAD has been suggested to occur more frequently during the winter months, when horses are stabled and exposed to larger amounts of respirable dust, endotoxins [104] and cold ambient temperatures [47], whereas eosinophilic-metachromatic IAD is suggested to occur during spring and summer, when the concentrations of pollen and aeroallergens are higher, befitting a type-1 hypersensitivity [95]. As such, differing inflammatory cell profiles may result from different aetiologies. The classic allergic Type-1 hypersensitivity involves a T-helper 2 lymphocyte response, characterized by IgE-mediated IL-4, IFN-γ, IL-5, IL-6, IL-13 and eotaxin-2 expression and eosinophil and mast cell recruitment, where as a non-allergic T-helper 1 lymphocyte response to airborne particles, other inhaled stimuli or viral disease result in IL-1β, IL-8, IFN-γ expression and neutrophilic infiltrate [72,78-80]. However, the dominant cytokine involved in each subtype of IAD remains elusive, as the dominant cytokine mRNA expressed with each subtype varies between studies.
A recent study demonstrated a significant association between respirable particulate exposure and BALF eosinophilic inflammation, supporting a role of hypersensitivity to aeroallergens [105]. Whether breed influences IAD phenotype is unclear, however the results of several studies suggest that Thoroughbreds are more likely to have eosinophilic-metachromatic inflammation, with or without neutrophilic inflammation, whereas Standardbred horses, of similar age, are more likely to have neutrophilic inflammation [72], a finding that appears to be supported by other reports [10,34,62,94,106]. Additionally, an indirect association between increasing age and neutrophilic inflammation has been reported [100] although a similar association was not found in other studies [34,72].

### 3.5. Differential diagnoses

The clinical findings of IAD are non-specific and common differential diagnoses include EIPH, RAO/severe equine asthma, obstructive upper airway disease, bacterial bronchitis and bronchopneumonia or pleuropneumonia, viral infections, lungworm and neoplasia [6].

EIPH and IAD commonly coexist, however the relationship between IAD and EIPH remains controversial [6]. Increased risk of EIPH has been associated with LAI as diagnosed by TA cytology [107]. Experimentally, LAI was associated with a significantly increased risk of EIPH [108] and instillation of autologous blood into the airways elicited a neutrophilic inflammatory response [90], suggesting inter-relationships of these two conditions.

Severe equine asthma (RAO and SPA-RAO) and IAD share a number of clinical, cytological and functional findings. While the lack of severe exercise intolerance or laboured breathing at rest in horses with IAD usually permits differentiation from RAO or SPA-RAO, these signs may be very subtle during periods of disease remission. In which case BALF cytology, pulmonary function testing or a mouldy hay challenge will assist with a definitive diagnosis [6]. All three disorders commonly have neutrophilic BALF, but the neutrophilia is less severe in IAD (<25% neutrophils) [35] and elevated BALF mast cells and eosinophils are not usually associated with RAO or SPA-RAO [6].
Multiple upper airway disorders may result in static or dynamic airway obstruction resulting in exercise intolerance or poor performance and in some horses, occasional coughing. These conditions can be distinguished from IAD by an abnormal respiratory noise during exercise, normal BALF cytology and normal tracheal mucus score. However, not all horses with upper airway obstructive disorders produce an abnormal respiratory noise [19,109]. Additionally, both IAD and abnormalities of the upper airways may be concurrent in performance horses [6,19,109], and there is evidence that horses with pharyngeal dysfunction or recurrent laryngeal neuropathy more commonly have concurrent LAI [19,70,110].

Bacterial or fungal pneumonia is usually associated with systemic signs including fever, malaise, weight loss and reduced appetite, leukocytosis and neutrophilia and septic TA fluid. However, there is some epidemiological evidence of a bacterial role in the development of IAD and excess tracheal mucus in young horses and horses recently introduced to training [59,101], therefore consideration of bacterial involvement is warranted.

Horses with viral respiratory tract infections often have more marked signs of disease, including fever, lethargy, cough and nasal discharge, than horses with IAD. Horses infected with lungworm, *Dictyocaulus arnfieldi*, often exhibit chronic coughing, increased respiratory effort and abnormal lung sounds, which are often indistinguishable from equine asthma. The degree of eosinophilic airway inflammation is typically more severe and persistent in horses with lungworm than IAD [6,111]. Thoracic neoplasia may present with various clinical signs, including chronic coughing, which may resemble IAD. Thoracic imaging, histopathology evaluations of pulmonary biopsies and cytology of respiratory fluids may assist with the diagnosis [3,6].

3.6. Management and treatment

Most treatment recommendations for IAD are aimed at environmental modification and anti-inflammatory drugs, but there is limited evidence-based data regarding the efficacy of treatment [6]. Environmental modification is aimed at decreasing exposure to respirable particles incriminated in the development of IAD [73,77,85], through
pasture turnout, the use of bedding and feeds with low dust and endotoxin concentrations [74,112] and improved ventilation [56]. Recently, feeding hay from ground-level instead of from a hay net was demonstrated to reduce exposure to respirable particulates [105].

Anti-inflammatory treatment recommendations for IAD are derived from those demonstrated to be of benefit to horses with RAO. Inhaled corticosteroids, (e.g. fluticasone and beclomethasone) have been demonstrated to improve pulmonary function in horses with RAO [113] and are likely beneficial for the treatment of IAD. Systemic corticosteroids commonly used to treat airway inflammation in horses include dexamethasone and prednisolone [6] and have been shown to rapidly and effectively reduce airway inflammation in horses with RAO [114]. Similar systemic corticosteroid treatment regimens have been recommended for horses with IAD [3].

Additional, less frequently used treatments include mast cell stabilizers such as sodium cromoglycate [94] and low dose interferon-α [88,89]. Recent advances in the treatment of horses with asthma include inhaled nanoparticles of cytosine-phosphate-guanosine oligonucleotides (CpG), modified soluble curcumin derivative and oral supplementation with omega-3 fatty acids [6]. Inhaled CpG oligonucleotides and modified soluble curcumin have been used in horses with RAO [115,116], however the relevance to IAD is unknown. In horses with asthma, administration of omega-3 fatty acids in combination with environmental modification resulted in greater clinical improvement compared to environmental modification alone [117].

The efficacy of bronchodilators in horses with IAD is unknown as controlled studies are lacking. In one study, aerosolized albuterol did not influence gas exchange or performance during treadmill exercise in both healthy horses and horses with IAD [118]. While bronchodilator treatment in horses with IAD remains questionable, it has been proposed that the increased mucociliary clearance associated with the use of clenbuterol may provide benefit to horses with IAD [119].
Treatment of concurrent disorders may also be necessary, such as antimicrobial administration in horses with bacterial airway colonisation and septic TA [3], or furosemide in horses with concurrent EIPH [120].

3.7. Monitoring treatment response

Monitoring the response to treatment in horses with IAD is difficult and there is currently no single accepted method. Although it has been previously recommended that repeat examination and reassessment of BALF be performed to confirm resolution of inflammation before horses with IAD return to intense exercise [56], this is no longer the case. Normalisation of BALF cytology may only occur with effective environmental modification as neutrophilia may persist despite corticosteroid treatment when air quality is unchanged [121,122]. Additionally, treatment with corticosteroids has resulted in reduced airway hyper-responsiveness without BALF cellular percentages and total nucleated cell count improvements [122,123], impeding our ability to monitor resolution of inflammation. Furthermore, there are limitations with the use of BAL as a monitoring tool: BAL is invasive, requires sedation and induces a transient local neutrophilia for at least 48-72 hours [45,46]. Additionally, absolute leukocyte numbers or percentages do not reflect cellular activity and active inflammation [122,124] or pulmonary function derangements [71,99]. Although a visual analogue scale (HOARSI) has proven useful for monitoring the response to treatment and management in horses with RAO (severe equine asthma) [123,125], this scoring system has shown little usefulness in horses with IAD [125]. As such, a method that is easy to use, non-invasive and reflects the level of airway inflammation would allow improved monitoring of treatment response and disease progression.

4. Syndrome of tracheal inflammation

Poor agreement between TA and BALF cytology has been demonstrated by multiple studies [24,26,61,96]. Given that TA and BALF collect secretions from different segments of the lower airways, these samples may allow detection of different conditions including the syndrome of tracheal inflammation (STI) and IAD [6,8,10]. As such, to best assess the lower airways cytologically, a combination of BAL and TA is advised [8,24,96].
STI is synonymous with various other descriptive terms such as tracheal IAD (trIAD) or IAD based on TA findings [8-10,97]. The definition of trIAD or STI is still variable, however this condition is generally accepted to represent a syndrome of lower airway inflammation characterised by a combination of increased tracheal mucus and neutrophilic inflammation identified by TA cytology with or without bacterial colonisation [8-10]. The term STI will be used throughout this thesis and is used to refer to literature using both STI and trIAD when describing airway inflammation diagnosed based on TA findings with or without detection of increased tracheal mucus during tracheobronchoscopy.

The reported prevalence of STI varies from 33% in young Thoroughbreds [85] to 7.1% in UK National Hunt horses that are older than 3 years [9]. Various case definitions have been used to diagnose STI, impeding comparisons between studies. Some authors consider TA neutrophils >20% with or without increased tracheal mucus [61,96,97] sufficient to diagnose STI, while others have suggested the use of a 3-point score for the absence or presence of increased tracheal mucus, increased proportions of neutrophils and total nucleated cell count in the TA [59]. This method likely underestimates milder inflammation and a 9-point system [82] may provide a more discriminatory means of grading tracheal inflammation. More recently, a modified version of this 9-point method has been devised to determine a tracheal inflammatory score (TIS) comprised of the sum of tracheal mucus (0-3), smear cell density (0-3) and neutrophil proportion scores (0-3) [9,126], with STI defined as a TIS ≥6/9 [9,82].

Despite attempts at defining STI, the clinical importance of the syndrome remains uncertain. While increased TA neutrophil percentage has been associated with coughing [29], other studies did not find an association between this parameter and clinical signs and need for treatment [9,127] or poor performance [17]. However, increased tracheal mucus has been associated with poor performance [17,102,128]. In another study, horses with higher TA neutrophil percentages after exercise had lower indices of pulmonary function during inspiration and expiration, suggesting a limit to breathing [28]. Collectively, these findings indicate that STI may be important clinically, however diagnostic criteria need to be established.
Although the tracheal mucus score used in the TIS is different to the more widely accepted tracheal mucus accumulation score by Gerber et al. [23], it is based on the same principle, while ensuring equal weighting of each component of the TIS. The modified 9-point TIS as described by Cardwell et al. [9] will be used throughout this thesis to diagnose STI:

- The tracheal mucus score was based on the presence of visible mucus within the tracheal lumen from 0 (none) to 3 (large pool at and beyond the thoracic inlet).
- The overall smear cell density was assessed at low power and scored from 0 (low) to 3 (high)
- The neutrophil proportion score was a semi-quantitative estimate: 0 (none, few cells or small numbers distributed diffusely, estimated 0-10% of nucleated cells), 1 (small number of aggregated cells, 11-25% of nucleated cells), 2 (moderate numbers, 26-50% of nucleated cells) and 3 (neutrophils are predominant cell type, > 50% of nucleated cells).

5. Exercise-induced pulmonary haemorrhage

Exercise-induced pulmonary haemorrhage (EIPH) occurs commonly in horses undertaking strenuous exercise including Standardbreds, Thoroughbreds and Quarter Horses, polo ponies and 3-day event horses [129-131]. The condition has considerable economic impact due to missed training days, impaired performance, cost of treatment and wastage [58]. The reported prevalence of EIPH depends on the population examined, the criteria for diagnosis and the frequency of examination. The prevalence of EIPH in Thoroughbreds after one race is 47-75% when diagnosed with detection of blood during endoscopic examination [58,132] while 100% of Standardbreds and Thoroughbreds examined endoscopically after three races had blood in their trachea at least once [132]. When using evaluation of haemosiderophages in BALF for diagnosis of EIPH, the prevalence is approximately 90% in racehorses [107,133].
5.1. Influence on performance

A significant negative association between EIPH and race performance in Thoroughbred racehorses has been demonstrated in several studies \[58,134,135\]. Using endoscopic grading of tracheal blood, horses with higher EIPH grades were less likely to win or place and more likely to finish a longer distance behind the winner \[58,135\]. Another study found that an inferior finishing position was associated with both recurrence and severity of EIPH \[134\]. In a long term, longitudinal observational study, no association between EIPH Grades 0, 1, 2 and 3 and long-term racing performance was found, while severe EIPH (Grade 4) was associated with measures of long term performance and a gradual decrease in total career earnings as EIPH grade increased \[136\].

5.2. Predisposing factors

Experimental mild pulmonary inflammation has been associated with a significantly increased risk of developing EIPH \[108\]. Additionally, a significant association between a tracheal inflammatory score and EIPH has been demonstrated \[107\], while another study demonstrated increased concentrations of inflammatory markers, including lipid peroxidases and platelet activating factor bioactivity, in BALF of EIPH positive group compared with control horses \[137\]. However, other studies did not find an association between haemosiderophage and neutrophil percentages in BALF from horses with IAD \[34,138\]. As such, the association between IAD and EIPH remains to be clarified. An association between EIPH and bacterial airway infection has not been found \[107\]. Other factors associated with increased risk of developing EIPH include shorter race distance, measures of racing intensity (time in training, lifetime starts and total earnings), ambient temperatures less than 20°C, environmental contamination evident in TA samples, prior episodes of EIPH and increasing age \[58,107,139,140\]. However, when the measures of racing intensity are included in the statistical analysis, the importance of age diminishes \[139\]. Increased incidence of EIPH, associated with measures of racing intensity and prior episodes of EIPH, suggest that repeated episodes of haemorrhage result in progressive pulmonary damage \[7\].
5.3. Pathogenesis
The most widely accepted hypothesis for the pathogenesis of EIPH is alveolar capillary wall stress failure subsequent to exercise-induced pulmonary hypertension, secondary to increased cardiac output, and chronic pulmonary vascular and interstitial remodelling [7,141,142]. Repeated bouts of regional (caudo-dorsal) pulmonary venous hypertension during strenuous exercise, associated with negative extravascular pressures, result in regional pulmonary vein remodelling. The resultant occlusion leads to regional pulmonary capillary hypertension, capillary stress failure and bleeding, haemosiderin accumulation, localised inflammation, bronchial circulation angiogenesis and lung fibrosis [92,142-144]. The reported gross lesions of EIPH includes bilateral, symmetrical dark red discolouration of the visceral pleura of the caudo-dorsal lung fields [7]. Histologically, scattered bronchiolitis, haemosiderophagel accumulation, alveolar and interstitial fibrosis, angiogenesis and remodelling of the small pulmonary veins characterised by accumulation of adventitial collagen, smooth muscle hyperplasia and reduced vascular luminal diameter, have been identified [143,145].

5.4. Diagnosis
The method of choice for the diagnosis of EIPH is endoscopic visualisation of blood within the tracheal lumen within 2 hours of exercise [146]. A grading system has been recommended to quantify the degree of haemorrhage; grade 0, no blood was visualised; grade 1, presence of 1 or more flecks of blood or ≤2 short (less than a quarter of the tracheal length), narrow (<10% of the tracheal surface area) streams of blood in the trachea or mainstem bronchi visible from the tracheal bifurcation; grade 2, a long (> half the tracheal length) stream of blood or >2 short streams occupying less than a third of the tracheal circumference; grade 3, multiple distinct streams of blood covering more than a third of the tracheal circumference but no blood pooling at the thoracic inlet; and grade 4, multiple coalescing streams of blood covering >90% of the tracheal surface with pooling of blood at the thoracic inlet [146]. However, the sensitivity of this scoring system has been questioned as the amount of blood mobilised by mucociliary clearance into the trachea after exercise varies and one horse may appear unaffected after one race but may have severe EIPH after subsequent races [7,132].
BALF cytology is quantitative and more sensitive than tracheal endoscopy for the diagnosis of EIPH [32] and does not have to be performed within a certain time of exercise [62]. However, this method only samples a small portion of the lung and the location of the bleed may be removed from the sampling site. In one study, all strenuously exercised horses exhibited EIPH, with both free erythrocytes and alveolar macrophages containing erythrocytes and erythrocyte breakdown products (haemosiderophages) detectable within the BALF [32]. These authors found that the number of erythrocytes increased significantly shortly after exercise and returned to pre-exercise values within one week, but the number of haemosiderophages did not increase until one week after exercise and remained increased for 3 weeks before returning to baseline by the fourth week post-exercise [32]. As such, the extent of EIPH can easily be overestimated because of the slow turnover time of haemosiderophages [147]. This prompted the adoption of a scoring system, using slides stained with Perl’s Prussian blue, to quantitatively evaluate the haemosiderin content of alveolar macrophages to obtain a total haemosiderin score (THS) [31]. The THS was significantly greater in horses with EIPH, compared to control horses and was strongly associated with the presence of large amounts of erythrocytes in BALF [31].

The THS is determined by counting and grading 300 alveolar macrophages according to the intensity of (blue) staining of macrophages on an ordinal scale (0-4) [148] and calculating the percentage of cells per grade, which are then multiplied by the numerical value of the grades and added. The maximum possible score is 400 and a cut-off THS of 75 has a 94% sensitivity and 88% specificity for EIPH [31]. More recently a simplified cytological definition of EIPH using BALF, adopted from Newton & Wood 2002 [107] has been suggested; BALF in which >20% of macrophages are haemosiderophages are classified as EIPH positive [10], but the validity of this method was not investigated in this study. Therefore, the THS remains the most reliable and objective assessment tool for EIPH diagnosis using BALF cytology.

Tracheal aspirates may also be used to diagnose EIPH by detection of haemosiderophages as >50% of macrophages [107]. Thoracic radiography may detect increased radio-opacity in the caudo-dorsal lung fields in some horses with EIPH,
however this modality has low sensitivity and specificity for EIPH [149]. Pulmonary scintigraphy has been described to evaluate ventilation/perfusion (V/Q) ratio and regional lung dysfunction in horses with EIPH [149] but is rarely used clinically. Despite all of the described diagnostic methods for EIPH, there is still no clinical or laboratory ‘gold standard’ method to provide an accurate, reproducible means of quantifying the severity of bleeding.

5.5. Treatment

Treatment of EIPH is aimed at reducing the prevalence and severity of the condition, as curative or preventative treatment remains unachievable at present. Furosemide is commonly used to treat EIPH as it decreases the occurrence and severity of bleeding [120,150], however the use of this drug is not allowed by racing jurisdictions in Australia and New Zealand. However, this agent does not stop bleeding in many treated horses [132]. Furosemide decreases pulmonary vascular pressure in horses during high-speed treadmill exercise, by decreasing plasma volume through its diuretic effects and possibly by venodilation [144]. Additional, less established, beneficial effects of Furosemide include bronchodilation and anti-inflammatory effects [151-153]. Furosemide is associated with improved performance in both Thoroughbred and Standardbred racehorses [154]. Use of other pharmacological interventions are not currently recommended [154].

A nasal dilator device (nasal strip) can be used to reduce the severity of, but not eliminate EIPH through dilation of the nares and decreasing negative inspiratory thoracic pressures and the magnitude of alveolar capillary transmural pressure differences [150,155,156].

Monitoring treatment response in horses with EIPH is challenging due to the limitations of the available diagnostics techniques. A non-invasive method to monitor treatment response and progression of EIPH would be invaluable.
6. Non-conventional diagnostic modalities

Most of the conventional diagnostic methods for direct assessment of the lower airways are invasive, often require sedation, are not without sequelae and do not provide information about the level of active airway inflammation. As BAL induces a transient neutrophilic airway inflammation for up to 72 hours [45,46], it is not suitable for frequent monitoring of horses. In addition, some horses with IAD may lack cytologic evidence of inflammation, but exhibit pulmonary dysfunction [71], moreover the presence of a certain cell type does not equate to cellular activity [124]. Therefore, alternative diagnostic and monitoring methods for LAI in horses are desirable.

6.1. Advanced imaging modalities

While computed tomography and magnetic resonance imaging provide information about soft tissue structures, the usefulness of these modalities to assess the lower respiratory tract of adult horses is precluded by size constraints of the table [157]. Both CT and MRI can be used to investigate pulmonary disease in foals [158], however application is restricted by equipment expense, limited availability and the need for general anaesthesia [3].

Scintigraphy with gamma-emitting radioisotopes such as krypton-81m or technetium-99m can be used to non-invasively assess pulmonary ventilation and perfusion in the horse [159]. Pulmonary scintigraphy permits evaluation of ventilation/perfusion (V/Q) ratios and regional lung function. Additionally, it may provide further insight into the pathogenesis and diagnosis of equine asthma or EIPH [149,160], although availability and cost may preclude the use of this modality to monitor disease.

6.2. Diagnostic modalities allowing direct pulmonary assessment

Non-invasive diagnostic methods available for direct assessment of the lower respiratory tract include pulmonary function testing and the analysis of volatile and non-volatile constituents in exhaled breath (EB) and exhaled breath condensate (EBC). These methods are used widely in human medical research [161-163] but little information regarding the use in equine medicine exists.
6.2.1. Pulmonary function testing

Multiple pulmonary function testing (PFT) methods have been developed for use in horses but many are invasive, require specialist equipment, are technically demanding and often require heavy sedation. Conventional lung mechanics involves the placement of an oesophageal balloon-tipped catheter within the thoracic oesophagus to measure pleural pressure changes, and a pneumotachograph, held in place with a face mask, to simultaneously measure flow at the nostrils [164]. While this technique has been considered the “gold standard” of PFT in the horse, it is moderately invasive and has low sensitivity for measurement of small airway obstruction [11].

Measurement of the maximal volume that can be exhaled in 1 second through forced expiratory manoeuvres, termed forced expiratory volume, is the principle technique for diagnosis of asthma in humans, however it requires patient compliance [165]. In horses, this technique requires heavy sedation [166] or even general anaesthesia, nasotracheal intubation, mechanical ventilation and exposure to a vacuum reservoir, which draws gas out of the lungs, inducing a single, forced exhalation. This method has been shown to differentiate between normal horses and those with IAD and RAO [166].

Forced oscillatory mechanics (FOM) are non-invasive methods that rely on the relationship between pressure and flow during the application of an external force [167,168]. Measurable pressure and flow, over and above that generated by the horse’s own respiratory system, is generated by forcing oscillations into the respiratory system via a facemask [167,168]. This technique frequently requires sedation, commonly induces glottic closure or excessive swallowing and measurement issues occur with altered breathing patterns, as with RAO, excitation or bronchoprovocation, however it is more sensitive than conventional PFT [63,168].

Flowmetric plethysmography (FP), involves measurement of changes in the cross-sectional area of the abdomen and thorax, which is used to detect changes in volume and flow at the level of the thorax, and to determine the relative contributions of chest and abdomen to breathing [169]. Flow from the nares is measured with a
pneumotachograph. This technique is non-invasive, suited to field-testing and typically does not require sedation, but it is not as sensitive as FOM [11].

PFT in human medicine often employs spirometric measurements of peak flow rates and time-flow relationships during a forced, maximal expiration [170]. Indices of pulmonary function based on measurements of breath-by-breath spirometry during maximal exercise, in horses, has been described [171]. Spirometry in horses at rest is often too insensitive to diagnose alterations of pulmonary function [172] and spirometry during exercise is a technical challenge. Therefore, spirometric PFT shortly after maximal exercise has been described [28,173]. This technique allows limits of breathing to be measured by analysis of relationships between relative gas flows at standardised relative times in single breaths after exercise [28]. More recently, a study of absolute and relative flow-time measures of pulmonary function during eupnoeic breathing and during CO₂ rebreathing demonstrated strong agreement of respiratory frequency and multiple measures of absolute peak expiratory flow and less variation during rebreathing [174]. However, significant differences between individual horses were observed, suggesting that while parameters with high repeatability/reliability may be useful to evaluate pulmonary function [174], further investigation of spirometry is required before application as a diagnostic technique.

Most PFT methods are not sensitive enough to detect small alterations of pulmonary function or mild airway obstruction/narrowing, as frequently occurs in IAD. There are two methods to increase the sensitivity of PFT: hyperventilation by chemical stimulation of ventilation using a hypercapnoeic rebreathing technique [65,174] or intravenous administration of lobeline [169], and bronchoprovocation. Bronchoprovocation is the measure of airway hyper-responsiveness (AHR), whereby one determines the dose of a nebulized bronchoconstrictor agent, such as histamine [11,100,175] or methacholine [168], that results in changes in certain respiratory parameters. These parameters are; an increase in respiratory system resistance when using FOM or conventional PFT [11,49,100] or increased $\Delta$flow by 35% when using FP [175]. The dose of bronchoconstrictor required to induce these changes, is lower in horses with LAI compared with healthy horses.
7. Exhaled breath condensate analysis

Exhaled breath (EB) and exhaled breath condensate (EBC) has been studied extensively in human medical research. Alterations in concentrations of several EB/EBC biomarkers have been found with inflammatory diseases of the lower airways, including asthma [161,176,177], chronic obstructive pulmonary disease (COPD) [162,178], bronchiectasis [178,179] and cystic fibrosis [163]. These studies have established the value of non-invasive approaches to investigate and monitor LAI. Collection of EB/EBC is non-invasive, easy to perform and can be repeated frequently as samples are not influenced by previous sampling, however biomarker concentrations may be influenced by methodological, physiological and environmental factors [180-182].

The gaseous phase of EB represents a mixture of various volatile molecules within the airways [183], of which the most frequently measured are nitric oxide (NO), carbon monoxide (CO), ethane and pentane [161,184-186]. EB collection is technically simple however analyses of most EB biomarkers require sophisticated equipment.

EBC describes sample collection by cooling of EB allowing sample acquisition in either a liquid or frozen state [187]. Measurement of EBC constituents is based on the hypothesis that aqueous aerosols contain a variety of components that reflect concentrations within the epithelial lining fluid (ELF) of the lower airways. EBC contains many non-volatile molecules, including cytokines, adenosine, arachidonic acid metabolites, 8-isoprostane, surfactant, lipids, histamine, serotonin and electrolytes [187], as well as water-soluble volatile molecules, including hydrogen peroxide (H₂O₂), ammonia and ethanol [188]. Volatile molecules form part of EBC due to diffusion and equilibration between the gaseous and aqueous phase of breath whereas the exact mechanism through which the non-volatile components enter EB remains unknown [189]. Previously, it was accepted that ELF became aerosolized during turbulent airflow [190,191] however, the formation of aerosols according to the bronchial fluid film burst (BFFB) model occurs when bubble bursting of the ELF occurs during opening of the bronchioles following exhalation [192]. This has recently been the more widely accepted mechanism of aerosol production [193,194]. The most commonly measured EBC parameters in veterinary medicine are H₂O₂, pH and leukotriene B₄ (LTB₄)
[195,196]. Reported normal values of these biomarkers in veterinary species are presented in Table 2.2, while the effect of pulmonary disorders on these biomarkers in both human and veterinary medicine are provided in Table 2.3.
<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Species</th>
<th>Collection method: material, collection temperature and breathing conditions</th>
<th>Analytical method</th>
<th>Reported values</th>
<th>Reference</th>
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<tr>
<td>Hydrogen peroxide</td>
<td>Horse</td>
<td>Stainless steel condenser, ice water at 0°C, conscious breathing</td>
<td>Spectrophotometric assay</td>
<td>0.2-0.6 μmol/L</td>
<td>Deaton et al. [126]</td>
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<tr>
<td></td>
<td>Dog</td>
<td>Plastic condenser, liquid nitrogen/ethanol slurry at -80°C, conscious breathing</td>
<td>Spectrophotometric assay</td>
<td>1.0-3.6 μmol/L</td>
<td>Wyse et al. [197]</td>
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<td></td>
<td>Cat</td>
<td>Stainless steel condenser, ice water at 0°C, plethysmography</td>
<td>Spectrophotometric assay</td>
<td>0.6-0.8 μmol/L</td>
<td>Kirschvink et al. [198]</td>
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<td>Calf</td>
<td>Teflon coated condenser (EcoScreen), -10°C to -20°C, spontaneous breathing</td>
<td>Amperometric biosensor (Eco-Check)</td>
<td>0.02-2.2 μmol/L</td>
<td>Knobloch et al. [180]</td>
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<td></td>
<td>Horse</td>
<td>Polypropylene condenser beaker, liquid nitrogen/ethanol slurry (-80°C) conscious breathing</td>
<td>Spectrophotometric assay</td>
<td>0.5-4.1 μmol/L</td>
<td>Duz et al. [195]</td>
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<td>Foal</td>
<td>Teflon coated condenser (EcoScreen), -10°C to -20°C, sedated spontaneous breathing</td>
<td>Chromogenophotometric based method (d-ROMs exhalation kit and FREE system)</td>
<td>21–32 μmol/L</td>
<td>Crowley et al. [199]</td>
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<tr>
<td>pH</td>
<td>Calf</td>
<td>Teflon coated condenser (EcoScreen), -10°C to -20°C, spontaneous breathing</td>
<td>ISFET electrode and ABL 550 blood gas analyser, non-de-aerated samples</td>
<td>5.43-6.46 (ISFET)</td>
<td>Knobloch et al. [180]</td>
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<td>5.34-6.37 (ABL550)</td>
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<td>Horse</td>
<td>Polypropylene condenser beaker, liquid nitrogen/ethanol slurry (-80°C) conscious breathing</td>
<td>Model pH 21 meter, non-de-aerated samples</td>
<td>5.36-6.61</td>
<td>Duz et al. [195]</td>
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<td>Horse</td>
<td>Polypropylene condenser tube, dry ice at -75°C, conscious breathing</td>
<td>Model pH 21 meter, neat samples</td>
<td>4.3–4.6 (pasture)</td>
<td>Whittaker et al. [200]</td>
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<td></td>
<td>Horse</td>
<td>Polypropylene condenser tube/beaker, dry ice at -75°C, liquid nitrogen-ethanol slurry (-80°C) and ice water 0°C, conscious breathing</td>
<td>Model pH 21 meter, neat and deaerated samples</td>
<td>4.61–5.02 (stabled)</td>
<td>Whittaker et al. [201]</td>
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<tr>
<td></td>
<td>Horse</td>
<td>Polypropylene condenser beaker, dry ice at -75°C, conscious breathing (pre- &amp; post-exercise)</td>
<td>Model pH 21 meter, neat and deaerated samples</td>
<td>Neat:* 6.48-7.26/ 7.32-7.66, 4.1-4.5/ 5.72-7.18, 5.25-6.43/ 5.74-6.78, Deaerated:* pH increased by 1.1/0.45 @ 0°C, 2.21/1.18 @ -75°C &amp; 1.79/1.51 @ -80°C</td>
<td>Cathcart et al. [202]</td>
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<tr>
<td>Leukotriene B4</td>
<td>Calf</td>
<td>Teflon coated condenser, -15°C to -20°C, conscious breathing</td>
<td>Enzyme-linked immunosorbent assay (ELIZA)</td>
<td>60–170 pg/ml</td>
<td>Reinhold et al. [196]</td>
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<td>Dog</td>
<td>Glass condenser, ice water 0°C, general anaesthesia</td>
<td>LTB4 Enzyme immunooassay (EIA)</td>
<td>15–94 pg/ml</td>
<td>Pietra et al. [203]</td>
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<td>Pig</td>
<td>Teflon coated condenser (EcoScreen), -10°C to -20°C, sedated spontaneous breathing</td>
<td>LTB4 Enzyme immunooassay (EIA)</td>
<td>20-180 pg/ml</td>
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*tube condenser/beaker condenser
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<tr>
<th>Pulmonary disorder</th>
<th>Biomarker</th>
<th>Effect</th>
<th>Comments</th>
<th>References</th>
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<tr>
<td>Asthma</td>
<td>pH</td>
<td>↓</td>
<td>Consistent finding between studies, correlates with disease severity and</td>
<td>Kostikas et al. [178], Antus et al. [206], Carraro et al. [207], Hunt et al. [208]</td>
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<td></td>
<td>H₂O₂</td>
<td>↑</td>
<td>H₂O₂ concentrations elevated in unstable steroid treated asthmatics and</td>
<td>Horváth et al. [209]</td>
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<td>steroid-naive compared to stable steroid treated patients</td>
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<td></td>
<td>LTB₄</td>
<td>↑</td>
<td>Increased in asthmatic patients with or without current corticosteroid</td>
<td>Kostikas et al. [176], Hanazawa et al. [210]</td>
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<td></td>
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<td>treatment</td>
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<td>COPD</td>
<td>H₂O₂</td>
<td>↑</td>
<td>H₂O₂ concentrations, reliably and with high sensitivity, increase in</td>
<td>Kostikas et al. [162]</td>
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<td></td>
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<td>patients with COPD.</td>
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<td></td>
<td>pH</td>
<td>↓</td>
<td>Decreased EBC pH in patients ventilated due to severe COPD, accompanied</td>
<td>Gessner et al. [211]</td>
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<td></td>
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<td>by a decrease in EBC concentrations of ammonia</td>
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<td>Cystic fibrosis</td>
<td>pH</td>
<td>↓</td>
<td>EBC pH reduced in CF patients with further reductions during exacerbations of disease</td>
<td>Ojoo et al. [212]</td>
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<td></td>
<td>H₂O₂</td>
<td>–</td>
<td>No difference detected between clinically stable CF patients and normal</td>
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<td>control patients</td>
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<td>Bronchiectasis</td>
<td>H₂O₂</td>
<td>↑</td>
<td>H₂O₂ concentrations increased in patients with bronchiectasis with strong correlation with disease severity</td>
<td>Loukides et al. [179]</td>
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<td>ARDS</td>
<td>H₂O₂</td>
<td>↑</td>
<td>Significant increase in EBC H₂O₂ concentrations in ventilated patients</td>
<td>Baldwin et al. [214]</td>
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<td></td>
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<td>which developed ARDS compared to those who did not</td>
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<td><strong>Veterinary medicine</strong></td>
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<td>RAO in horses</td>
<td>H₂O₂</td>
<td>↑</td>
<td>Significant increases in horses with RAO compared to healthy controls</td>
<td>Deaton et al. [126]</td>
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<td></td>
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<td></td>
<td>– No difference after environmental antigen exposure compared to healthy</td>
<td>Wyse et al. [185], Deaton et al. [215], Wyse et al. [216]</td>
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<td>horses</td>
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<td>– No difference between horses with RAO or IAD and healthy horses</td>
<td>Duz et al. [195]</td>
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<td>– Non-significant trend for EBC acidification in horses with RAO/IAD</td>
<td>Duz et al. [195]</td>
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<td>compared to healthy horses</td>
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<td>↑ Stabling of horses causes significant increase in EBC pH</td>
<td>Whittaker et al. [200]</td>
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<td>↑ High intensity exercise causes significant increase in EBC pH</td>
<td>Cathcart et al. [202]</td>
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<td></td>
<td>LTB₄</td>
<td>↑</td>
<td>Increased concentrations in horses with RAO. Samples collected in standing,</td>
<td>Fey et al. [217]</td>
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<td>intubated horses (healthy horses not examined)</td>
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<td>Rhodococcus equi in foals</td>
<td>H₂O₂</td>
<td>↑</td>
<td>Significant increase in EBC H₂O₂ concentrations in foals with ultrasound</td>
<td>Crowley et al. [199]</td>
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<td>detectable lung consolidation on <em>R. equi</em> endemic farms compared to control foals (without lung consolidation)</td>
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<tr>
<td>Bacterial pneumonia in calves</td>
<td>LTB₄</td>
<td>↑</td>
<td>Increases detected after experimental <em>Pasteurella multocida</em> infection</td>
<td>Reinhold et al. [196]</td>
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<tr>
<td>Viral pneumonia in calves</td>
<td>LTB₄</td>
<td>↑</td>
<td>Increases detected after experimental bovine respiratory syncytial virus infection</td>
<td>Reinhold et al. [196]</td>
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<td>Mycoplasma pneumonia in calves</td>
<td>pH</td>
<td>↑</td>
<td>EBC acidification in calves with mild (clinically latent) experimental Mycoplasma pneumonia</td>
<td>Schröder [218]</td>
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<tr>
<td>Feline lower airway inflammation</td>
<td>H₂O₂</td>
<td>↑</td>
<td>Increased after an allergen-challenge in <em>Ascaris suum</em>-sensitised cats in an experimental study</td>
<td>Kirschvink et al. [198]</td>
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<tr>
<td>Clamydia suis pneumonia in pigs</td>
<td>LTB₄</td>
<td>↑</td>
<td>Increased after experimentally induced <em>Chlamydia suis</em> pneumonia</td>
<td>Reinhold et al. [204]</td>
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CF, cystic fibrosis; COPD, chronic obstructive pulmonary disease; H₂O₂, hydrogen peroxide; IAD, inflammatory airway disease; NH₃, ammonia; RAO, recurrent airway obstruction; ARDS, Acute respiratory distress syndrome.
7.1. pH

EBC acidification has been reported in humans [178,208,219] and calves [218] in response to LAI, suggesting that pH may be useful to detect acid-base disturbances of the ELF caused by inflammation. EBC pH is not a specific biomarker but rather a complex parameter reflecting the acid-base balance of the respiratory tract and may be influenced by volatile molecules including volatile acids, ammonia and CO₂ and non-volatile molecules, including cations and anions [189]. Airway pH is regulated by multiple factors including proton pump activity [220], glutaminase secretion and resultant ammonia production by respiratory epithelium [221], surfactant secretion and buffering from proteins and bicarbonate [222] as well as acid and base equivalents secreted from airway glands [223]. Airway acidification mechanisms in response to pulmonary disease are unknown but may be due to volatile acid production by activated or increased numbers of granulocytes [178], up-regulation of epithelial proton pump activity, micro-aspiration of gastric contents [222] and decreased production of ELF buffers, specifically down-regulation of glutaminase activity in response to exposure to pro-inflammatory cytokines resulting in reduced production of ammonia [221].

7.1.1. pH alterations in response to disease and subject variables

EBC pH in asthmatics is significantly lower compared with healthy humans and treatment with corticosteroids restores normal pH values, suggesting that pH is a reliable indicator of airway inflammation [208]. Airway acidification has also been demonstrated in COPD [178,219], cystic fibrosis [163], bronchiectasis [178] and acute lung injury [211]. EBC pH has also been demonstrated to be significantly correlated with eosinophilic and neutrophilic airway inflammation in humans [178].

A study in calves, with mild experimental Mycoplasma infection, demonstrated a decrease of EBC pH indicating that EBC acidification might be an early marker of subclinical pulmonary inflammation [218]. Similarly, a study in horses demonstrated a non-significant trend for lower EBC pH in horses with LAI compared with control horses [195]. High intensity exercise has been shown to result in a significant increase in EBC pH in neat EBC in a study of racing Thoroughbred horses [202], but the
mechanism for these changes remain unknown. Positive correlations between EBC pH and ambient and exhaled ammonia concentrations have been demonstrated in humans [224] and healthy ponies [200]. Ammonia is produced by airway epithelial cell-associated glutaminase and likely contributes to airway homeostasis by acting as a buffer [221]. Upregulation of glutaminase and subsequent ammonia production may occur in response to acidic stress [221]. In a study of racehorses, animals bedded on wood shavings, compared to paper bedding, had larger increases in EBC pH post-exercise; the stables with wood shavings had poorer ventilation and possibly greater ambient ammonia concentrations, but ambient ammonia was not measured [202]. The impact of elevated ambient ammonia concentrations on equine airway epithelium, glutaminase activity and its association with LAI remains to be established [202]. EBC pH has been demonstrated to be independent of variables of spontaneous breathing in both humans and animals [180,202,225].

7.1.2. Variability, influencing factors and measurement considerations

EBC pH is easily measured and has been shown to be repeatable with a low intra-subject coefficient of variation (CV) [195,225], minimal inter- and intra-day variation and excellent intra-sample repeatability [195]. However, a study in calves demonstrated a significant circadian variation, with EBC pH increasing throughout the day [180]. Additionally, collection temperature, has been shown to influence pH of equine EBC, with EBC collected as a liquid resulting in a significantly higher pH when compared to frozen EBC, likely due to evaporation of volatile components of EBC or on-going biochemical processes [201]. Less variation in sample pH collected as a liquid and analysed within 2 hours compared to EBC collected frozen, was found in one study [201]. Conversely, studies evaluating the influence of different condensing temperatures in healthy human subjects concluded that EBC pH was not influenced by collection temperature [225,226]. However, other authors suggest that when EBC is collected from subjects with acidic EBC, such as occurs with asthma or COPD, freezing during sample collection results in reduced volatile acid capture resulting in increased EBC pH [227]. Therefore, the ideal collection temperature for measurement of EBC pH remains unknown.
In humans, an influence of environmental temperature and humidity was found, with increased EBC pH during periods of dry, warm weather and reductions in EBC pH during cold, humid weather [181]. However, environmental effects on EBC pH were not found in horses [202], nor calves [180]. The temperature at which EBC pH is measured has been shown to significantly influence EBC pH values; pH was significantly higher at measurement temperatures of 37°C compared with 23°C [228]. Thus, it is recommended that the temperature at which pH measurements in EBC are performed be standardised and declared. Storage of EBC at -80°C has been shown to have little effect on pH in deaerated EBC in human and neat and deaerated EBC in equine studies [201,229], suggesting that the pH of EBC is stable when frozen for up to two years [225]. However, Davis and Hunt [227] found that EBC samples with low pH that have been frozen and the container opened before thawing, resulted in an increase in pH compared with the initial measurement. The authors hypothesised that this is due to sublimation of volatile acids from the frozen EBC and loss of these components when the container is opened and therefore recommend that all EBC samples should be allowed to thaw and agitated to enable resuspension of the volatile acids before opening the container.

In 2005, the American Thoracic Society/European Respiratory Society (ATS/ERS) task force on EBC [187] stated that deaeration of EBC should be performed to enhance the stability of pH measurement. This is achieved by removal of volatiles, specifically CO₂, by deaeration with an inert gas such as argon, nitrogen or oxygen, which raises pH by approximately 1 unit [229]. Some authors consider deaeration of EBC with argon [225] or standardisation of sample PaCO₂ [230] necessary to obtain reliable pH measurements, whereas others argue that neat samples reflect the “biological” pH of the airway surface, as CO₂ is a relevant component of EBC [180,231-233]. In studies of EBC in horses, variable results have been documented with regards to deaeration. In some studies intra-sample repeatability of pH was better using neat samples than with argon gas deaeration [195,202]. However, in another study with a larger sample size, deaeration of EBC resulted in lower variation of pH compared to neat samples [201]. As a consensus between human and veterinary literature has not been reached, measurement of pH in both neat and deaerated pH may be required initially. Factors
such as gender, age, volume of collected EBC and duration of EBC collection have not been demonstrated to influence EBC pH [225,227].

7.2. Hydrogen peroxide

H₂O₂ is produced by the conversion of superoxide anions O₂⁻ to H₂O₂ by superoxide dismutase [234,235] during the respiratory burst of activated inflammatory cells, such as neutrophils, eosinophils and macrophages in response to oxidative stress [236]. Increased concentrations of EBC H₂O₂ are indicative of increased reactive oxygen species (ROS) production and/or impaired removal. In humans, the epithelial cells of the conducting airways are postulated to be a source of EBC H₂O₂, as concentrations were flow dependent [182]. This theory is supported by the findings of a study that collected EBC by fractional sampling to separate collection of EBC originating from the proximal airways compared to the distal airways/alveoli by adjusting resistance to breath flow [237]. This study demonstrated that the concentration of H₂O₂ in the EBC collected from the proximal airways was 2.6 times higher than that in the alveolar fraction in all 26 subjects of variable respiratory health [237].

7.2.1. H₂O₂ alterations in response to disease

In human medical research, EBC H₂O₂ concentrations have been shown to correlate with the presence and severity of asthma [177,238], response to treatment in asthmatics [239], bronchiectasis [179,240], COPD [241], pneumonia [242] and multiple other disorders. A relationship between the number of eosinophils and neutrophils in sputum and increased EBC H₂O₂ concentrations has also been demonstrated [161,240].

In horses with RAO, the concentration of H₂O₂ in EBC was positively correlated with BALF neutrophil percentage and TIS [126]. Similarly, a positive correlation between EBC H₂O₂ concentration and BALF neutrophil and eosinophil percentages in healthy and Ascaris suum (AS)-sensitised cats, respectively, as well as a significant increase in EBC H₂O₂ concentrations after an allergen challenge in the AS-sensitised cats, has been reported [198]. Recently, a significant increase in EBC H₂O₂ concentration in foals with R. equi pneumonia, compared with control foals was demonstrated, while there were no significant differences in traditional measures of inflammation (leukocyte count and
neutrophil count in whole blood and plasma fibrinogen concentration) between these two groups [199]. It is likely that the concentrations of H$_2$O$_2$ in EBC reflect the degree of replication and cellular activity of *R. equi* [199]. Therefore, EBC H$_2$O$_2$ and may be used as a monitoring tool to aid diagnostic and therapeutic decision-making in foals with *R. equi* pneumonia. Additionally, EBC H$_2$O$_2$ concentrations have been found to increase significantly in sheep during periods of heat stress and with reduced dietary selenium (an anti-oxidant and free radical scavenger) intake [243]. However, in other studies, increases in EBC H$_2$O$_2$ concentration could not be demonstrated in RAO horses exposed to an environmental challenge [215,216]. Similarly, Duz *et al.* [195] was unable to detect an increase in H$_2$O$_2$ concentration in horses with LAI compared with control horses. The latter study demonstrated a large coefficient of variation in H$_2$O$_2$ concentration within individual subjects due to large intra- and inter-day variability [195], which may explain the lack of significant difference between healthy and diseased.

The source of exhaled H$_2$O$_2$ in horses with RAO is postulated to be neutrophils, based on the strong correlations between H$_2$O$_2$ concentration and neutrophil number and percentage [126]. Increased concentrations of H$_2$O$_2$ in EBC may depend, in part, on neutrophil activity, which cannot be determined from cell counts. Therefore EBC H$_2$O$_2$ concentration may be a more accurate measure of respiratory tract inflammatory status than BALF cell counts or percentages and TA inflammatory scores [126].

### 7.2.2. Variability, influencing factors and measurement considerations

Many studies have found large intra- and inter-subject variability in EBC H$_2$O$_2$ concentrations. In human studies, large CVs for EBC H$_2$O$_2$ concentration have been found, in both healthy individuals and patients with COPD [244]. This has been speculated to be as a result of circadian rhythms and expiratory flow rates [244]. However, despite this variation, EBC H$_2$O$_2$ concentrations are often higher in patients with asthma [239], viral respiratory diseases [245], COPD [246] and bronchiectasis [179] compared with healthy subjects. Similarly, substantial intra-and inter-day variation in EBC H$_2$O$_2$ concentrations has been found in control horses and horses with LAI [195]. In a study of healthy dogs, highly variable H$_2$O$_2$ concentrations within and between individuals were reported [197]. However, in another study, high within-
animal reproducibility of \( \text{H}_2\text{O}_2 \) concentrations in calves was demonstrated when the influence of food intake and time of day was taken into consideration [180]. In the same study, inter-animal variability of EBC \( \text{H}_2\text{O}_2 \) concentration remained high [180], indicating that monitoring of this biomarker within individual animals may be more meaningful.

The concentration of \( \text{H}_2\text{O}_2 \) in EBC is likely influenced by several factors that are responsible for, in part, the reported variation. Knobloch et al. [180] demonstrated that EBC \( \text{H}_2\text{O}_2 \) concentrations increased after feeding in healthy calves (foregut fermenters). Whether a similar influence of feeding on EBC \( \text{H}_2\text{O}_2 \) concentrations occurs in horses (hindgut fermenters) is unknown, however feeding was not associated with EBC \( \text{H}_2\text{O}_2 \) variations in humans [247]. Significant diurnal variation and circadian rhythms of increasing EBC \( \text{H}_2\text{O}_2 \) concentrations throughout the day have been demonstrated in calves and humans with and without COPD [180,244]. In another study of healthy humans, peaks in EBC \( \text{H}_2\text{O}_2 \) occurred at 12:00 and 24:00 [246]. Diurnal and seasonal variations in ambient levels of \( \text{H}_2\text{O}_2 \) have been well documented, with higher ambient concentrations of \( \text{H}_2\text{O}_2 \) in the late afternoon than in early morning, and higher levels in summer than winter, respectively, likely due to the strong positive correlation between ambient \( \text{H}_2\text{O}_2 \) and UV-radiation [248]. Furthermore, correlations between ambient and EBC \( \text{H}_2\text{O}_2 \) have been demonstrated [180,233], leading to the recommendations that an inhalation filter system be used to reduce the inhaled fraction of \( \text{H}_2\text{O}_2 \). Alternatively, the ambient \( \text{H}_2\text{O}_2 \) concentration could be determined to correct the measured EBC \( \text{H}_2\text{O}_2 \) concentration [180,233]. PALL® and P3-type inhalation filters have been demonstrated to reduce, but not eliminate, the inspired \( \text{H}_2\text{O}_2 \) concentration [180,249]. In addition, the use of P3-type inhalation filters reduced inter-subject variation of EBC \( \text{H}_2\text{O}_2 \) [249], suggesting that previously documented high levels of inter-subject variation is influenced by inhaled \( \text{H}_2\text{O}_2 \).

Breathing patterns and respiratory rate should be considered for interpretation of EBC \( \text{H}_2\text{O}_2 \) measurements, as \( \text{H}_2\text{O}_2 \) concentrations have been demonstrated to decrease with increasing respiratory flow rates [182] and increasing tidal volumes in healthy human subjects [250]. However, EBC \( \text{H}_2\text{O}_2 \) was not influenced by variables of spontaneous breathing in healthy calves [180]. Nevertheless, standardisation of EBC
H$_2$O$_2$ concentration to EB volume has been recommended, as the volume of EBC collected per volume of EB is not constant in spontaneously breathing animals [251,252] and this method may avoid the confounding effects of respiration on biomarker concentrations [180,251].

In one study, a positive correlation between patient age and EBC H$_2$O$_2$ concentrations in non-smokers was demonstrated [246] however; no association with age was found in other studies. Potential influences of ambient temperature and relative humidity on EBC H$_2$O$_2$ concentration have not been reported. Collection temperature may have an effect, as Goldoni et al. [253] demonstrated that the absolute concentration EBC H$_2$O$_2$ (concentration per volume of EBC collected) increased as condenser temperatures decreased. However, lower condenser temperatures also resulted in increased condensate volume and lower, as measured, EBC H$_2$O$_2$ concentration due to dilution effects. Additionally, collection time may influence EBC H$_2$O$_2$ concentration as it has been demonstrated that the detectable fraction of a H$_2$O$_2$ standard decreases as collection time increases [198]. Additionally, the decomposition rate of H$_2$O$_2$ increases as breath temperature and sample time increased [254]. Therefore, to allow comparison of EBC H$_2$O$_2$ results between studies, standardisation of EBC collection is required.

### 7.2.3. H$_2$O$_2$ measurement - considerations and recommendations

Due to the instability of H$_2$O$_2$ at room temperature as a result of photo- and thermo-sensitivity, it is recommended that EBC for H$_2$O$_2$ analysis be collected frozen or analysed immediately if collected as a liquid [187]. In one study, H$_2$O$_2$ concentrations in EBC samples remained stable for up to 40 days when stored at -70°C [255]. Other recommendations made to standardise H$_2$O$_2$ collection and measurements include; the expression of H$_2$O$_2$ concentration per 100L of exhaled breath during EBC collection instead of per litre condensate, the use of an inspiratory filter to minimise the amount of H$_2$O$_2$ inhaled during EBC collection or measurement of ambient H$_2$O$_2$ to correct measured EBC H$_2$O$_2$ concentrations [180,233].

Spectrophotometry (also known as a colorimetric assay) has been used for determination of EBC H$_2$O$_2$ concentrations. However, this method lacks sensitivity
when applied to EBC samples as measured concentrations in healthy and diseased individuals are close to the lower limit of detection, which may add to the variability of results [256-258]. The use of more sensitive methods, including fluorescence, chemiluminescence and amperometric biosensor detection, is advised [255,259,260] in an effort to obtain more reliable H$_2$O$_2$ results. Both chemiluminescence [260] and amperometric biosensors [261] are highly sensitive and reported to have detection limits (LoD) of 0.01 µmol/L and 0.049 µmol/L respectively [261,262]. Fluorescence spectrophotometry or more simply, fluorescence detection (FLD) by bench-top analyser [249,259] or using flow injection analysis [263,264] are more sensitive than the traditional spectrometry methods with average LoD of 0.0034-0.04 µmol/L and are more precise than chemiluminescence and biosensor methods [259,263,264]. As H$_2$O$_2$ is highly volatile and does not fluoresce spontaneously, FLD quantitation of H$_2$O$_2$ utilises an indirect method that relies on the formation of a fluorescent dimer. The conversion of a non-fluorescing substrate, such as 4-hydroxyphenylacetic acid (4-HPAA) or homovanillic acid (HVA) into a stable, fluorescent dimer is caused by the reduction-oxidation coupling reaction between H$_2$O$_2$ and horseradish peroxidase (HRP) [265]. Improved repeatability has been demonstrated when using HPAA as a substrate instead of HVA [263].

7.3. **Leukotriene B$_4$**

LTB$_4$, an arachidonic acid metabolite derived from phospholipids of cell membranes, is released by macrophages, activated neutrophils, mast cells, eosinophils and epithelial cells [266-268]. It acts as a non-specific pro-inflammatory mediator by recruiting and activating leukocytes and stimulating pro-inflammatory cytokine production [269]. Specifically, LTB$_4$ is a potent chemo-attractant of neutrophils and eosinophils [270,271] and activator of neutrophils [267]. Released LTB$_4$ results in contraction of respiratory smooth muscle with local airway narrowing, increased vascular permeability and associated oedema and increased mucus secretion [189,272].

7.3.1. **LTB$_4$ alterations in response to disease**

In human medicine, increased EBC LTB$_4$ concentrations have been found in smokers [273], cystic fibrosis [274], COPD and asthmatics [176]. Additionally, an association
between EBC LTB$_4$ and LAI was demonstrated by decreased concentrations of LTB$_4$ in subjects with COPD and cystic fibrosis after treatment [274,275]. Reinhold et al. [196] demonstrated a strong linear correlation between the number of neutrophils in BALF and the concentration of LTB$_4$ in EBC of healthy calves. This study also demonstrated increased LTB$_4$ concentration in subjects after induction of bronchopneumonia caused by gram-negative bacteria or bovine respiratory syncytial virus (BRSV) [196]. However, the absolute concentrations of LTB$_4$ after bacterial infection were still within the reference ranges for healthy calves, suggesting that LTB$_4$ may not be a useful biomarker for bacterial pneumonia [196]. Significant negative correlation between LTB$_4$ concentration and lung compliance and positive correlation with the degree of airway hyper-responsiveness were found in the calves with bacterial pleuropneumonia and BRSV-induced bronchiolitis, respectively [196]. These findings demonstrate a relationship between the severity of lung inflammation and pulmonary function derangements with the concentration of EBC LTB$_4$. A study in pigs demonstrated a tendency of EBC LTB$_4$ concentrations to increase with *Chlamydia suis* pneumonia [204]. LTB$_4$ was found in EBC of horses with RAO, however healthy horses were not examined and the usefulness of this analyte as a biomarker of LAI could not be determined [217]. Additionally, LTB$_4$ has been measured in EBC collected from anaesthetised dogs, but information regarding the disease status of the animals was not provided [203]. As such, the association between LTB$_4$ concentrations and LAI remains ill-defined.

### 7.3.2. Variability, influencing factors and measurement considerations

Large inter-subject variation (CV up to 0.8) [276] with considerably less intra-subject inter-day variation (CV 0.03 to 0.25) [196] has been demonstrated in calves. Conversely, a study in COPD patients revealed substantial within- (CV 0.48) and between-day (CV 0.76) variation with wide limits of agreement, demonstrating little reproducibility [277]. The influence of environmental and animal factors on the concentration of LTB$_4$ in EBC has not been evaluated previously. However, correction of LTB$_4$ concentration to the amount of LTB$_4$ exhaled per 100L EB$_i$ has been demonstrated to reduce the level of variation [233]. In the face of large inter-subject variability, it may be preferable to perform follow-up studies where each animal acts as its own control, rather than group comparison [233].
7.3.3. LTB₄ analysis - considerations and recommendations

The physiologic range of EBC LTB₄ concentrations in humans (0-220 pg/ml) [274,278,279] and that reported for veterinary species (Table 2) is low, necessitating pre-analysis concentration [189]. This may be done using lyophilisation/freeze drying [280], solid phase extraction (SPE) [281,282], nitrogen flow evaporation [283,284] or vacuum evaporation [285]. Better recovery has been reported for the use of SPE compared with freeze-drying [284,286], likely because freeze-drying results in increased degradation of lipid mediators such as leukotrienes [286].

Various methods have been described for the determination of LTB₄ concentrations, including commercially available enzyme immunoassay (EIA) [204,217], enzyme-linked immunosorbent assay (ELISA) [196], gas chromatography/mass spectrometry (GC/MS) [287], liquid chromatography/mass spectrometry (LC/MS) [288] and liquid chromatography with spectrophotometry (LC-UV) [280]. EIA is the most commonly used technique as it was previously recommended as the technique of choice [187] based on a study that demonstrated reasonable specificity of EIA, in human EBC [281]. This study coupled EIA with reverse-phase high performance liquid chromatographic separation of different leukotrienes prior to concentrations determination [281]. However, most studies that have used EIA have not included this step. Additionally, the manufacturer reported LoD of 13 pg/mL is above the reported lower range of concentrations for healthy humans (10 pg/mL) and veterinary species (Table 2)¹. Moreover, these EIA methods are not manufactured for EBC, increasing the risk of cross reactivity and false positive results [289]. In fact, the high levels of within-assay variation reported for LTB₄ (CV 0.18) [277], has been attributed to the low sensitivity of EIA [189]. As such, it is now recommended to use the more selective GC/MS or LC/MS methods for determination of LTB₄ concentrations in EBC [189,283,287]. These methods have comparable or better sensitivity to EIA and provide additional structural information, removing the confounding effect of false positive results [189]. Detection of LTB₄ using GC, regardless of the associated detector, requires laborious sample derivatisation prior to analysis [287], whereas LC is much faster as it does not require this step. Multiple LC/MS methods, including the use of ion trap MS, triple quadruple

MS and tandem MS has been described [283,284,290] and often have greater sensitivity than EIA. Additionally, LC-MS is not influenced by the matrix of EBC [284], unlike the EIA methods [289].

LTB₄ has been shown to be stable when stored at -80°C for up to 28 days, but significant reductions of EBC LTB₄ concentrations occur after this period [233].

### 7.4. EBC collection

Multiple different methods have been used to collect EBC in human and veterinary medicine. EBC has been collected in conscious, spontaneously breathing animals, such as calves [180,196], pigs [204], dogs [197] and horses [195,199,201,202] using tightly fitting facemasks (Fig. 2.2). One-way valves within the masks ensure that rebreathing does not occur whilst preventing contamination of expired samples with environmental air. The use of filters at the inspiratory valves is recommended to minimise inspiration of environmental particulates, bacteria, ammonia and H₂O₂, which may influence EBC analytes [180,249]. Multiple different condensation systems with varying materials have been used, such as stainless steel [126], Teflon [217] and polypropylene [202] as well as varying condensation temperatures ranging from 0°C to -100°C [201]. Studies comparing the influence of different collection methods have found that the type of material [291] and collection temperature [201] significantly influence the concentration of the EBC biomarkers, precluding comparison of results of different studies. Highly standardised collection methods are desirable but despite the establishment of an EBC task force in human medicine [187], this has yet to be achieved. The current study will employ collection methods that have been used successfully for the collection of EBC pH and H₂O₂ in horses previously [195,201,202].
Figure 2.2

A line diagram adapted from Cathcart et al. [202], Cathcart et al. [205]. A modified face mask (A) allowed collection of a sample of EB in a gas impermeable Tedlar bag (B) and EBC by directing EB to a chilled condensation chamber (D). Re-breathing and contamination of EBC by environmental air is minimized with the use of inspiratory (Vi) and expiratory (Ve) one-way valves. Coolant (liquid nitrogen-ethanol slurry) within the condensation chamber, in close apposition to the condenser surface (f).
8. Conclusion

Exhaled breath condensate may allow earlier detection of inflammation of respiratory tissues, as well as by-products of inflammatory processes, compared to techniques that measure functional or structural changes [196], such as respiratory cytology and endoscopy. Additionally, a non-invasive method for the assessment of equine lower airways is desirable for welfare implications and frequent, simple sampling. Exhaled breath condensate analysis, including pH measurements and H$_2$O$_2$ and LTB$_4$ concentrations, could offer such a non-invasive alternative or adjunct to BAL and TA for diagnosis of LAI and also permit frequent monitoring of treatment response in these disease processes. To date there are no or few published reports on the use of EBC parameters in horses with IAD, STI and EIPH.
9. Aims and objectives of this study

The research questions for this study were:

1. What is the consistency of EBC pH measurements and \( \text{H}_2\text{O}_2 \) and LTB\(_4\) concentrations in horses and do animal, environmental and analysis factors influence these biomarkers?

2. Is EBC useful for the diagnosis of lower airway inflammation in horses?

3. Can a superior method be developed for the determination of \( \text{H}_2\text{O}_2 \) concentration in EBC?

The research objectives were to:

1. Evaluate the intra- and inter-day consistency of EBC pH and \( \text{H}_2\text{O}_2 \) and LTB\(_4\) concentrations.

2. Determine if animal and environmental factors influence these EBC biomarker concentrations.

3. In horses with and without non-infectious LAI
   3.1. Compare EBC pH measurements and \( \text{H}_2\text{O}_2 \) and LTB\(_4\) concentrations between horses with and without various categories of LAI.
   3.2. Determine if correlations between EBC biomarkers and conventional diagnostic testing results, including airway fluid cytology and airway endoscopic findings, exist.

4. Develop a superior method for EBC \( \text{H}_2\text{O}_2 \) concentration determination.

10. Hypotheses of this study

The hypotheses of this study were that EBC pH would be highly consistent, however EBC \( \text{H}_2\text{O}_2 \) and LTB\(_4\) concentrations will have low levels of consistency. EBC biomarkers will be altered in response to lower airway inflammation. The concentrations of \( \text{H}_2\text{O}_2 \) and LTB\(_4\) in EBC will be higher and EBC pH measurements will be lower in horses with LAI.
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Short Communication

The consistency and influence of environmental and animal factors on exhaled breath condensate hydrogen peroxide, pH and leukotriene B₄ in horses.

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Abstract

This study was performed to determine the consistency of exhaled breath condensate (EBC) hydrogen peroxide ($\text{H}_2\text{O}_2$), pH and leukotriene B$_4$ (LTB$_4$) measurements in asymptomatic horses and to define the influence of environmental and animal factors on these variables. Intra- and inter-day consistency for both $\text{H}_2\text{O}_2$ and pH measurements were adequate, with intraclass correlation coefficients $\geq 0.8$, whereas the consistency for LTB$_4$ was poor. $\text{H}_2\text{O}_2$ was influenced by ambient temperature ($T_A$), humidity, time of day and collection location (all $P < 0.01$), while pH was influenced by respiratory rate during EBC collection and $T_A$ (both $P < 0.001$). The consistency of EBC $\text{H}_2\text{O}_2$ and pH measurements may be sufficient for use as diagnostic biomarkers in horses. However, the influence of identified environmental and animal factors should be considered.

Keywords: EBC biomarker; Equine; $\text{H}_2\text{O}_2$; LTB$_4$; Respiratory
Analysis of exhaled breath condensate (EBC) hydrogen peroxide (H$_2$O$_2$), pH and leukotriene B$_4$ (LTB$_4$) is used in human medical research to characterise pulmonary inflammatory diseases, including asthma (Horvath et al., 2005). EBC constituents are present in low concentrations that reflect those within the epithelial lining fluid of the lower airways (Reinhold and Knobloch, 2010). However, results may be influenced by methodological, physiological and environmental factors including ambient temperature ($T_A$), relative humidity (RH), respiratory rate (RR) and collection or analysis methods (Horvath et al., 2005; Kullmann et al., 2008).

Associations between EBC biomarkers and pulmonary inflammation have been reported in animals (Reinhold and Knobloch, 2010), but specific information on horses is limited. Equine asthma or collectively, non-infectious lower airway inflammatory conditions, is common in horses and diagnosis is typically based on endoscopy and bronchoalveolar lavage fluid (BALF) cytology (Couëtil et al., 2016). These methods are invasive, require sedation and do not measure active inflammation. Measurement of EBC biomarkers could offer a non-invasive alternative to conventional methods. The aims of this study were to assess (1) intra- and inter-day consistency of EBC H$_2$O$_2$, pH and LTB$_4$ measurements and (2) the influence of animal and environmental factors on these biomarkers.

Ten horses (Group 1) were used to determine the consistency of EBC pH, H$_2$O$_2$ and LTB$_4$ and for investigation of animal and environmental factors influencing H$_2$O$_2$. Investigation of animal and environmental factor influences on pH and LTB$_4$ were determined using 20 horses (Group 2). All horses were considered free of respiratory disease (asymptomatic) based solely on history and clinical examination. The study was approved by Charles Sturt University’s Animal Care and Ethics Committee (ACEC approval number 14/082; Approval date 14 October 2014).

The EBC collection system (Fig. 3.1) was adapted from described methods (Duz et al., 2009). Environmental and animal factors at the time of EBC collection were recorded. Biomarker consistency was assessed by collection of EBC on five occasions from each horse (Fig. 3.2). Tracheobronchoscopic examination and collection of BALF were performed after EBC collection on day 3. Flow injection analysis with
fluorescence detection allowed determination of $\text{H}_2\text{O}_2$ concentrations, while $\text{LTB}_4$ concentrations were determined using liquid chromatography with mass-spectrometry. A bench-top pH meter was used to measure pH at room temperature in neat EBC and after deaeration using argon gas.

Variance components used in the consistency analysis were estimated using two-way mixed effects model ANOVA. For each biomarker, consistency was calculated with the intraclass correlation coefficient (ICC) using special case 3A[C,1] (the consistency definition using single measurements) (McGraw and Wong, 1996), with thresholds of $\geq 0.9$ accepted as high consistency and $\geq 0.8$ as adequate consistency. Effects of environmental and animal factors on biomarkers were assessed by univariate and multivariable analyses using linear mixed models. Data were analysed using ASReml-R; with significance set at $P<0.05$. Detailed materials and methods are in Supplementary items 3.1 and 3.2.

$\text{H}_2\text{O}_2$ and $\text{LTB}_4$ were expressed using concentrations in EBC ($\mu\text{mol}/\text{L}$ and $\text{pg}/\text{mL}$, respectively) and concentrations standardised to the average total volume of exhaled breath ($\text{EB}_{\text{TV}}$) per collection: pmol/100 L EB and pg/100L EB, respectively (Supplementary item 3.1 and 3.2). Respiratory effort, depth and rate were unchanged during sample collection ($P = 0.072$). All horses were normal on endoscopic inspection of the upper respiratory tract and trachea. BALF cytological findings from all horses are provided in Supplementary item 3.2.

Six horses in Group-1, and 11 in Group-2, had cytological evidence of airway inflammation (Couëtil et al., 2016). Concentrations of biomarkers for each collection period are shown in Fig. 3.2. All EBC $\text{H}_2\text{O}_2$ concentrations were above the limit of detection (LoD) and $\text{LTB}_4$ concentrations below the LoD (Supplementary item 3.1) were assigned a value of zero for the statistical analysis. ICC results are provided in Table 3.1. Results of the multivariable analyses for $\text{H}_2\text{O}_2$ and pH are in Table 3.2 and predicted effects are in Supplementary item 3.3. There were no significant associations between environmental or animal factors and $\text{LTB}_4$. Several interaction terms for $\text{H}_2\text{O}_2$ and pH were significant (Supplementary item 3.4).
Figure 3.1
Photographs of equipment used for collection of exhaled breath condensate (EBC) from horses in this study. EBC was collected by condensation of expired breath.
A custom-built facemask (A) was connected via flexible corrugated tubing with an internal diameter of 35 mm (B), to an airtight EBC condensation chamber (C). The facemask, constructed of a modified grazing muzzle, included two one-way non-rebreathing valves (D; Salford Valve, Cranlea) with an internal diameter of 35 mm, positioned over the nares with inspiratory PALL filters (E; Protex PF30S, PALL) attached. The insert provides a close-up image of the valves and inspiratory filters.
An elastic rubber diaphragm (F) was attached to the facemask to create and airtight seal around the horse’s muzzle. The condensation chamber (C) was constructed of three 50 mL polypropylene tubes arranged in parallel (insert), immersed in an ethanol-liquid nitrogen slurry, cooling the condensation surface to less than -50 °C. Two one-way valves (G; Aeromask valve, Trudell Medical) at the outlet of the condensation chamber prevented contamination of the EBC sample by environmental air. Collection time was standardised to 15 min.
These findings extend the understanding of EBC analysis in horses. The consistency of EBC $H_2O_2$ and pH were adequate to high, thus these biomarkers may be worth considering for non-invasive respiratory assessment in horses. Substantial intra- and inter-day variations in $H_2O_2$ in horses has been reported previously (Duz et al., 2009), while acceptable repeatability was reported in cats (Kirschvink et al., 2005) and humans (Van Beurden et al., 2002). In our study, $H_2O_2$ was standardised to $EB_{TV}$, as the volume of EBC collected per volume of EB is not constant in spontaneously breathing animals (Reinhold and Knobloch, 2010). The ICC for $H_2O_2$ in the current study was higher for $EB_{TV}$, suggesting that standardisation improves consistency, as previously proposed for calves (Knobloch et al., 2008). Minimal variability in pH has been reported in horses (Duz et al., 2009) and humans (Horvath et al., 2005). Our results indicate that both neat and deaerated EBC provided adequate consistency in pH, although deaerated EBC provided slightly superior inter-day consistency in pH, similar to a previous study (Whittaker et al., 2012). Moreover, our findings indicate that $LTB_4$ is unsuitable for lower airway assessment, as consistency was poor. Horses were not categorised based on BALF cytology results, as the study aim was to determine EBC biomarker consistency in horses without clinical signs of respiratory disease. Previous studies in horses and humans (Duz et al., 2009; Van Beurden et al., 2002) demonstrated that EBC biomarker variability was not affected by respiratory health status, similar to the results of this study.
Figure 3.2

Exhaled breath condensate (EBC) hydrogen peroxide (H$_2$O$_2$, A and B), pH (C and D) and leukotriene B$_4$ (LTB$_4$, E and F) for Group-1 horses. EBC was collected from each horse on three occasions on day one at 08:00, 12:00 and 16:00 to assess intra-day consistency and at 12:00 for three days to assess inter-day consistency. Results are shown as median and mean (cross), quartiles (box), with whiskers calculated using the Tukey method.
Table 3.1
The intra- and inter-day consistency, expressed as intraclass correlation coefficient (ICC) and 95% confidence interval (CI) of exhaled breath condensate (EBC) H$_2$O$_2$, pH and leukotriene B$_4$ (LTB$_4$) as measured per volume of condensate and standardised according to the total estimated volume of exhaled breath (EB) during EBC collection.

<table>
<thead>
<tr>
<th>EBC biomarker</th>
<th>Intra-day consistency</th>
<th>Inter-day consistency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ICC</td>
<td>95% CI</td>
</tr>
<tr>
<td>H$_2$O$_2$ µmol/L</td>
<td>0.969</td>
<td>0.765 to 0.975</td>
</tr>
<tr>
<td>H$_2$O$_2$ pmol/100 L EB</td>
<td>0.976</td>
<td>0.810 to 0.980</td>
</tr>
<tr>
<td>pH neat</td>
<td>0.861</td>
<td>0.271 to 0.880</td>
</tr>
<tr>
<td>pH deaerated</td>
<td>0.860</td>
<td>0.268 to 0.879</td>
</tr>
<tr>
<td>LTB$_4$ pg/mL</td>
<td>0.599</td>
<td>-0.196 to 0.601</td>
</tr>
<tr>
<td>LTB$_4$ pg/100L EB</td>
<td>0.608</td>
<td>-0.186 to 0.613</td>
</tr>
</tbody>
</table>

The impact of environmental factors on EBC biomarkers in horses is poorly understood. In our study, H$_2$O$_2$ increased with increasing T$_A$ and decreased with increasing RH, while changes due to the time of day were dependent on interactions with other factors. Diurnal variation in H$_2$O$_2$ has been reported for humans (Van Beurden et al., 2002) and calves (Knobloch et al., 2008), however, the influences of T$_A$ or RH have not been reported and likely reflect the inverse relationship between T$_A$ and RH in our climatic region. Interactions between time of day and collection location and T$_A$ may be due, in part, to the positive correlation between UV-radiation and atmospheric H$_2$O$_2$, with increased ambient H$_2$O$_2$ in the afternoon (Reinhold and Knobloch, 2010). Correlations between ambient and EBC H$_2$O$_2$ have been demonstrated (Knobloch et al., 2008). In our study, EBC collected in yards was associated with significantly higher H$_2$O$_2$ concentrations than indoors. While this may reflect an influence of UV-radiation on ambient and EBC H$_2$O$_2$, it remains speculative, as ambient samples were not collected. Additionally, caution is warranted with interpretation of the interaction terms (Supplementary item 3.4) as our study was not sufficiently powered to allow adequate evaluation of such interaction terms. Considering the demonstrated influences of environmental factors on EBC H$_2$O$_2$, the difference between ambient conditions in Australia compared with Scotland (Duz et
al., 2009; Wyse et al., 2005) may have contributed to the disparity in consistency between the current study and previous reports in horses (Duz et al., 2009; Wyse et al., 2005).

In this study, increased RR and $T_A$ were associated with increased pH. Kullmann et al. (2008) reported an influence of $T_A$ on pH and suggested that this factor may contribute to biomarker variability. However, in other studies neither ambient nor ventilation parameters were associated with EBC pH (Knobloch et al., 2008).

In conclusion, the consistency of EBC $\text{H}_2\text{O}_2$ and pH were adequate to high, suggesting that these biomarkers may be useful for lower airway assessment in horses. However, $T_A$, humidity, time of day, collection location and RR during EBC collection may influence these biomarkers, necessitating standardisation when collecting samples longitudinally.
Table 3.2
Animal and environmental factors identified as influencing EBC biomarkers. Results of univariate and multivariable analyses and brief descriptions of observed effects are presented.

<table>
<thead>
<tr>
<th>Covariates</th>
<th>Univariate analysis a</th>
<th>Multivariable analysis a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P-value</td>
<td>Predicted effect</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H2O2 μmol/L</td>
</tr>
<tr>
<td><strong>H2O2 μmol/L</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ambient temperature</td>
<td>&lt; 0.001</td>
<td>curvilinear increase with temperature</td>
</tr>
<tr>
<td>Ambient humidity</td>
<td>0.018</td>
<td>linear decrease with humidity</td>
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<tr>
<td>Feeding within 1 h of EBC collection</td>
<td>0.052</td>
<td></td>
</tr>
<tr>
<td>Collection location</td>
<td>0.069</td>
<td></td>
</tr>
<tr>
<td>Time of day</td>
<td>0.142</td>
<td></td>
</tr>
<tr>
<td><strong>H2O2 pmol/100L EB</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collection location</td>
<td>0.006</td>
<td>higher when collected in yard vs. crush</td>
</tr>
<tr>
<td>Ambient humidity</td>
<td>&lt; 0.001</td>
<td>linear decrease with humidity</td>
</tr>
<tr>
<td>Ambient temperature</td>
<td>&lt; 0.001</td>
<td>curvilinear increase with temperature</td>
</tr>
<tr>
<td>Time of day</td>
<td>0.005</td>
<td>linear increase from am to pm</td>
</tr>
<tr>
<td>Feeding within 1 h of EBC collection</td>
<td>0.047</td>
<td>higher in horses that have been fed</td>
</tr>
<tr>
<td>Metachromatic IAD</td>
<td>0.129</td>
<td></td>
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<tr>
<td><strong>pH neat</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average RR during collection</td>
<td>0.006</td>
<td>linear increase with RR</td>
</tr>
<tr>
<td>Ambient temperature</td>
<td>&lt; 0.001</td>
<td>linear increase with temperature</td>
</tr>
<tr>
<td>Ambient humidity</td>
<td>&lt; 0.001</td>
<td>linear decrease with humidity</td>
</tr>
<tr>
<td>Collection location</td>
<td>&lt; 0.001</td>
<td>higher when collected in yard vs. crush</td>
</tr>
<tr>
<td>Time of day</td>
<td>0.143</td>
<td></td>
</tr>
<tr>
<td>Eosinophilic IAD</td>
<td>0.063</td>
<td></td>
</tr>
<tr>
<td>Metachromatic IAD</td>
<td>0.142</td>
<td></td>
</tr>
<tr>
<td>BAL neutrophil %</td>
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<td>linear increase with BAL neutrophil %</td>
</tr>
<tr>
<td>BAL eosinophil %</td>
<td>0.089</td>
<td></td>
</tr>
<tr>
<td><strong>pH deaerated</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average RR during collection</td>
<td>0.006</td>
<td>linear increase with RR</td>
</tr>
<tr>
<td>Ambient temperature</td>
<td>&lt; 0.001</td>
<td>curvilinear increase with temperature</td>
</tr>
<tr>
<td>Ambient humidity</td>
<td>&lt; 0.001</td>
<td>linear decrease with humidity</td>
</tr>
<tr>
<td>Collection location</td>
<td>&lt; 0.001</td>
<td>higher when collected in yard vs. crush</td>
</tr>
<tr>
<td>Eosinophilic IAD</td>
<td>0.058</td>
<td></td>
</tr>
<tr>
<td>BAL neutrophil %</td>
<td>0.008</td>
<td>linear increase with BAL neutrophil %</td>
</tr>
<tr>
<td>BAL eosinophil %</td>
<td>0.057</td>
<td></td>
</tr>
</tbody>
</table>

a The effect of the covariate on the EBC biomarker is only provided for covariates with P<0.05
b Refer to the supplementary item 3 for figures of the predicted effects
c Mean (95% confidence intervals)
d Refer to supplementary item 4 for more information regarding interaction terms
Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Acknowledgements

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Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tvjl.2017.07.005

The original full-length manuscript can be found as Appendix 1, pg. 236.
References


Supplementary material

Supplementary item 3.1
A detailed description of the analysis methods used to determine exhaled breath condensate (EBC) hydrogen peroxide (H$_2$O$_2$), pH and leukotriene B$_4$ (LTB$_4$) concentrations.

**EBC H$_2$O$_2$ measurement**

H$_2$O$_2$ concentrations were determined with the use of flow injection analysis with fluorescence detection (FIA-FD) using an indirect method during which a fluorescent dimer of 4-hydroxyphenyl acetic acid (HPAA, Sigma Aldrich) was formed by the redox coupling reaction between H$_2$O$_2$ in EBC and horseradish peroxidase (HRP, Sigma Aldrich) (Svensson et al., 2004).

One of three 50 mL polypropylene tubes collected from each horse was thawed at 2 °C over 80 min. A working solution of reaction mixture consisting of 560 µl HRP (5 units/mL), and 1400 µL of 3mM 4-HPAA substrate, both prepared in 100 mM phosphate buffer, was prepared fresh for each batch. Fifty microliters of working solution were added to 500 µL of EBC and allowed to react for 60 minutes on ice. A 200 µL aliquot was transferred to a glass low volume insert (Phenomenex) in an autosampler for analysis by FIA-FD. A six-point calibration curve (0 to 5 µmol/L), which had good linearity ($r^2 = 0.997$), was prepared for every analysis using the same procedure as for the samples. All solutions were prepared on ice to limit temperature fluctuation and associated sample degradation. Samples and standards were analysed in three duplicate blocks to minimise the influence of reaction time on the fluorescent reaction. Run time was 1 min with injection overlap after 0.8 min. The lower limit of detection (LoD) was 0.05 µmol/L with an intra-assay coefficient of variation of 1.3%.

The flow injection was performed using an Agilent 1290 UPLC with a mobile phase consisting of instrument grade water:methanol (56:44, v/v) with a 0.3 ml/min flow rate and 2 µL injection volume. The auto-sampler was maintained at 4 °C and samples were kept in the dark. The excitation and emission wavelengths were 285 and 400 nm, respectively.
H$_2$O$_2$ was expressed using direct concentrations in EBC (µmol/L) and concentrations standardised to the estimated total volume of exhaled breath (EBTV) per collection (pmol/100 L EB). EBTV was estimated from the respiratory rate throughout EBC collection and published tidal volumes at rest (van Erck-Westergren et al., 2005), followed by biomarker expression per 100L of EB.

**EBC pH measurement**

After thawing, samples were mixed to resuspend sublimated volatile acids (Davis and Hunt, 2012). The pH of neat EBC was measured using a bench top pH meter (HI 2211 pH/ORP meter, Hanna Instruments) and a micro bulb pH-electrode (HI 1083B, Hanna Instruments), which was calibrated prior to use. PH was recorded as soon as the reading stabilised, as Borrill et al. (2005) reported ongoing fluctuations in pH measured in EBC maintained at room temperature, regardless of sample characteristics. The reading was deemed stable once there was less than 0.01 units fluctuation within 1 minute. Subsequently, EBC was deaerated using argon gas and pH was recorded when the reading stabilised. The duration of deaeration was dependant on sample volume. Each sample was measured in triplicate at room temperature (21.23 ± 0.57 °C).

**EBC LTB$_4$ measurement**

LTB$_4$ concentrations were determined using liquid chromatography with triple quadrupole tandem mass-spectrometry (LC-QQQ) adapted from Montuschi et al. (2005), accounting for processing losses with the use of a LTB$_4$-d$_4$ (Sapphire Bioscience) as an internal standard. 100 µL of 0.1 µg/mL LTB$_4$-d$_4$ solution was added to each EBC sample, resulting in a final concentration of 10 ng/mL. Spiked EBC samples were concentrated five-fold, using evaporation in a vacuum chamber (OVL-570, Gallenkamp) at room temperature (21.23 ± 0.57 °C) with a constant nitrogen flow. Residues were resuspended in 200 µL instrument grade methanol.

Analysis was performed with an Agilent 1260 LC connected to a 6410 triple quadrupole mass spectrometer using positive electrospray ionisation (ESI+). The liquid chromatography was done using a Phenomenex Kinetex XB-C18 100A column (50 x 4.6 mmm x 2.6 µm) and a linear gradient with water-methanol-formic acid (70:30:0.1, v/v) that was changed to 100% methanol over four minutes. A 12-point calibration curve
over the range of 0-5 ng/ml was prepared using the peak area ratios for LTB₄/LTB₄-d₄. The calibration curve had good linearity ($r^2 = 0.995$) and the LoD was 3.22 pg/mL. Samples and standards were analysed in duplicate and all working solutions were prepared fresh for each batch.

The optimised MS source parameters were: gas temperature (350°C), gas flow rate (13 l/min), nebuliser pressure (40 psi) and capillary voltage (4000 V). Multiple reaction monitoring (MRM) was used for quantitative analysis of LTB₄ in EBC. The $m/z$ 359 $\rightarrow$ m/z 341 and $m/z$ 363 $\rightarrow$ m/z 345 transitions were used to detect LTB₄ and LTB₄-d₄, respectively (Montuschi et al., 2005).

As for H₂O₂, LTB₄ was expressed using direct concentrations in EBC (pg/mL) and concentrations standardised to the average EBTV per collection (pg/100L EB).

References


Supplementary item 3.2
Expansion of information regarding animal selection, exhaled breath condensate collection (EBC), bronchoalveolar lavage fluid (BALF) cytological results and statistical analysis.

Animals
Sample size calculations ($\alpha = 0.05$, power of 0.8) were based on previously reported findings (Cathcart et al., 2013; Duz et al., 2009). To detect a difference of 1 $\mu$mol/L for $\text{H}_2\text{O}_2$ and 1 pH unit, 10 horses and 9 horses were required in Group 1, respectively. For the determination of animal and environmental influences on EBC biomarkers, a required sample size of 19 was determined for Group 2. Group 1 consisted of seven mares and three geldings; six Thoroughbreds, three Standardbreds and one Quarter Horse; mean age was 7.6 years (range: 4-15). Group 2 consisted of 14 mares and six geldings; 10 Standardbreds, nine Thoroughbreds and one Quarter Horse; mean age was 6.7 years (range: 4-15). The mean ± SD of body weight was 519.9 ± 30.7 kg. Prior to the study, the horses were at pasture, and housed in yards with access to Lucerne hay during the study.

EBC collection and handling
All EBC samples were frozen during collection and stored at -80 °C until analysis. Relative humidity (RH), $T_a$, food consumption within one hour of EBC collection and respiratory rate (RR) during collection was recorded. The average RR during collection was determined by recording the RR at 5 min intervals, starting at the initiation of EBC collection or whenever the horse’s RR appeared to change. A limitation of the current study was the unavailability of an expiratory spirometer with a large enough internal diameter (that would not add excessive resistance to breathing) to accurately measure total exhaled breath volume ($\text{E}_{\text{TV}}$) during EBC collection. As a compromise $\text{E}_{\text{TV}}$ was estimated from RR throughout EBC collection and published tidal volume at rest for light breed horses (van Erck-Westergren et al., 2005). During EBC collection, horses were restrained in a crush in a well-ventilated building or in an outdoor, roofed yard with rubber flooring, depending on temperament. Between collections, the facemask and tubing were rinsed with deionised water and dried. Intra-day biomarker consistency was assessed by collection
of EBC samples from each horse on three occasions on day one at 08:00, 12:00 and 16:00. To determine inter-day consistency, a single sample obtained at 12:00 for three consecutive days was used. A randomised crossover design was used to assess the influence of environmental factors.

**Respiratory fluid collection**

After EBC collection on day 3, each horse was sedated with xylazine (0.3 to 0.5 mg/kg BW IV, Ilium Xylazine, Troy Laboratories). BALF was then obtained by blind placement of a commercial BAL catheter (BAL300, SurgiVet), instillation of 300 mL of sterile isotonic saline and retrieval by aspiration. Pooled BALF was analysed within three hours of collection. Differential cell counts were performed on 300 leukocytes and results were recorded as the relative percentages of neutrophils, mast cells and eosinophils in BALF. Mild equine asthma was diagnosed if BALF cytology had a differential cell count of ≥10% neutrophils or ≥5% mast cells or ≥5% eosinophils or any combination of these (Couëtil et al., 2016). Six horses in Group-1 and 11 in Group-2 were diagnosed with mild equine asthma by BALF cytology (Table 3.3)

**Statistical methods**

Variance components used in the consistency analysis were estimated using two-way mixed effects ANOVA model with horses as the random term and collection period as a fixed term. The intra- and inter-day consistency for each EBC biomarker was calculated with the intraclass correlation coefficient (ICC) using special case 3A[C,1] (consistency definition using single measurements) and confidence intervals were estimated (McGraw and Wong, 1996). Thresholds of ≥0.9 as high consistency and ≥0.8 as adequate consistency were adopted for interpretation of ICC results (Bartko, 1966).

For assessment of environmental and animal factors on biomarkers, univariate analyses, using linear mixed models, were performed and variables with $P<0.2$ were included in multivariable models. Horses were included as a random term and all other variables included, as determined by the univariate linear models, were included as fixed terms. The model with the lowest AIC/BIC (Akaike’s Information Criterion/Bayesian Information Criterion) (Anderson and Burnham, 2002), was
analysed using a linear mixed model with restricted maximum likelihood. Multivariable models including two-way interaction terms were also explored with a backward elimination method, with the highest P-value term (P>0.05) removed at each step. Assumptions were that residuals in the model were normally distributed, had constant variance and were independent. The model assumptions were evaluated using scatter plots, histograms and the Shapiro-Wilk test for normality. Factor level variances for categorical variables were tested using the Brown-Forsythe test and weighted least squares analysis was used to account for the observed differences in variance between factor levels. Predicted means and 95% confidence intervals (CIs) were calculated for variables retained in the multivariable analyses. Data were analysed using ASReml-R (Butler et al., 2007), with significance set at P<0.05.
Table 3.3

The bronchoalveolar lavage fluid (BALF) cytological findings of horses in Groups-1 and 2. The presence (Y) or absence (N) of mild equine asthma as classified by BALF cytology (Couëtil et al., 2016) is indicated. Group-2 consists of horses from Group-1 and 10 additional horses.

<table>
<thead>
<tr>
<th>Horse ID</th>
<th>Mild equine asthma</th>
<th>BALF cellular percentages (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Neutrophil</td>
<td>Eosinophil</td>
</tr>
<tr>
<td>Group-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>N</td>
<td>4.0</td>
<td>0.3</td>
</tr>
<tr>
<td>2</td>
<td>N</td>
<td>4.0</td>
<td>3.0</td>
</tr>
<tr>
<td>3</td>
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References


Supplementary item 3.3

Figures demonstrating the predicted effect of environmental and animal factors significantly associated with exhaled breath condensate (EBC) hydrogen peroxide ($H_2O_2$) and pH in the multivariable analysis.

**Figure 3.3.**

Graphical representation of the predicted $H_2O_2$ ($\mu$mol/L) response to ambient temperature ($^{\circ}$C). There is a linear increase of $H_2O_2$ ($\mu$mol/L) as the ambient temperature increases. The shaded area represents 95% confidence intervals (CI).
Figure 3.4

Graphical representation of the predicted concentration of H$_2$O$_2$ (pmol/100L EB) in EBC when collected in the crush or the outside yard. H$_2$O$_2$ (pmol/100L EB) is significantly higher (P<0.001) when collected in the yard compared with the crush. The whiskers indicated the 95% CI.
Graphical representation of the predicted response of $H_2O_2$ (pmol/100L EB) to ambient humidity (%). There is a linear decrease of $H_2O_2$ (pmol/100L EB) as the relative ambient humidity increases. The shaded area represents 95% CI.
Figure 3.6
Graphical representation of the predicted response of pH, in deaerated EBC, to respiratory rate during collection (breaths/min). There is a linear increase of pH in deaerated EBC as the respiratory rate increases. The shaded area represents 95% CI.
Graphical representation of the predicted response of pH, in deaerated EBC, to ambient temperature (°C). There is a curvilinear increase of pH in deaerated EBC as the ambient temperature increases. The shaded area represents 95% CI.
Supplementary item 3.4

Table 3.4

Animal and environmental factors identified to influence exhaled breath condensate (EBC) hydrogen peroxide (H$_2$O$_2$) and pH in combination (interaction terms). Results are presented as $P$-values following multivariable analysis, including interaction terms and using stepwise backward elimination, with a brief description of the observed effect.

<table>
<thead>
<tr>
<th>Interaction terms</th>
<th>$P$-value</th>
<th>Predicted effect</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>H$_2$O$_2$ μmol/L</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Time of day and ambient temperature $^a$ | 0.01 | Linear decrease from am to pm at temperatures ranging from 21 to 31 °C  
Linear increase from am to pm at temperatures ranging from 31 to 35 °C  
Stays most constant from am to pm at 21 °C and 32 °C |
| **H$_2$O$_2$ pmol/100L EB** |          |                  |
| Time of day and collection location $^b$ | 0.002 | Linear increase from am to pm when collected in the crush  
Stays nearly constant from am to pm when collected in the yard |
| **pH neat** |          |                  |
| Time of day and collection location $^c$ | 0.012 | Linear decrease from am to pm when EBC is collected in the crush  
Linear increase from am to pm when EBC is collected in the yard |
| **pH deaerated** |          |                  |
| Ambient temperature and collection location $^d$ | 0.033 | Slight curvilinear decrease below 10 °C and increase above 10 °C when EBC is collected in the crush  
Curvilinear increase with a plateau at 30 °C when EBC is collected in the yard |

$^a$ See Fig. 3.8 for the graphical representation of the influence of time of day and ambient temperature on H$_2$O$_2$ μmol/L  
$^b$ See Fig. 3.9 for the graphical representation of the influence of time of day and collection location on H$_2$O$_2$ pmol/100L EB  
$^c$ See Fig. 3.10 for the graphical representation of the influence of time of day and collection location on pH in neat EBC  
$^d$ See Fig. 3.11 for the graphical representation of the influence of ambient temperature and collection location on pH in neat EBC
Figures

Figures demonstrating the predicted effect of the interaction terms associated with EBC H₂O₂ and pH in the multivariable analysis.

**Figure 3.8**

Graphical representation of the predicted H₂O₂ (µmol/L) concentrations throughout the day at different ambient temperatures (°C). There is a linear decrease from am to pm at temperatures ranging from 21 to 31 °C with a linear increase from am to pm at temperatures ranging from 31 to 35 °C. H₂O₂ (µmol/L) stays most constant all day at 21 °C and 32 °C. The shaded area represents 95% confidence intervals (CI).
Figure 3.9

Graphical representation of the predicted $\text{H}_2\text{O}_2$ (pmol/100L EB) concentration throughout the day when EBC is collected in an indoor crush compared with a covered outside yard. There is a linear increase from am to pm when collected in the crush, though $\text{H}_2\text{O}_2$ (pmol/100L EB) stays nearly constant throughout the day when collected in the yard. The shaded area represents 95% CI. Crush = EBC collection in the indoor crush, Yard = EBC collection in the outside yard.
Figure 3.10

Graphical representation of the predicted pH in neat EBC throughout the day when EBC is collected in an indoor crush compared with a covered outside yard. There is a linear decrease from am to pm when collected in the crush, however pH increases throughout the day when collected in the yard. The shaded area represents 95% CI. Crush = EBC collection in the indoor crush, Yard = EBC collection in the outside yard.
Graphical representation of the predicted pH in deaerated EBC in response to ambient temperature when collected in the crush or the outside yard. When EBC is collected in the crush, there is slight curvilinear decrease below 10 °C above that there is a curvilinear increase. When EBC is collected in the yard, there is a curvilinear increase in pH that plateaus at 30 °C. The shaded area represents 95% CI.

Figure 3.11
CHAPTER 4

Manuscript submitted for publication to the Equine Veterinary Journal (under review)
Exhaled breath condensate hydrogen peroxide, pH and leukotriene B₄ are associated with lower airway inflammation and airway cytology in the horse.

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Summary

**Background:** Exhaled breath condensate (EBC) analysis is a non-invasive method to assess the lower respiratory tract. In humans, EBC hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), pH and leukotriene B\textsubscript{4} (LTB\textsubscript{4}) are useful for detection and monitoring of inflammatory lung diseases, including asthma.

**Objectives:** To determine associations between EBC biomarkers and cytological and endoscopic definitions of lower airway inflammation whilst controlling for sampling and environmental variables.

**Study Design:** Prospective, cross-sectional study

**Methods:** Clinical, endoscopic and airway cytological findings from 47 horses were compared with EBC pH and concentrations of H\textsubscript{2}O\textsubscript{2} and LTB\textsubscript{4} by univariate and multivariable analyses. Dichotomous (presence / absence of airway inflammation) and continuous outcome variables (differential cell counts in tracheal aspirate and bronchoalveolar lavage fluid, BALF) were evaluated and potential effects of collection and methodological factors were included.

**Results:** EBC pH and H\textsubscript{2}O\textsubscript{2} concentrations were higher in horses with lower airway inflammation and both were positively associated with the percentage of neutrophils in BALF (P<0.05). Mast cell percentage in BALF was negatively associated with EBC pH, and BALF eosinophil percentage was positively associated with EBC LTB\textsubscript{4} (P<0.05). Ambient temperature, relative humidity and assay methodology (vial type and batch) significantly impacted some analytes.

**Main Limitations:** Airway inflammation is challenging to characterise due to a variety of clinical and cytological phenotypes. Although research methodology was designed to overcome this limitation, numbers of horses were small in some categories.

**Conclusions:** EBC pH and H\textsubscript{2}O\textsubscript{2} concentrations are altered by airway inflammation, suggesting a role for these biomarkers in the diagnosis and monitoring of airway disease. Environmental and methodological factors can influence these biomarkers and should be considered in the interpretation of results.

**Keywords:** Asthma; Bronchoalveolar lavage; Equine; H\textsubscript{2}O\textsubscript{2}; Inflammatory airway disease; LTB\textsubscript{4}
Introduction

Non-infectious lower airway inflammatory (LAI) conditions of horses are common causes of poor performance and wastage [1]. These conditions include the heterogenous syndrome of mild-moderate equine asthma (previously termed inflammatory airway disease, IAD), the syndrome of tracheal inflammation (STI) and exercise-induced pulmonary haemorrhage (EIPH) [2-4]. While EIPH and IAD often coexist [5], the pathophysiological relationship between these conditions remains unclear [3]. The clinical presentation of LAI conditions is variable and has been related to differing inflammatory cell populations and inflammatory mediators in bronchoalveolar lavage fluid (BALF) [6,7]. Diagnosis of these LAI conditions is based on tracheobronchoscopy, tracheal aspirate (TA), BALF cytology and/or pulmonary function testing [1,3,8-10]. These conventional methods are invasive, often require sedation and do not quantitate active inflammation [11,12].

Analysis of biomarkers in exhaled breath condensate (EBC) is a non-invasive method for direct pulmonary assessment in human medical research [13]. EBC is collected by condensing expired breath to a liquid or frozen state [13]. Constituents of EBC are likely present in concentrations that reflect those within the epithelial lining fluid (ELF) of the lower airways [14]. However, biomarker concentrations may be influenced by methodological, physiological and environmental factors [15-18]. Acidification of EBC has been reported in humans with asthma, COPD and bronchiectasis [19]. Similarly, LAI has been associated with EBC acidification in calves [20] and horses [21]. The concentration of hydrogen peroxide (H$_2$O$_2$) in human EBC has been correlated with the severity and treatment response of several inflammatory diseases, including asthma [22]. Increased EBC H$_2$O$_2$ concentrations have been reported in horses with recurrent airway obstruction (RAO) [23] and foals with *Rhodococcus equi* pneumonia [24]. However, in other studies, no association between H$_2$O$_2$ and LAI in horses was found [25,26]. Leukotriene B$_4$ (LTB$_4$) is pro-inflammatory eicosanoid [27] and increased concentrations in EBC from humans with COPD and asthma have been reported [28]. EBC LTB$_4$ has been associated with LAI in calves with bacterial and viral pneumonia [29], pigs with *Chlamydia suis* pneumonia [30] and horses with RAO [31]. These findings suggest that these EBC biomarkers may be useful for the detection of LAI.
Measurement of specific EBC biomarkers could offer a non-invasive method for diagnosis and monitoring of LAI in horses. To date, reports evaluating EBC biomarkers in horses are scarce [21,23,31] and further investigation is warranted. Given the challenges in identifying and classifying LAI, this study used current recommendations for cytological and endoscopic definitions to diagnose the various syndromes [3,8-10]. The aim of this study was to identify associations between EBC pH, H₂O₂ and LTB₄ concentrations and presenting complaints, airway endoscopic and cytology findings (including case definitions of LAI), while controlling for environmental, animal and methodological factors. Our hypotheses were that EBC H₂O₂ and LTB₄ concentrations would be higher and EBC pH would be lower in horses with LAI.

**Materials and methods**

**Animals**

Forty-seven horses were included in this prospective, cross-sectional study. Sample size calculations (α = 0.05, power of 0.8) indicated group sizes of 16 were required to detect a 0.5 unit difference in mean pH and 1 µmol/L difference in H₂O₂ [21]. Twenty-seven horses were presented to the Veterinary Clinical Centre (VCC), Charles Sturt University (CSU) with complaints of respiratory disease and/or poor performance unrelated to musculoskeletal or cardiac abnormalities. Twenty asymptomatic sedentary horses were recruited from the CSU research herd. Client owned horses consisted of 19 geldings and 8 females; 14 Standardbreds, 6 Thoroughbreds, 4 Australian stock horses and 3 Quarter horses; mean age was 4.6 years (range: 2-10). Research horses consisted of 14 mares and 6 geldings; 10 Standardbreds, 9 Thoroughbreds, and 1 Quarter horse; mean age was 6.7 years (range: 4-15). Consent for inclusion in the study was obtained from all owners.

**EBC collection and handling**

A previously described EBC collection system (Fig 4.1) was used [18]. EBC was collected prior to all other procedures. Horses were restrained in a crush or a stable, in a well-ventilated building, depending on temperament. All EBC samples were frozen during collection and stored at -80°C until analysis. Samples were batched based on previously described stability periods for each biomarker [14,32,33]. Relative humidity (RH), ambient temperature (Tₐ), time of collection and respiratory rate (RR) during
collection were recorded for all samples. The average RR during EBC collection was determined by recording the RR at 5 min intervals, starting at the initiation of EBC collection or whenever the horse’s RR appeared to change. \( \text{EBC}_{TV} \) was estimated from the respiratory rate throughout EBC collection and published tidal volumes at rest \[34\], followed by biomarker expression per 100L of EB. EBC was collected from horses throughout the year. The facemask and tubing were rinsed with deionised water and air-dried after each collection.

**Upper and lower airway endoscopy and respiratory fluid collection**

After EBC collection, each horse was sedated with xylazine (0.3 to 0.5 mg/kg BW i.v. Ilium Xylazine\(^a\)). Video-endoscopy was used to examine the trachea and grade mucus \[35\] and blood \[10\]. A trans-endoscopic tracheal aspirate (TA) sample was obtained using a single lumen catheter (Equivet endoscope flushing catheter\(^b\)) and infusing 20 mL of sterile 0.9% NaCl solution. BALF was then obtained by blind placement of a commercial BAL catheter (BAL300\(^c\)), instillation of 300 mL of sterile isotonic saline and retrieval by aspiration; samples were deemed adequate if surfactant was present \[12\]. Aliquots of pooled BALF and TA fluid were processed within three hours of collection by cytocentrifugation and Wrights-Giemsa staining for cytological analysis. Differential cell counts were performed on 300 leukocytes and macrophage morphology was graded (Supplementary Item 4.1). A second BALF slide was stained using Perl’s Prussian Blue stain and a total haemosiderin score (THS) was determined using weighted grading of 300 macrophages \[9\]. Results were recorded as the relative percentages of the total leukocytes in BALF and TA fluid and were used to allocate horses into LAI categories.
Figure 4.1

Photographs of equipment used for collection of EBC from horses in this study. A custom-built facemask (A) was connected via flexible corrugated tubing with an internal diameter of 35 mm (B), to an airtight EBC condensation chamber (C). The facemask, constructed of a modified grazing muzzle, included two one-way non-rebreathing valves (D; Salford Valve, Cranlea) with an internal diameter of 35 mm, positioned over the nares with inspiratory PALL filters (E; Protec PF30S, PALL) attached. The insert provides a close-up image of the valves and inspiratory filters. An elastic rubber diaphragm (F) was attached to the facemask to create an airtight seal around the horse’s muzzle. The condensation chamber (C) was constructed of three 50 mL polypropylene tubes arranged in parallel (insert), immersed in an ethanol-liquid nitrogen slurry, cooling the condensation surface to less than -50°C. Two one-way valves (G; Aeromask valve, Trudell Medical) at the outlet of the condensation chamber prevented contamination of the EBC sample by environmental air. Collection time was standardised to 15 min.

EBC pH measurement

EBC samples for pH determination were stored for <11 months [33]. After thawing, samples were mixed to re-suspend sublimated volatile acids [36]. The pH of neat EBC was measured using a bench top pH meter (HI 2211 pH/ORP meter⁴) and a micro bulb pH-electrode (HI 1330B⁵), both calibrated prior to use. Subsequently, EBC was deaerated using argon gas and pH was recorded when the reading stabilised.
Measurements were done in either a 5 ml flat bottom vial or a 1.5 ml conical centrifuge vial (Eppendorf safe-lock vial) depending on the total volume of EBC available for pH measurement. Each sample was measured in triplicate at room temperature (21.23 ± 0.57 °C).

**EBC H₂O₂ measurement**

Flow injection analysis with fluorescence detection allowed indirect determination of H₂O₂ concentrations (Supplementary item 4.1). In brief, EBC was incubated with horseradish peroxidase (HRP), causing the non-fluorescing 4-hydroxyphenyl acetic acid (4-HPAA) to produce a fluorescent dimer as a result of H₂O₂ in EBC [37].

**EBC LTB₄ measurement**

LTB₄ concentrations were determined using liquid chromatography and triple quadrupole tandem mass-spectrometry (LC-MS/MS) adapted from Montuschi et al. [38] (Supplementary item 4.1). In brief, LTB₄-d₄ was added to EBC as an internal standard before sample concentration under vacuum and analysis by LC-MS/MS.

H₂O₂ and LTB₄ were expressed using direct concentrations (μmol/L and pg/mL, respectively) and concentrations standardised to the estimated total volume of exhaled breath (EBTV) per collection: pmol/100 L EB and pg/100L EB, respectively.

**Case definitions**

The study population comprised symptomatic and asymptomatic horses. Inflammatory airway conditions were diagnosed using recommended clinical, endoscopic and cytologic criteria [3,8-10], and concurrent expression of multiple abnormalities was considered. The following definitions were applied:

- **IAD**: BALF cytology thresholds were adapted from Couëtil et al. [3] with ≥10% neutrophils or ≥5% mast cells or ≥5% eosinophils or any combination of these. For statistical analysis, horses were further categorised as neutrophilic, metachromatic, eosinophilic, ‘mixed’ (combination of ≥2 subtypes) and ‘any’ (any subtype) IAD.
• EIPH: tracheobronchoscopic blood grading ≥1 [10], and/or BALF cytology with ≥20% haemosiderophages [1] and/or total haemosiderin score (THS) ≥75 in BALF [9].
• Lower airway disease (LAD): IAD and/or EIPH based on BALF cytology
• STI: tracheal inflammation score (TIS) ≥6/9; the 9-point inflammation score comprised the sum of tracheal mucus (0-3), smear cell density (0-3) and neutrophil proportion scores (0-3) [1,8].

Statistical methods
Concentrations of EBC biomarkers were compared with each case definition and with presenting complaints as dichotomous outcome variables (affected / unaffected) by two-sample t-tests or linear regression models with unequal variances applied, as required. Associations between EBC biomarkers and continuous and ordinal outcome variables (TA and BALF differential counts and cytology scores, TIS and THS) were assessed using Pearson’s or Spearman’s correlation coefficients, as appropriate. Multivariable linear regression models were used to identify associations between EBC biomarkers and case definitions or BALF leukocyte types, including animal, environmental and methodological factors. Variables with P<0.1 identified in univariate, pair-wise comparisons were included in multivariable models. The final model was derived by backward step-wise elimination, with the highest P-value term (P>0.05) removed at each step. Interactions were not considered due to sample size limitations. Assumptions were that residuals were normally distributed, had constant variance and were independent. The model assumptions were evaluated using scatter plots and histograms. Non-normally distributed data were transformed to the natural logarithmic scale prior to analysis, as required. Inverse variance weights were fitted if the variance of the EBC biomarker differed across the two levels of the disease indicator. Predicted means and 95% confidence intervals (CIs) were calculated for variables retained in multivariable analyses. Data were analysed using Stata 14; with significance set at P≤0.05.
Results

Study population

Physical examination, including pulmonary auscultation before and during a rebreathing procedure, did not reveal any abnormalities in 45 horses. One horse started coughing and another horse had a marked increase in RR during the rebreathing procedure. Presenting complaints of the 27 client-owned horses included poor performance (16), coughing (8), nasal discharge (3). Fifteen (of 27) had more than one presenting complaint. Horses from the CSU research herd had no abnormalities on physical examination. The population mean (± SD) body weight was 486.28 ± 59.31 kg.

Twenty-three, 15 and nine of 47 horses had IAD, EIPH and STI, respectively. Thirty-two horses were also included in the LAD group: six had both EIPH and IAD. Details of endoscopy, respiratory fluid analysis findings and presenting complaints are presented in Supplementary item 4.2. EBC samples from 46, 45 and 30 horses were available for pH, LTB₄ and H₂O₂ analyses respectively.

Environmental and animal factors

Mean $T_A$ and RH during EBC collection were 18.70 ± 7.52 °C and 54.47 ± 14.81%, respectively. Changes in respiratory effort and depth were not observed during sample collection; however, RR during collection (15.28 ± 3.80 breaths/min) was lower than prior to sampling (19.45 ± 10.93 breaths/min) (P=0.005). The estimated total volume of exhaled breath (EB_TV) per collection was 1,283.23 ± 319.36 L and the volume of EBC collected was 4.21 ± 1.28 mL. The mean volume of TA fluid and BALF retrieved was 16.6 ± 3.5 mL and 221.4 ± 26.4 mL, respectively.

Univariate pair-wise comparisons

Results of comparisons between phenotypes of LAI and EBC pH, H₂O₂ and LTB₄ are shown in Table 4.1. Horses with neutrophilic, eosinophilic or mixed IAD had significantly increased EBC pH. Direct H₂O₂ was significantly increased in three horses with mixed IAD (P<0.001) but was not increased in horses with other forms of LAI, and no effect was observed for direct or standardised LTB₄ concentrations. No association was observed between poor performance, coughing or nasal discharge and EBC pH. Horses with coughing as a presenting complaint, had lower direct and standardised
EBC H₂O₂ concentrations, (P = 0.013 and P = 0.037, respectively) and higher direct LTB₄ concentrations (P=0.024). Horses that presented with poor performance had lower direct and standardised EBC LTB₄ concentrations (P = 0.045 and P = 0.047, respectively). No associations with nasal discharge were present. EIPH was associated with a significantly lower mean standardised EBC LTB₄ concentration (P=0.042, Supplementary item 4.3).

Significant correlations between EBC biomarkers and endoscopy, BALF and TA cytological analysis are provided in Table 4.2. Correlations between EBC biomarkers and environmental and methodological factors are provided in Supplementary item 4.4.

**Multivariable linear regression models**

Factors retained in the multivariable analyses for EBC pH, H₂O₂ and LTB₄ between horses with and without LAI are shown in Table 4.3. Associations between BALF cellular components and these EBC biomarkers and any significant confounding factors are also provided (figures demonstrating the effect of confounding factors are provided in supplementary item 4.5). The pH of neat EBC was increased in horses with IAD (with or without EIPH) (Fig 4.2a) and a positive curvilinear association with BALF neutrophil percentage was identified (Fig 4.2b). A negative curvilinear association between pH measured in deaerated EBC and BALF mast cell percentage was observed (Fig 4.2c). Ambient humidity confounded interpretation of pH (neat and deaerated), and vial type affected pH in deaerated samples. No associations were observed for direct H₂O₂ concentration, however standardised H₂O₂ (pmol/100L EB) was significantly higher in horses with IAD (with and without EIPH) (Fig 4.3a), and a positive curvilinear association with BALF neutrophil percentage was identified (Fig 4.3b). Analysis batch was a confounding factor for interpretation of standardised H₂O₂. A positive curvilinear association between LTB₄ concentration and BALF eosinophil percentage was identified (Fig 4.4). Determination of LTB₄ concentration was confounded by Tₐ and RH.
Table 4.1

Univariate analysis comparing exhaled breath condensate (EBC) pH, hydrogen peroxide (H$_2$O$_2$) and leukotriene B$_4$ (LTB$_4$) in horses with or without defined categories of LAI.

<table>
<thead>
<tr>
<th>LAI category</th>
<th>Number of affected (unaffected) horses</th>
<th>Mean ± SD in affected horses</th>
<th>Mean difference between affected and unaffected horses</th>
<th>95% CI (mean difference)$^d$</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td></td>
<td>pH neat</td>
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<tr>
<td>LAD</td>
<td>31(15)</td>
<td>6.79 ± 0.43</td>
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<td>0.15 to 0.70</td>
<td>0.004</td>
</tr>
<tr>
<td>Neutrophilic IAD</td>
<td>9(37)</td>
<td>7.01 ± 0.44</td>
<td>0.45</td>
<td>0.09 to 0.81</td>
<td>0.019</td>
</tr>
<tr>
<td>Eosinophilic IAD</td>
<td>6(40)</td>
<td>7.05 ± 0.39</td>
<td>0.45</td>
<td>0.05 to 0.86</td>
<td>0.033</td>
</tr>
<tr>
<td>Metachromatic IAD</td>
<td>12(34)</td>
<td>6.68 ± 0.47</td>
<td>0.04</td>
<td></td>
<td>0.824</td>
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<tr>
<td>Mixed IAD$^a$</td>
<td>4(42)</td>
<td>7.18 ± 0.35</td>
<td>0.57</td>
<td>0.05 to 1.09</td>
<td>0.038</td>
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<tr>
<td>Any IAD$^b$</td>
<td>23(23)</td>
<td>6.82 ± 0.47</td>
<td>0.33</td>
<td>0.07 to 0.60</td>
<td>0.016</td>
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<tr>
<td>STI</td>
<td>9(30)</td>
<td>6.89 ± 0.41</td>
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<tr>
<td>EIPH any$^c$</td>
<td>14(32)</td>
<td>6.68 ± 0.39</td>
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<td>0.799</td>
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<td></td>
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<td>pH deaerated</td>
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<tr>
<td>LAD</td>
<td>31(15)</td>
<td>7.63 ± 0.53</td>
<td>0.37</td>
<td>0.02 to 0.72</td>
<td>0.039</td>
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<tr>
<td>Neutrophilic IAD</td>
<td>9(37)</td>
<td>7.87 ± 0.55</td>
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<td>0.01 to 0.89</td>
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<tr>
<td>Eosinophilic IAD</td>
<td>6(40)</td>
<td>7.95 ± 0.38</td>
<td>0.50</td>
<td>0.10 to 0.91</td>
<td>0.020</td>
</tr>
<tr>
<td>Metachromatic IAD</td>
<td>12(34)</td>
<td>7.43 ± 0.57</td>
<td>-0.12</td>
<td></td>
<td>0.547</td>
</tr>
<tr>
<td>Mixed IAD$^a$</td>
<td>4(42)</td>
<td>7.83 ± 0.56</td>
<td>0.35</td>
<td></td>
<td>0.306</td>
</tr>
<tr>
<td>Any IAD$^b$</td>
<td>23(23)</td>
<td>7.67 ± 0.57</td>
<td>0.31</td>
<td></td>
<td>0.067</td>
</tr>
<tr>
<td>STI</td>
<td>9(30)</td>
<td>7.78 ± 0.48</td>
<td>0.34</td>
<td></td>
<td>0.123</td>
</tr>
<tr>
<td>EIPH any$^c$</td>
<td>14(32)</td>
<td>7.49 ± 0.49</td>
<td>-0.04</td>
<td></td>
<td>0.841</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>H$_2$O$_2$ μmol/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAD</td>
<td>22(8)</td>
<td>0.62 ± 0.49</td>
<td>0.12</td>
<td></td>
<td>0.511</td>
</tr>
<tr>
<td>Neutrophilic IAD</td>
<td>6(24)</td>
<td>0.75 ± 0.71</td>
<td>0.20</td>
<td></td>
<td>0.495</td>
</tr>
<tr>
<td>Eosinophilic IAD</td>
<td>6(24)</td>
<td>0.82 ± 0.46</td>
<td>0.28</td>
<td></td>
<td>0.185</td>
</tr>
<tr>
<td>Metachromatic IAD</td>
<td>6(24)</td>
<td>0.69 ± 0.28</td>
<td>0.12</td>
<td></td>
<td>0.447</td>
</tr>
<tr>
<td>Mixed IAD$^a$</td>
<td>3(27)</td>
<td>0.98 ± 0.10</td>
<td>0.43</td>
<td>0.21 to 0.66</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Any IAD$^b$</td>
<td>15(15)</td>
<td>0.71 ± 0.54</td>
<td>0.24</td>
<td></td>
<td>0.148</td>
</tr>
<tr>
<td>STI</td>
<td>8(19)</td>
<td>0.58 ± 0.54</td>
<td>-0.11</td>
<td></td>
<td>0.561</td>
</tr>
<tr>
<td>EIPH any$^c$</td>
<td>9(21)</td>
<td>0.48 ± 0.46</td>
<td>-0.15</td>
<td></td>
<td>0.418</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>H$_2$O$_2$ pmol/100L EB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAD</td>
<td>22(8)</td>
<td>163.59 ± 2.37</td>
<td>0.51</td>
<td></td>
<td>0.426</td>
</tr>
<tr>
<td>Neutrophilic IAD</td>
<td>6(24)</td>
<td>169.79 ± 4.67</td>
<td>0.35</td>
<td></td>
<td>0.607</td>
</tr>
<tr>
<td>Eosinophilic IAD</td>
<td>6(24)</td>
<td>275.43 ± 1.09</td>
<td>1.46</td>
<td></td>
<td>0.112</td>
</tr>
<tr>
<td>Metachromatic IAD</td>
<td>6(24)</td>
<td>235.70 ± 0.76</td>
<td>1.02</td>
<td></td>
<td>0.217</td>
</tr>
<tr>
<td>Mixed IAD$^a$</td>
<td>3(27)</td>
<td>439 ± 0.207</td>
<td>2.73</td>
<td></td>
<td>0.080</td>
</tr>
<tr>
<td>Any IAD$^b$</td>
<td>15(15)</td>
<td>194.26 ± 2.14</td>
<td>1.10</td>
<td></td>
<td>0.100</td>
</tr>
<tr>
<td>STI</td>
<td>8(19)</td>
<td>110.74 ± 2.73</td>
<td>-0.86</td>
<td></td>
<td>0.189</td>
</tr>
<tr>
<td>EIPH any$^c$</td>
<td>9(21)</td>
<td>105.21 ± 2.67</td>
<td>-0.38</td>
<td></td>
<td>0.534</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LTB$_4$ pg/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAD</td>
<td>30(15)</td>
<td>16.37 ± 23.75</td>
<td>-5.33</td>
<td></td>
<td>0.107</td>
</tr>
<tr>
<td>Neutrophilic IAD</td>
<td>9(36)</td>
<td>24.97 ± 32.39</td>
<td>8.53</td>
<td></td>
<td>0.421</td>
</tr>
<tr>
<td>Eosinophilic IAD</td>
<td>5(40)</td>
<td>16.20 ± 12.16</td>
<td>-2.20</td>
<td></td>
<td>0.353</td>
</tr>
<tr>
<td>Type</td>
<td>Count</td>
<td>Mean ± SD</td>
<td>Median</td>
<td>p-value</td>
<td></td>
</tr>
<tr>
<td>---------------------</td>
<td>-------</td>
<td>-----------</td>
<td>--------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>Metachromatic IAD</td>
<td>12(33)</td>
<td>16.61 ± 23.67</td>
<td>2.10</td>
<td>0.181</td>
<td></td>
</tr>
<tr>
<td>Mixed IAD&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4(41)</td>
<td>17.95 ± 14.14</td>
<td>0.18</td>
<td>0.858</td>
<td></td>
</tr>
<tr>
<td>Any IAD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22(23)</td>
<td>19.69 ± 26.69</td>
<td>3.02</td>
<td>0.821</td>
<td></td>
</tr>
<tr>
<td>STI</td>
<td>9(29)</td>
<td>15.17 ± 2.53</td>
<td>0.91</td>
<td>0.186</td>
<td></td>
</tr>
<tr>
<td>EIPH any&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14(31)</td>
<td>4.16 ± 3.15</td>
<td>-1.34</td>
<td>0.051</td>
<td></td>
</tr>
</tbody>
</table>

**LTB₄ pg/100L EB**

<table>
<thead>
<tr>
<th>Type</th>
<th>Count</th>
<th>Mean ± SD</th>
<th>Median</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAD</td>
<td>30(15)</td>
<td>5.41 ± 7.56</td>
<td>-0.50</td>
<td>0.159</td>
</tr>
<tr>
<td>Neutrophilic IAD</td>
<td>9(36)</td>
<td>9.47 ± 11.15</td>
<td>4.87</td>
<td>0.278</td>
</tr>
<tr>
<td>Eosinophilic IAD</td>
<td>5(40)</td>
<td>6.59 ± 6.03</td>
<td>1.13</td>
<td>0.396</td>
</tr>
<tr>
<td>Metachromatic IAD</td>
<td>12(33)</td>
<td>4.96 ± 5.94</td>
<td>-0.84</td>
<td>0.402</td>
</tr>
<tr>
<td>Mixed IAD&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4(41)</td>
<td>8.08 ± 6.51</td>
<td>2.75</td>
<td>0.432</td>
</tr>
<tr>
<td>Any IAD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22(23)</td>
<td>6.61 ± 8.45</td>
<td>2.01</td>
<td>0.834</td>
</tr>
<tr>
<td>STI</td>
<td>9(29)</td>
<td>5.59 ± 1.80</td>
<td>0.71</td>
<td>0.124</td>
</tr>
<tr>
<td>EIPH any&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14(31)</td>
<td>1.75 ± 1.57</td>
<td>-0.88</td>
<td>0.042</td>
</tr>
</tbody>
</table>

EBC, exhaled breath condensate; EIPH, exercise-induced pulmonary haemorrhage; H₂O₂, hydrogen peroxide; IAD, inflammatory airway disease; LAD, lower airway disease (IAD and/or EIPH); LTB₄, leukotriene B₄; STI, syndrome of tracheal inflammation.

<sup>a</sup>Mixed IAD, a combination of ≥2 IAD subtypes; <sup>b</sup>Any IAD, any IAD subtype; <sup>c</sup>EIPH any: EIPH diagnosed by any of the described methods; <sup>d</sup>95% CI are only provided for significant categories. Significant results are presented in bold.
Table 4.2

Significant correlations between results of bronchoalveolar lavage fluid (BALF) and tracheal aspirate (TA) cytology, tracheal inflammation score (TIS), tracheal mucus accumulation scores, BALF macrophage morphology and exhaled breath condensate (EBC) pH, hydrogen peroxide ($H_2O_2$) and leukotriene B$_4$ (LTB$_4$).

<table>
<thead>
<tr>
<th>Conventional diagnostic parameter</th>
<th>Number of horses included in analysis</th>
<th>Correlation coefficient</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pH neat</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAL neutrophil %</td>
<td>46</td>
<td>$r = 0.41$</td>
<td>0.005</td>
</tr>
<tr>
<td>BAL eosinophil %</td>
<td>46</td>
<td>$r = 0.37$</td>
<td>0.011</td>
</tr>
<tr>
<td>TIS</td>
<td>38</td>
<td>$r = 0.34$</td>
<td>0.032</td>
</tr>
<tr>
<td>TA mast cell %</td>
<td>38</td>
<td>$r = -0.42$</td>
<td>0.006</td>
</tr>
<tr>
<td>BAL macrophage morphology</td>
<td>46</td>
<td>$r = 0.40$</td>
<td>0.006</td>
</tr>
<tr>
<td><strong>pH deaerated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAL eosinophil %</td>
<td>46</td>
<td>$r = 0.35$</td>
<td>0.015</td>
</tr>
<tr>
<td>TIS</td>
<td>38</td>
<td>$r = 0.33$</td>
<td>0.036</td>
</tr>
<tr>
<td>TA mast cell %</td>
<td>38</td>
<td>$r = -0.40$</td>
<td>0.010</td>
</tr>
<tr>
<td>BAL macrophage morphology</td>
<td>46</td>
<td>$r = 0.31$</td>
<td>0.037</td>
</tr>
<tr>
<td><strong>$H_2O_2 , \mu mol/L$</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAL macrophage morphology</td>
<td>30</td>
<td>$r_s = 0.40$</td>
<td>0.046</td>
</tr>
<tr>
<td><strong>LTB$_4 , pg/mL$</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TA neutrophil %</td>
<td>38</td>
<td>$r_s = 0.76$</td>
<td>0.017</td>
</tr>
<tr>
<td>BAL neutrophil %</td>
<td>45</td>
<td>$r_s = 0.45$</td>
<td>0.012</td>
</tr>
<tr>
<td>Tracheal mucus accumulation score</td>
<td>45</td>
<td>$r_s = 0.43$</td>
<td>0.015</td>
</tr>
<tr>
<td><strong>LTB$_4 , pg/100L EB$</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAL neutrophil %</td>
<td>45</td>
<td>$r_s = 0.45$</td>
<td>0.014</td>
</tr>
<tr>
<td>Tracheal mucus accumulation score</td>
<td>45</td>
<td>$r_s = 0.38$</td>
<td>0.034</td>
</tr>
</tbody>
</table>

$r$=Pearson’s correlation coefficient; $r_s$=Spearman’s rank correlation coefficient.
Table 4.3

Significant findings of the multivariable analysis evaluating exhaled breath condensate (EBC) pH, hydrogen peroxide ($\text{H}_2\text{O}_2$) and leukotriene B$_4$ (LTB$_4$) in horses with or without lower airway inflammation (LAI) as well as the association of these EBC biomarkers with bronchoalveolar lavage fluid (BALF) leukocyte percentage (%).

<table>
<thead>
<tr>
<th>LAI category or BALF leukocyte percentage</th>
<th>Number of affected (unaffected) horses or total number of horses</th>
<th>P-value</th>
<th>Effect (95% CI)$^c$</th>
<th>Significant environmental and methodological factors associated with the EBC biomarker and LAI category or BALF leukocyte in the multivariable model</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH neat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAD</td>
<td>31(15)</td>
<td>0.007</td>
<td>EBC pH in horses with LAD was 0.33 unit higher (0.10 to 0.56)</td>
<td></td>
</tr>
<tr>
<td>Any IAD$^a$</td>
<td>23(23)</td>
<td>0.04</td>
<td>EBC pH in horses with IAD was 0.11 unit higher (0.01 to 0.47)</td>
<td></td>
</tr>
<tr>
<td>BALF Neutrophil %</td>
<td>n = 46</td>
<td>0.01</td>
<td>pH increases by 0.23 units for each 1.72% increase in neutrophil % (0.06 to 0.40)</td>
<td></td>
</tr>
<tr>
<td>pH deaerated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BALF Mast cell %</td>
<td>n = 46</td>
<td>0.041</td>
<td>pH decreases by 0.20 units for each 1.72% increase in mast cell % (0.01 to 0.40)</td>
<td></td>
</tr>
</tbody>
</table>

H$_2$O$_2$ pmol/100L EB

| LAE                                      | 22(8)                                                   | 0.05   | EBC H$_2$O$_2$ pmol/100L EB in horses with LAD was 0.73 pmol/100L EB higher (0.001 to 1.98) |                                                                                  |
| Mixed IAD$^b$                             | 3(27)                                                   | 0.033  | EBC H$_2$O$_2$ pmol/100L EB in horses with IAD was 0.78 pmol/100L EB higher (0.05 to 2.01) |                                                                                  |
| BALF Neutrophil %                        | n = 30                                                   | 0.033  | EBC H$_2$O$_2$ pmol/100L EB increases by 0.54 pmol/100L EB for each 1.72% increase in neutrophil % (0.003 to 1.37) |                                                                                  |
| BALF Macrophage %                        | n = 30                                                   | 0.044  | EBC H$_2$O$_2$ pmol/100L EB decreases by 0.31 pmol/100L EB for each 1% increase in macrophage % (0.001 to 0.69) |                                                                                  |

LTB$_4$ pg/mL

| BALF Eosinophil %                        | n = 45                                                   | 0.035  | EBC LTB$_4$ pg/mL increases by 0.74 pg/mL for each 1.72% increase in eosinophil % (0.04 to 1.91) |                                                                                  |

LTB$_4$ pg/100L EB

| BALF Eosinophil %                        | n = 45                                                   | 0.027  | EBC LTB$_4$ pg/100L EB increases by 0.52 pg/100L EB for each 1.72% increase in eosinophil % (0.05 to 1.19) |                                                                                  |

| P-value | Effect (95% CI)$^c$ |                                                                                  |
|---------|--------------------|                                                                                  |
|<0.001  | EBC pH decreases by 0.02 units for each 1% increase in RH (0.01 to 0.02) |                                                                                  |
| 0.001   | EBC pH decreases by 0.01 units for each 1% increase in RH (0.01 to 0.02) |                                                                                  |
|<0.001  | EBC pH decreases by 0.02 units for each 1% increase in RH (0.01 to 0.02) |                                                                                  |
|<0.001  | EBC pH decreases by 0.01 units for each 1% increase in RH (0.01 to 0.02) |                                                                                  |
|<0.001  | EBC pH decreases by 0.01 units for each 1% increase in RH (0.01 to 0.02) |                                                                                  |
|<0.001  | EBC pH decreases by 0.01 units for each 1% increase in RH (0.01 to 0.02) |                                                                                  |
|<0.001  | EBC pH decreases by 0.01 units for each 1% increase in RH (0.01 to 0.02) |                                                                                  |

$^a$Any IAD, any IAD subtype; $^b$Mixed IAD, a combination of ≥2 IAD subtypes; $^c$Supplementary item 4.5 for graphs of predicted effects.

BALF, bronchoalveolar lavage fluid; EBC, exhaled breath condensate; H$_2$O$_2$, hydrogen peroxide; LAD, lower airway disease (IAD and/or EIPH); LTB$_4$, leukotriene B$_4$; RH, relative humidity; $T_a$, ambient temperature.
Predicted pH measured in neat (a, b) and deaerated (c) exhaled breath condensate (EBC) following the multivariable analysis (Table 4.3). Data are presented as means and 95% confidence intervals and are averaged across ambient humidity range (30-80%) (a-c) and vial type (c). a) Horses without (‘No’) and with (‘Yes’) lower airway disease (LAD: IAD and/or EIPH) and any IAD subtype. The predicted change in EBC pH at different bronchoalveolar lavage fluid (BALF) neutrophil (b) and mast cell percentages (c).
Figure 4.3 a, b
Predicted H$_2$O$_2$ concentrations standardised to the average volume of exhaled breath according to the multivariable analysis (Table 4.3). a) Predicted H$_2$O$_2$ for horses without ('No') and with ('Yes') lower airway disease (LAD: IAD and/or EIPH) and those with mixed IAD. b) Predicted H$_2$O$_2$ concentrations at different bronchoalveolar lavage fluid (BALF) neutrophil percentages according to the multivariable analysis. Data are presented as back-transformed means and 95% confidence intervals and are averaged across all batches.
Figure 4.4 a, b

Predicted direct LTB₄ concentrations (a) and LTB₄ concentrations standardised to the average volume of exhaled breath (b) at different bronchoalveolar lavage fluid (BALF) eosinophil percentages according to the multivariable analysis (Table 4.3). Data are presented as back-transformed means and 95% confidence intervals and are averaged across the range of ambient temperatures (15-30°C) and ambient humidity (30-80%).

Discussion

The results of this study indicate that EBC H₂O₂ concentrations and pH are higher in horses with LAI. Both these biomarkers and EBC LTB₄ are associated with various BALF leukocyte percentages, suggesting that analysis of EBC provides information relevant to the diagnosis and pathogenesis of LAI. Importantly, the study findings demonstrate that environmental and analysis factors can influence these biomarkers and should be considered when interpreting results.

The mean pH of neat EBC was slightly higher than previous reports in horses with and without LAI [21], calves [15] and pigs [39]. Mean pH of deaerated EBC was comparable to previous reports in healthy horses, humans and dogs [13,40]. The average change in pH after deaeration was approximately 1 unit, similar to a previous study [41]. Gas standardisation of EBC with argon [33] or CO₂ partial pressure [42] is considered necessary by some authors to obtain reliable pH measurements, whereas others argue that neat samples reflect the biological pH of the airway surface, as CO₂ is a relevant component of EBC [15]. Previous studies have demonstrated superior consistency of pH measurement in deaerated samples [18,40]. However, rather than increasing
measurement precision, deaeration might artificially reduce true biologic variation and decrease associations between EBC pH and LAI categories, as seen in the current study.

The results did not support our hypothesis of EBC acidification in horses with LAI. This contrasts with previous studies in people [19] and calves [20] that report EBC acidification in subjects with LAI and airway neutrophilia. The pH of EBC in horses with LAI has been reported only once previously, where a trend for lower pH in horses with LAI was observed [21]. Therefore, it remains uncertain whether airway acid-base dysregulation occurs with respiratory disease in horses. It is possible that acid-base dysregulation does not occur in horses with milder forms of LAI, as in this study, or that other components not measured in the current study, such as exhaled and environmental ammonia [43], prevented airway acidification. Alternatively, methodological factors such as condenser temperatures [36,40] or environmental conditions, such as RH and TA, may have influenced EBC pH. The absence of association between presenting complaints and pH was likely due to the often, subclinical nature of LAI in this study.

Associations between EBC pH and respiratory fluid leukocyte percentages in horses have not been reported previously. A positive association between EBC pH and BALF neutrophil percentage was identified. Neutrophils and eosinophils contain a variety of substances within lysosomes, including hypochlorous and hypohalous acids, which function to neutralise and digest bacteria and foreign particles. The release of these substances would be expected to result in a reduction in local pH [44] and therefore increased numbers of these leukocytes in BALF would be expected to be associated with decreased EBC pH. However, BALF cell percentages do not necessarily equate to cellular activity [11]. In addition, leukocyte changes observed in the current study may not have been of sufficient magnitude to cause airway acidification. A negative association was observed between EBC pH and mast cell percentage in TA and BALF. To the authors’ knowledge, this association has not previously been reported in any species and may reflect increased volatile acid liberation secondary to inflammatory mediator release associated with metachromatic inflammation. Cellular pathways mediating the observed changes in EBC pH remain to be determined.
In this study, environmental variables significantly influenced EBC biomarkers, particularly pH, as has been found previously [18]. A negative correlation between RH and EBC pH, and a positive correlation between TA and EBC pH were found during univariate analysis, however only RH was retained in the final multivariable model. This likely reflects the inverse relationship between TA and RH in our climatic region, and these factors may have contributed to the pH findings in this study. Unfortunately, the effect of season was not assessed in this study. Vial type influenced pH measured in deaerated EBC, which may reflect reduced surface area of EBC exposed to argon gas and reduced deaeration efficacy in a narrow, conical vial.

As the volume of EBC collected per volume of EB is not constant in spontaneously breathing animals [39], H$_2$O$_2$ and LTB$_4$ were expressed as measured and following standardisation to EB volume to avoid the confounding effects of respiration on biomarker concentrations [15,39]. In support of this recommendation, we have previously found improved consistency in EBC biomarker results expressed as EB$_{TV}$ [18]. A limitation of our study was the lack of accurate measurement of EB$_{TV}$ by spirometry. Instead, EB$_{TV}$ was estimated from RR, throughout EBC collection, and published tidal volumes at rest [34]. The mean RR during EBC collection was significantly lower than prior to sampling. Determination of the cause was outside the scope of this study but may have been due to increased mechanical dead-space stimulating marginal increased depth of respiration [45] with compensatory reduction in respiratory frequency.

Concentrations of H$_2$O$_2$ in EBC were similar to previous reports in horses [23,26]. Horses with IAD, with and without EIPH, had higher concentrations of EBC H$_2$O$_2$ than horses without IAD. This association supports our hypothesis and is consistent with the findings of studies in humans with LAI [13,46]. However to date, the association in horses with LAI has been unclear. Deaton et al. [23] found that EBC H$_2$O$_2$ was greater in horses with RAO compared to control horses. Similarly, EBC H$_2$O$_2$ was greater in foals with *R. equi* pneumonia than in control foals [24]. However, other studies did not demonstrate an association between EBC H$_2$O$_2$ and LAI [21,25]. The current study is the first to demonstrate an association between H$_2$O$_2$ and mild pulmonary
inflammation in horses; however, caution is warranted with interpretation of the biological significance as there were only three horses in the mixed IAD group.

Hydrogen peroxide is produced from the conversion of superoxide anions $\text{O}_2^-$ by superoxide dismutase [47] during the respiratory burst of activated neutrophils, eosinophils and macrophages in response to oxidative stress [48]. A positive association between $\text{H}_2\text{O}_2$ and BALF neutrophil percentage was observed in the current study, as has been previously reported in horses with RAO [23] and healthy cats [49]. A similar relationship between the number of neutrophils in sputum and increased EBC $\text{H}_2\text{O}_2$ concentrations has been demonstrated in humans [22]. Additionally, our study demonstrated a significant positive correlation with BAL macrophage morphology but a negative association with BALF macrophage percentage, suggestive of an association between EBC $\text{H}_2\text{O}_2$ and macrophage activity rather than with the number of macrophages present.

Multivariable analysis demonstrated and accounted for a batch effect on $\text{H}_2\text{O}_2$ concentration. This has not been reported in previous equine studies and is likely due to the indirect nature of the $\text{H}_2\text{O}_2$ analysis method resulting in inconsistency between batches. Analysis is based on the conversion of a substrate (HPAA) to a fluorescent dimer in the presence of both $\text{H}_2\text{O}_2$ and an enzyme (HRP) [37]. As enzyme activity is responsible for utilisation of $\text{H}_2\text{O}_2$ and creation of the fluorescent dimer, any variation in enzyme purity or activity may affect measured $\text{H}_2\text{O}_2$ concentrations. Additionally, fluorescence detection can suffer from spectral interferences, such as reabsorption of fluoresced light, as well as quenching of fluorescence by species such as chloride and sulphide [50]. Other environmental, animal or methodological covariates were not significantly associated with EBC $\text{H}_2\text{O}_2$ in multivariable analysis.

Mean EBC LTB$_4$ in the current study was substantially lower than in horses with RAO [31], healthy calves [29] and humans [13]. These results may reflect species variation and/or a study population with milder airway inflammation. Differences in EBC LTB$_4$ concentration attributable to LAI were not observed. Previously reported poor consistency of LTB$_4$ in EBC [18] likely contributed to the absence of demonstrable associations between EBC LTB$_4$ and categories of LAI. However multivariable analysis
demonstrated a positive association between BALF eosinophil percentage and EBC LTB₄ concentration. The macrophages, activated neutrophils, mast cells, eosinophils and epithelial cells release LTB₄, which acts as a non-specific pro-inflammatory mediator by activating neutrophils [51] and causing eosinophilic chemotaxis [52]. Univariate analyses revealed significantly increased EBC LTB₄ concentrations in horses with a history of coughing, and positive correlations with tracheal mucus accumulation score, BALF and TA neutrophil percentages. Similarly, in a previous study, a strong linear relationship between EBC LTB₄ and BALF neutrophil percentage in healthy calves was found [29]. The observed associations between LTB₄ and tracheal mucus accumulation score, TA neutrophil percentage and coughing have not been reported previously. These findings demonstrate an association between LTB₄ and the degree of LAI, including eosinophilic inflammation, suggesting LTB₄ may be a useful biomarker of LAI. Environmental factors did not affect LTB₄ concentration in a previous study [18]. However, in the current study, both T_A and RH demonstrated a significant negative association with EBC LTB₄ in the multivariable analysis and should be considered as confounding variables for this analyte.

A strength of the current study was the ability to evaluate the effects of different LAI phenotypes in data analysis, which allowed elucidation of multiple novel associations. This reflects the complex nature of inflammation of the lower airways in horses and suggests that EBC biomarkers may provide a non-invasive method through which the pathophysiology, progression or response to treatment of LAI conditions, such as equine asthma, can be monitored. However, there is inherent difficulty in diagnosing LAI due to the diversity of clinical and cytological phenotypes, which likely represent a number of related or unrelated clinical syndromes, with multiple inclusion criteria based on variable clinical signs, changes in airway cytology and changes in pulmonary function [3,8,53]. As unequivocal inclusion criteria for mild asthma in horses have yet to be established [3], a case-control approach was not used in the current study. Instead, case definitions, based on clinical and cytological findings were used to investigate associations between airway inflammation and EBC biomarkers. Additionally, a current clinical limitation is the reliance on BALF cytology to diagnose IAD. Horses with IAD may not necessarily have BALF cytological abnormalities despite compromised pulmonary function [3,54] and cytological differential counts may not
necessarily reflect total cell numbers or activation [11]. Increased EBC H$_2$O$_2$ or LTB$_4$ concentrations are indicative of increased production and/or impaired removal of inflammatory mediators and may, therefore, better reflect pulmonary leukocyte activity and be more useful indicators of respiratory tract inflammatory activity than airway cytology or endoscopy. Additionally, the inclusion of horses affected only by EIPH may have influenced our results, however the frequent simultaneous presence of EIPH and IAD [1,3,5], precluded the exclusion of EIPH from this study. The number of samples available for measurement of each EBC biomarker was determined by various laboratory constraints, including, but not limited to a faulty UHPLC resulting in the inability to test some EBC samples before exceeding the recommended storage period for EBC H$_2$O$_2$, laboratory error and laboratory equipment malfunctions.

In conclusion, this study demonstrated that EBC H$_2$O$_2$ concentrations and pH are higher in horses with LAI. Additionally, both H$_2$O$_2$ and pH had a positive association with BALF neutrophil percentage, while EBC LTB$_4$ demonstrated a positive association with BALF eosinophil percentage. Mast cell percentage in BALF was negatively associated with EBC pH. These EBC biomarkers, with EBC pH demonstrating the most promise, may be useful indicators of LAI phenotypes and could potentially provide more information on respiratory tract inflammatory activity than conventional diagnostic modalities. However, environmental and methodological factors can influence these biomarkers, necessitating standardisation when collecting and analysing samples.
Conflict of interest statement
None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Ethical animal research
The study was approved by the CSU Animal Care and Ethics Committee (protocol number: 14/082).

Sources of funding
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Authorship
S. du Preez, S.L. Raidal and K.J Hughes contributed to the conception and design of the study. S. du Preez, S.L. Raidal and K.J Hughes contributed to the acquisition and interpretation of data. G.S. Doran contributed substantially to the method development for EBC analysis and acquisition of the data. M Prescott contributed substantially to the analysis and interpretation of data. S. du Preez composed the article. All authors contributed to the critical revision of the article and approved the final manuscript for publication.

Manufacturers
a Troy Laboratories, Glendenning, New South Wales, Australia
b Kruuse, Langeskov, Denmark
c SurgiVet, Dublin, Ohio, United States of America
d Hanna Instruments, Keysborough, Victoria, Australia

e Eppendorf South Pacific, North Ryde, NSW, Australia

f Sigma-Aldrich, Sydney, New South Wales, Australia

Sapphire Bioscience, Redfern, New South Wales, Australia

h StataCorp, College Station, Texas, United States of America
Supplementary material

**Supplementary item 4.1**

The grading system used for semi-quantitative grading of bronchoalveolar lavage fluid (BALF) macrophage morphology, method used for determination of total haemosiderin score and a detailed description of the analysis methods used to determine exhaled breath condensate (EBC) hydrogen peroxide (H$_2$O$_2$) and leukotriene B$_4$ (LTB$_4$) concentrations.

**BALF macrophage morphology grading [55]**

1. small alveolar macrophages with no cytoplasmic vacuole
2. mostly small alveolar macrophages with some larger cells present
3. mostly larger alveolar macrophages with only some vacuolated
4. mostly large, heavily vacuolated alveolar macrophages

The images were taken at 400X magnification.
Total haemosiderin score (THS) of alveolar macrophages [9,56]

Alveolar macrophages were graded [56]:

Grade 0  absence of blue coloration in the cytoplasm of alveolar macrophages
Grade 1  faint blue staining in the cytoplasm
Grade 2  dense blue colour in a minor portion of the cytoplasm or medium colour intensity throughout the cell
Grade 3  deep blue staining in most of the cytoplasm
Grade 4  cell filled with haemosiderin, dark blue throughout the cytoplasm.

The THS was determined by counting 300 alveolar macrophages and grading them as above, followed by conversion to percentages per grade, multiplying the percentages with the numerical value of the grade and adding these values together for the final THS. The maximum possible score is 400 (if 100% of the alveolar macrophages have a grade of 4). Doucet and Viel [9] determined that a cut-off THS of 75 would provide a 94% sensitivity and 88% specificity for EIPH in their population.
**EBC H₂O₂ measurement**

H₂O₂ concentrations were determined with the use of flow injection analysis with fluorescence detection (FIA-FD) using an indirect method during which a fluorescent dimer of 4-hydroxyphenyl acetic acid (HPAA, Sigma Aldrich) was formed by the redox coupling reaction between H₂O₂ in EBC and horseradish peroxidase (HRP, Sigma Aldrich) [37].

EBC samples for H₂O₂ concentration determination were stored at -80°C for no longer than 40 days [32]. One of three 50 mL polypropylene tubes collected from each horse was thawed at 2 °C over 80 min. A working solution of reaction mixture consisting of 560 µl HRP (5 units/mL), and 1400 µL of 3mM 4-HPAA substrate, both prepared in 100 mM phosphate buffer, was prepared fresh for each batch. Fifty microliters of working solution were added to 500 µL of EBC and allowed to react for 60 minutes on ice. A 200 µL aliquot was transferred to a glass low volume insert (Phenomenex) in an auto-sampler vial for analysis by FIA-FD. A six-point calibration curve (0 to 5 µmol/L), which had good linearity (r² = 0.997), was prepared for every analysis using the same procedure as for the samples. All solutions were prepared on ice to limit temperature fluctuation and associated sample degradation. Samples and standards were analysed in three duplicate blocks to minimise the influence of reaction time on the fluorescent reaction. Run time was 1 min with injection overlap after 0.8 min. The lower limit of detection (LoD) was 0.05 µmol/L with an intra-assay coefficient of variation of 1.3%.

The flow injection was performed using an Agilent 1290 UPLC with a mobile phase consisting of instrument grade water:methanol (56:44, v/v) with a 0.3 ml/min flow rate and 2 µL injection volume. The auto-sampler was maintained at 4 °C and samples were kept in the dark. The excitation and emission wavelengths were 285 and 400 nm, respectively.
**EBC LTB₄ measurement**

LTB₄ concentrations were determined using liquid chromatography with triple quadrupole tandem mass-spectrometry (LC-MS/MS) adapted from Montuschi et al. [38], accounting for processing losses with the use of a LTB₄-d₄ (Sapphire Bioscience) as an internal standard. EBC samples for LTB₄ concentration determination were stored at -80°C for no longer than 28 days [14]. 100 µL of 0.1 µg/mL LTB₄-d₄ solution was added to each EBC sample, resulting in a final concentration of 10 ng/mL. Spiked EBC samples were concentrated five-fold, using evaporation in a vacuum chamber (OVL-570, Gallenkamp) at room temperature (21.23 ± 0.57°C) with a constant nitrogen flow. Residues were resuspended in 200 µL instrument grade methanol.

Analysis was performed with an Agilent 1260 LC connected to a 6410 triple quadrupole mass spectrometer using positive electrospray ionisation (ESI+). The liquid chromatography was done using a Phenomenex Kinetex XB-C18 100A column (50 x 4.6 mm x 2.6 µm) and a linear gradient with water-methanol-formic acid (70:30:0.1, v/v) that was changed to 100% methanol over four minutes. A 12-point calibration curve over the range of 0-5 ng/ml was prepared using the peak area rations for LTB₄/LTB₄-d₄. The calibration curve had good linearity ($r^2 = 0.995$) and the LoD was 3.22 pg/mL. Samples and standards were analysed in duplicate and all working solutions were prepared fresh for each batch.

The optimised MS/MS source parameters were: gas temperature (350°C), gas flow rate (13 l/min), nebuliser pressure (40 psi) and capillary voltage (4000 V). Multiple reaction monitoring (MRM) was used for quantitative analysis of LTB₄ in EBC. The $m/z$ 359 $\rightarrow$ $m/z$ 341 and $m/z$ 363 $\rightarrow$ $m/z$ 345 transitions were used to detect LTB₄ and LTB₄-d₄, respectively [38].
## Supplementary item 4.2

### Table 4.4

Details of endoscopic examination, respiratory fluid analysis and presenting complaints.

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The highlights designate horses that were diagnosed with IAD (BALF neutrophil ≥10%, mast cell ≥5% and eosinophil ≥5% of the total leukocyte count) or EIPH based on endoscopic evaluation or BALF haemosiderophage ≥20% or THS ≥75 or STI based on TIS of ≥6/9. NA - data not available as TA was not done.

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<td>4.7</td>
<td>0</td>
<td>10.0</td>
<td>7</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>sedentary horse</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>6</td>
<td>4.0</td>
<td>3.0</td>
<td>3.3</td>
<td>0</td>
<td>2.0</td>
<td>41</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>sedentary horse</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>4</td>
<td>7.0</td>
<td>3.7</td>
<td>2.0</td>
<td>0</td>
<td>2.0</td>
<td>1.5</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>sedentary horse</td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>10</td>
<td>8.3</td>
<td>0.3</td>
<td>0.7</td>
<td>0</td>
<td>0.0</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>sedentary horse</td>
<td></td>
</tr>
</tbody>
</table>
Supplementary item 4.3

Table 4.5

Univariate analysis results demonstrating the association of exhaled breath condensate (EBC) pH, hydrogen peroxide (H$_2$O$_2$) and leukotriene B$_4$ (LTB$_4$) with presenting complaints.

<table>
<thead>
<tr>
<th>LAI category or presenting complaint</th>
<th>Number of affected (unaffected) horses in each category</th>
<th>Mean ± SD in affected horses</th>
<th>Mean difference between affected and unaffected horses</th>
<th>95% CI (mean difference)$^a$</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH neat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poor performance</td>
<td>16(30)</td>
<td>6.75 ± 0.39</td>
<td>0.12</td>
<td>-0.415</td>
<td>0.617</td>
</tr>
<tr>
<td>Coughing</td>
<td>9(37)</td>
<td>6.60 ± 0.32</td>
<td>-0.09</td>
<td>-0.452</td>
<td></td>
</tr>
<tr>
<td>Nasal discharge</td>
<td>4(42)</td>
<td>6.77 ± 0.21</td>
<td>0.10</td>
<td>-0.452</td>
<td>0.452</td>
</tr>
<tr>
<td></td>
<td>pH deaerated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poor performance</td>
<td>16(30)</td>
<td>7.53 ± 0.44</td>
<td>0.01</td>
<td>0.968</td>
<td></td>
</tr>
<tr>
<td>Coughing</td>
<td>9(37)</td>
<td>7.55 ± 0.43</td>
<td>0.04</td>
<td>0.869</td>
<td></td>
</tr>
<tr>
<td>Nasal discharge</td>
<td>4(42)</td>
<td>7.49 ± 0.34</td>
<td>-0.03</td>
<td>0.877</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H$_2$O$_2$ μmol/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poor performance</td>
<td>11(19)</td>
<td>0.38 ± 0.36</td>
<td>-0.31</td>
<td>0.061</td>
<td></td>
</tr>
<tr>
<td>Coughing</td>
<td>8(22)</td>
<td>0.32 ± 0.23</td>
<td>-0.34</td>
<td>0.132</td>
<td></td>
</tr>
<tr>
<td>Nasal discharge</td>
<td>3(27)</td>
<td>0.48 ± 0.14</td>
<td>-0.11</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H$_2$O$_2$ pmol/100L EB</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poor performance</td>
<td>11(19)</td>
<td>84.27 ± 2.47</td>
<td>-0.96</td>
<td>0.132</td>
<td></td>
</tr>
<tr>
<td>Coughing</td>
<td>8(22)</td>
<td>61.51 ± 2.29</td>
<td>-1.73</td>
<td>0.14</td>
<td>NA</td>
</tr>
<tr>
<td>Nasal discharge</td>
<td>3(27)</td>
<td>127.64 ± 0.58</td>
<td>0.14</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LTB$_4$ pg/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poor performance</td>
<td>16(29)</td>
<td>4.48 ± 2.82</td>
<td>-1.27</td>
<td>0.02 to 4.07</td>
<td>0.045</td>
</tr>
<tr>
<td>Coughing</td>
<td>8(37)</td>
<td>15.60 ± 0.74</td>
<td>1.02</td>
<td>0.10 to 2.69</td>
<td>0.024</td>
</tr>
<tr>
<td>Nasal discharge</td>
<td>3(42)</td>
<td>8.45 ± 3.68</td>
<td>0.14</td>
<td>0.123</td>
<td>0.995</td>
</tr>
<tr>
<td></td>
<td>LTB$_4$ pg/100L EB</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poor performance</td>
<td>16(29)</td>
<td>1.90 ± 1.49</td>
<td>-0.79</td>
<td>0.01 to 2.18</td>
<td>0.047</td>
</tr>
<tr>
<td>Coughing</td>
<td>8(37)</td>
<td>4.79 ± 0.67</td>
<td>0.46</td>
<td>0.123</td>
<td>0.887</td>
</tr>
<tr>
<td>Nasal discharge</td>
<td>3(42)</td>
<td>2.98 ± 1.77</td>
<td>-0.08</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

$^a$95% CI are only provided for significant categories. Significant results are presented in bold.

NA - data not available as sample sizes too small for statistical analysis.
**Supplementary item 4.4**

**Table 4.6**

Univariate analysis results demonstrating the association of exhaled breath condensate (EBC) pH, hydrogen peroxide (H$_2$O$_2$) and leukotriene B$_4$ (LTB$_4$) with pertinent environmental and methodological covariates.

<table>
<thead>
<tr>
<th>Environmental or methodological covariates</th>
<th>Correlation coefficient or Association</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pH neat</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ambient temperature</td>
<td>$r = 0.47$</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Relative humidity</td>
<td>$r = -0.50$</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>pH deaerated</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ambient temperature</td>
<td>$r = 0.53$</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Relative humidity</td>
<td>$r = -0.50$</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Respiratory rate during collection</td>
<td>$r = 0.31$</td>
<td>0.030</td>
</tr>
<tr>
<td>pH Vial used$^a$</td>
<td>pH mean (SD) was 0.79 (0.12) units lower when measured in Eppendorf tube</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>H$_2$O$_2$ μmol/L</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ambient temperature</td>
<td>$r = 0.77$</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Relative humidity</td>
<td>$r = -0.54$</td>
<td>0.001</td>
</tr>
<tr>
<td>Analysis batch$^b$</td>
<td>mean(SD) was 0.62(0.32) μmol/L lower in batch 8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>H$_2$O$_2$ pmol/100L EB</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ambient temperature</td>
<td>$r = 0.75$</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Relative humidity</td>
<td>$r = -0.45$</td>
<td>0.009</td>
</tr>
<tr>
<td>Analysis batch$^b$</td>
<td>Mean (SD) was 6.56 (1.15) pmol/100L EB lower in batch 8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>LTB$_4$ pg/mL and LTB$_4$ pg/100L EB</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>no significant associations</td>
<td></td>
</tr>
</tbody>
</table>

$^a$EBC pH measurements were done in either a 5 ml flat bottom vial or a 1.5 ml conical centrifuge vial (Eppendorf safe-lock vial$^e$) depending on the total volume of EBC available for pH measurement.

$^b$Analysis batch; please refer to page 151 for elaboration on the effect of batch on H$_2$O$_2$ and LTB$_4$ concentration.
Supplementary item 4.5

Figures demonstrating the predicted effect of each significant lower airway inflammation (LAI) category or bronchoalveolar lavage fluid (BALF) leukocyte percentage on exhaled breath condensate (EBC) pH, hydrogen peroxide ($H_2O_2$) and leukotriene B$_4$ (LTB$_4$) in the multivariable analysis. These effects are provided in conjunction with any significant confounding factors.

Figure 4.5

Predicted pH measured in neat exhaled breath condensate (EBC) following the multivariable analysis. Predicted linear decrease in EBC pH in response to increasing ambient humidity for horses without (blue) and with (red) lower airway disease (LAD: IAD and/or EIPH). Data are presented as means and 95% confidence intervals.
Figure 4.6 a, b, c
Predicted pH measured in neat (a) and deaerated (b, c) exhaled breath condensate (EBC) following the multivariable analysis. The black lines represent the means and 95% confidence intervals. a) Predicted curvilinear increase in EBC pH in response to increasing BALF neutrophil percentage. The pH measurements decrease as ambient humidity (blue lines) increases. Predicted decrease in EBC pH in response to increasing BALF mast cell percentages. b) The pH measurements decrease as ambient humidity (blue lines) increases and c) the vial type in which EBC is deaerated influences the measured pH.
Figure 4.7 a, b, c

Predicted H₂O₂ concentrations standardised to the average volume of exhaled breath according to the multivariable analysis. a) Predicted H₂O₂ for horses with (red) and without (blue) lower airway disease (LAD: IAD and/or EIPH) and those with ≥2 subtypes of IAD for batch 4, 5 and 8 (the other batch numbers were too low for inclusion in analysis). Data are presented as means and 95% confidence intervals. b) Predicted curvilinear increase in H₂O₂ in response to increasing BALF neutrophil percentage, c) predicted curvilinear decrease in H₂O₂ in response to increasing BALF mast cell percentage. The differences in batch are presented as blue lines and the black lines represent the means and 95% confidence intervals.
Figure 4.8 4 a, b
Predicted LTB4 concentrations standardised to the average volume of exhaled breath according to the multivariable analysis. Predicted curvilinear increase in LTB4 in response to increasing BALF eosinophil percentage. The predicted LTB4 concentrations decrease as ambient temperature (a) and ambient humidity (b) increases. The differences at various ambient temperatures and humidity’s are presented as blue lines and the black lines represent the means and 95% confidence intervals.
References


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CHAPTER 5

Manuscript prepared in preparation for submission to *Journal of Chromatography B*
Short Communication

Sensitive and selective analysis of $\text{H}_2\text{O}_2$ in exhaled breath condensate using LC-MS/MS.

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Abstract

Changes in the concentration of hydrogen peroxide (H$_2$O$_2$) in exhaled breath condensate (EBC) occur in inflammatory lung diseases in humans and animal species. The concentration of H$_2$O$_2$ in EBC can vary widely and is often close to the lower limit of detection of commonly used detection methods. The following work reports an innovative, sensitive and more selective liquid chromatography tandem mass-spectrometry (LC-MS/MS) method for the determination of EBC H$_2$O$_2$. The new method was more sensitive (LLoQ of 0.093 μM) than previously published routine methods. The intra- and inter-day repeatability, expressed as coefficients of variation (CV), were 1.0-3.2% and 1.7-2.3%, respectively. Several LC columns were compared for analysis and a runtime of < 2 min using isocratic elution was achieved. While the new method was 1.5-3.7 times more sensitive than existing methods, it also provided superior selectivity and repeatability compared with both fluorescence and biosensor detection methods while providing high sample throughput. Additionally, comparing peak areas of multiple standards and the gradients of standard curves for EBC versus instrument grade water allowed evaluation of the influence of sample matrix. Both measures were significantly different (P<0.001), which demonstrates a significant matrix effect, emphasising the importance of matrix evaluation and consideration prior to analysis of incurred samples.

*Keywords*: EBC; fluorescence; horse; horseradish peroxidase; hydrogen peroxide; mass-spectrometry
1. Introduction

Exhaled breath condensate (EBC)\(^2\) is collected by condensing expired breath to liquid or solid states using a cryogenic trap [1]. EBC constituents are likely present in concentrations that reflect those within the epithelial lining fluid of the lower airways [2] and contain many non-volatile molecules, including cytokines, arachidonic acid metabolites and surfactant as well as water-soluble volatile molecules including ammonia and hydrogen peroxide (H\(_2\)O\(_2\)) [1]. Changes in the concentrations of EBC biomarkers occur in inflammatory lung diseases in humans and animal species. H\(_2\)O\(_2\) is produced during the respiratory burst of activated inflammatory cells in response to oxidative stress [3]. The concentration of H\(_2\)O\(_2\) in EBC has been correlated with the severity of several inflammatory lung diseases, including asthma [4] in humans, Ascaris suum-sensitised cats [5] and equine asthma [6, 7]. Concentrations of H\(_2\)O\(_2\) in EBC vary widely and are often close to the lower limit of detection (LoD) of many methods, driving the need for improved sensitivity and selectivity in analytical methods to identify subtle concentration differences.

Commonly used methods to determine H\(_2\)O\(_2\) concentrations in EBC include spectrophotometry [6], chemiluminescence [8], fluorescence detection [9] and bench top biosensors [10]. Of these, fluorescence detection (FLD) and flow injection analysis (FIA) with FLD have superior sensitivity. As H\(_2\)O\(_2\) is highly volatile and does not fluoresce spontaneously, FLD quantitation of H\(_2\)O\(_2\) utilises an indirect method that relies on the formation of a fluorescent dimer. The conversion of a non-fluorescing monomer, 4-hydroxyphenylacetic acid (4-HPAA) into a stable, fluorescent dimer, 2,2'-dihydroxybiphenyl-5,5'-diacetate (DHPA), is caused by the redox coupling reaction between H\(_2\)O\(_2\) and horseradish peroxidase (HRP) [11]. Although some studies have found the combination of HRP and HPAA to be highly selective for H\(_2\)O\(_2\) [12], others have documented interference when measuring H\(_2\)O\(_2\) by fluorescence, due to the presence of other oxidative substances such as sulphide, resulting in suppression of

\(^2\) Abbreviations: EBC, exhaled breath condensate; DHPA, 2,2'-dihydroxybiphenyl-5,5'-diacetate; FIA, flow injection analysis; FLD, fluorescence detection; HRP, horseradish peroxidase; 4-HPAA, 4-hydroxyphenylacetic acid
the observed fluorescence [13]. While the presence of sulphide in EBC is unknown, the presence of substances altering the level of fluorescence remains plausible. As such, a method that does not rely on fluorescence, but allows direct quantitation of the DHPA, could offer a more reliable and specific method for quantitation of low concentrations of \( \text{H}_2\text{O}_2 \) in EBC.

Tandem mass spectrometry (MS/MS), which is renowned for superior sensitivity and selectivity may provide additional sensitivity over existing methods. As \( \text{H}_2\text{O}_2 \) has considerable volatility combined with a low molecular weight, direct detection by LC-MS/MS is unlikely to provide improved sensitivity over fluorescence. For this reason, analysis of the fluorescing DHPA is a more likely target for LC-MS/MS analysis, which has not been reported in the literature. The aims of this study were to develop a superior method for the measurement of \( \text{H}_2\text{O}_2 \) in EBC and to evaluate the effect of solution matrix on results. We hypothesised that a novel, sensitive and more selective method for determination of \( \text{H}_2\text{O}_2 \) concentrations in EBC could be developed using LC-MS/MS and that the use of EBC as sample matrix would be dissimilar to instrument grade water.

2. Materials and methods

2.1. Reagents and chemicals

\( \text{H}_2\text{O}_2 \) (30% in water), 4-HPAA, HRP and formic acid (FA) were supplied by Sigma-Aldrich (NSW, Australia). Methanol was supplied by Mallinckrodt (NSW, Australia). All chemicals were high purity or HPLC-grade. Unless otherwise stated, all stock solutions and standards were prepared in instrument grade water.

2.2. Stock solutions and standard curve preparation

4-HPAA was dissolved to a concentration of 10 mM and HRP to an activity of 250 U/mL and stored at 2 °C during use and at -80 °C between batch analyses. A 10 mM \( \text{H}_2\text{O}_2 \) working standard was prepared and diluted to provide a series of standards across the 0.01-5 \( \mu \text{M} \) range in either instrument grade water or pooled EBC. The 10 mM working standard was quantified prior to each analysis by measuring absorbance at 240 nm using a Cary 50 CONC UV-Visible spectrophotometer (Varian - Agilent Technologies, Santa Clara, CA, USA) and the concentration calculated using \( \varepsilon_{240} = 43.6 \text{ M}^{-1}\text{cm}^{-1} \) [14].
EBC was collected from five horses and pooled. EBC was heated to 40 °C for at least 5 h, based on the decomposition rate of H₂O₂ [15], to eliminate native H₂O₂ and allow spiking with H₂O₂ standards.

2.3. Dimerization procedure

HRP stock was diluted to an activity of 5 U/mL and 4-HPAA to a concentration of 3 mM, both in 100 mM phosphate buffer. The working solution consisted of 560 µL HRP and 1400 µL 4-HPAA and was prepared fresh for each batch. One part working solution of enzyme and substrate was added to 10 parts H₂O₂ standard in water or EBC (e.g. 20 µL working solution: 200 µL standard) in an Eppendorf™ centrifuge tube (1.5 mL), mixed by repeated inversion and allowed to react for 60 min at 2 °C. Methanol was added at a ratio of 1 part: 3 parts standard to precipitate proteins and inactivate the enzyme. Each tube was vortex mixed (1 min) then centrifuged (21,000 x g, 5 min) and the supernatant transferred to a glass, low volume insert (Phenomenex, NSW, Australia) in an auto-sampler vial for analysis.

2.4. Chromatography and detection

Analysis was performed using an Agilent Technologies (Santa Clara, CA, USA) 1260 Series LC with binary pump, degasser, column heating block, auto-sampler, DAD (205 nm), 1260 fluorescence detector (FLD) and 6470 quadrupole MS/MS controlled by MassHunter software. The MS/MS and FLD were interchanged on the LC as needed because the FLD flow cell was susceptible to back pressure, preventing concurrent use. To allow direct comparison between LC column efficiency and the more commonly used FIA method, the LC column was replaced with a union to allow the LC to be used for FIA. Since the use of LC has not been reported for the analysis of H₂O₂, four columns were evaluated for optimal performance in conjunction with MS/MS. These included Agilent Poroshell 120 SB-C18 (100mm x 2.1 x 2.7 µm) (SB100), Agilent Poroshell 120 SB-C18 (50mm x 2.1 x 2.7 µm) (SB50), Phenomenex Kinetex 100A Biphenyl (50mm x 3 x 2.6 µm) (BP) and Agilent Poroshell HPH-C18 (50mm x 3.0 x 2.7 µm) (HPH) columns. As run times were less than 2 min, isocratic elution was used, with a 0.3 mL/min flow rate and 6 µL injection volume. The SB100, SB50 and BP columns used instrument grade water (0.1% FA) and methanol (30:70, v/v) as mobile phases,
while the HPH column used 10 mM ammonium carbonate (pH 9.5) and methanol (30:70, v/v). Each standard was analysed a minimum of four times.

FLD used excitation and emission wavelengths of 285 and 400 nm, respectively, with PMT voltage of 10 V. The MS/MS used ESI- for both HPAA and DHPA. The optimised MS/MS source parameters were: gas temperature (250 °C) and flow (13 L/min), sheath gas temperature (250 °C) and flow (12 L/min), nebuliser pressure (25 psi), capillary voltage (negative: 1500 V) and nozzle voltage (negative: 2000 V). Ion transitions (m/z) of 301.3 → 257.3 and 213.3 were used for DHPA, while 151 → 107 and 79.1 were used for 4-HPAA. Fragmentor voltage, collision energy and cell accelerator voltage were 105/10/2 (DHPA) and 92/10/1 (4-HPAA), respectively.

2.5. Data analysis
Analytes were quantified using peak areas. The limit of detection (LoD) and lower limit of quantitation (LLoQ) were determined for both FIA and LC analysis utilising FLD and MS/MS using instrument grade water or EBC as a matrix. The limit of blank (LoB) was determined as the mean of several injections of a blank plus 1.645 x SD_{blank}. Similarly, the LoD was determined as the LoB plus 1.645 x SD_{low concentration sample} [16] and the LLoQ was calculated by performing multiple injections of a low concentration standard and calculated as the mean plus 10 x SD, provided the coefficient of variation (CV) was less than 20% [17]. Data were expressed as mean and SD. Pair wise comparisons between variables were done using t-tests, adjusting for equality of variance using the F-test. Matrix comparisons were performed by comparing gradients of each, using t-tests for differences between two independent regression coefficients [18]. Pearson’s correlation coefficient, t-test and linear regression analysis were used to evaluate the relationship between LC-MS/MS and LC-FLD.

3. Results and discussion
3.1. LC column comparison
H\textsubscript{2}O\textsubscript{2} in EBC is quantified indirectly by dimerization of 4-HPAA. The fluorescence of DHPA can be measured using a bench-top spectrophotometer or flow injection analysis [9, 14]. The use of LC for the separation of DHPA from 4-HPAA has not been reported, even though it has the potential to improve sensitivity. Consequently, a
comparison of four LC columns was undertaken, consisting of three different stationary phases. 4-HPAA and DHPA could not be separated using any of the four LC columns tested due to relatively short retention times on all columns, so efforts were focussed on optimisation of peak shape and symmetry. The BP column showed the poorest performance, resulting in a split peak, which eliminated it from further studies (Fig. 5.1a). The HPH column produced a single peak with a baseline width of approximately 0.3 min (Fig. 5.1b) but had a peak height that was smaller than the taller of the split peaks for the BP column. This suggested that the buffer, or the pH of the buffer might have suppressed ionisation. Two SB-C18 columns with identical column dimensions (except length) were also trialled. The SB100 showed an improved peak height and shape (Fig. 5.1c) compared with the HPH and BP columns, while the SB50 produced a narrower DHPA peak (Fig. 5.1d) that was approximately 25% taller than the SB100 column, making it the best performer of the columns tested. As a result, all work was performed using the SB50 column.

**Figure 5.1**

Chromatograms demonstrating the peak shape and quality, retention time, peak height and area associated with each column for a standard of the same concentration of H$_2$O$_2$ using LC-MS/MS. a) BP, b) HPH, c) SB100, d) SB50 and e) the two extracted transitions for LC-MS/MS with the SB50 column and EBC as matrix.

The relatively short retention time of DHPA suggested that it is not retained by the SB50 column and inclusion of a LC-column is of little use. However, when the column was removed from the LC system, allowing FIA operation, the peak shape of DHPA became distorted and resulted in extensive tailing (Fig. 5.2 a-d). These results suggest that
the SB-C18 stationary phase interacted sufficiently with DHPA to limit tailing and allow focusing of the sample plug (Fig. 5.2 e-h) compared to the absence of an LC-column, while the longer SB100 column was detrimental to peak shape by allowing more longitudinal diffusion to occur. As a result, the SB50 was identified as the most efficient column for DHPA analysis (Fig. 5.1 e). Additionally, the relatively narrow bore of the column limited longitudinal diffusion, resulting in lower LoD and LLoQ values for FLD and MS/MS when using the SB50 column (Table 5.1) compared with FIA.

**Figure 5.2**
Chromatograms demonstrating the peak shape and quality, retention time, peak height and area associated with detection of DHPA, by fluorescence detection (a, c, e, g) and MS/MS (b, d, f, h) using flow injection (a to d) and LC using the SB50 column (e to h).
Table 5.1
Comparison of the LoB, LoD and LLoQ (μM) for H₂O₂ quantitation for each method, FLD and MS/MS, utilising either flow injection (FIA) or chromatography (LC), using column SB50, with either EBC or instrument grade water as the standard curve matrix.

<table>
<thead>
<tr>
<th></th>
<th>FIA</th>
<th>LC</th>
<th>FIA</th>
<th>LC</th>
<th>FIA</th>
<th>LC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matrix</td>
<td>EBC</td>
<td>Water</td>
<td>EBC</td>
<td>Water</td>
<td>EBC</td>
<td>Water</td>
</tr>
<tr>
<td>LoB</td>
<td>0.049</td>
<td>0.007</td>
<td>0.031</td>
<td>0.040</td>
<td>0.054</td>
<td>0.018</td>
</tr>
<tr>
<td>LoD</td>
<td>0.157</td>
<td>0.021</td>
<td>0.046</td>
<td>0.075</td>
<td>0.074</td>
<td>0.042</td>
</tr>
<tr>
<td>LLoQ</td>
<td>0.157</td>
<td>0.119</td>
<td>0.122</td>
<td>0.262</td>
<td>0.104</td>
<td>0.193</td>
</tr>
</tbody>
</table>

3.2. Effect of sample matrix
To identify whether the matrix affected the quantitation of DHPA by FLD and MS/MS, a comparison between instrument grade water and EBC was made, using H₂O₂ spiked additions. The LoD and LLoQ for both LC-MS/MS and LC-FLD were lower when using EBC as the standard curve matrix (Table 5.1) and variation at low concentrations was higher for water than EBC (e.g. at a H₂O₂ concentration of 0.027 μM, the CV for EBC was 6.1% compared with 12.4% for water). Comparison of the standard curve gradients, when using instrument grade water or EBC as the matrix was significantly different when using LC-MS/MS (EBC, 954.1; water, 1531.2; P<0.001), LC-FLD (EBC, 50.5; water, 53.8; P = 0.05 to <0.001), FIA-MS/MS (EBC, 491.6; water, 751; P<0.005) and FIA-FLD (EBC, 75.6; water, 129.9; P<0.001). Pair wise comparison of chromatogram peak areas (Fig. 5.2) revealed a significant difference between EBC and water (P<0.001) for both LC-MS/MS and LC-FLD (Table 5.2).
Table 5.2

Summary data used for the pair wise comparison of the peak area (PA) of two standards (standard A, 0.72 μM and standard B, 2.88 μM H₂O₂) using either pooled EBC or instrument grade water as a matrix.

<table>
<thead>
<tr>
<th></th>
<th>HPLC-MS/MS</th>
<th></th>
<th>HPLC-FLD</th>
<th></th>
<th>FIA-MS/MS</th>
<th></th>
<th>FIA-FLD</th>
<th></th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean PA</td>
<td>SD</td>
<td>Mean PA</td>
<td>SD</td>
<td>Mean PA</td>
<td>SD</td>
<td>Mean PA</td>
<td>SD</td>
<td></td>
</tr>
<tr>
<td>A - EBC</td>
<td>466.0</td>
<td>9.1</td>
<td>31.0</td>
<td>1.4</td>
<td>396.5</td>
<td>12.2</td>
<td>100.5</td>
<td>0.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>A - water</td>
<td>1173.5</td>
<td>30.6</td>
<td>54.3</td>
<td>2.6</td>
<td>807.5</td>
<td>4.1</td>
<td>148.9</td>
<td>2.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>B - EBC</td>
<td>1536.8</td>
<td>16.0</td>
<td>99.1</td>
<td>2.9</td>
<td>1291.3</td>
<td>37.8</td>
<td>253.7</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>B - water</td>
<td>3164.0</td>
<td>100.6</td>
<td>146.6</td>
<td>4.7</td>
<td>2014.0</td>
<td>33.7</td>
<td>365.3</td>
<td>3.2</td>
<td></td>
</tr>
</tbody>
</table>

These results differ from previous studies, which did not find a significant difference when comparing water and EBC matrices for standard curve preparation [9, 14]. This may be due, in part, to the inability to duplicate exact analytical conditions between previous and current studies. Additionally, the viscosity and pH of a solution has been reported to modulate diffusion of small molecules in solution, which may slow the diffusion of H₂O₂ to the binding site on HRP to form the reactive enzyme intermediate, which results in the formation of the stable dimer, DHPA [19]. Therefore, it is likely that activation of HRP and formation of DHPA would occur more readily in water than EBC due to the lower viscosity of water. Though the pH of standards prepared in water or EBC was not measured, it is reasonable to assume the solution pH differed, which may further have impacted the efficient function of HRP. The presence of other molecules in EBC may also interact with either 4-HPAA or HRP, resulting in inhibition of the production of DHPA in EBC compared with using water as matrices. The cause of the difference between matrices remains speculative, as determination of the cause was beyond the scope of this study however, it is apparent that pooled EBC should be used as the matrix for standard curve preparation to ensure reliable results for incurred EBC samples are attained.

3.3. Comparison of LC-MS/MS and LC-FLD

Comparison of the H₂O₂ concentrations using LC-MS/MS vs HPLC-FLD, using linear regression analysis revealed strong correlations with each other when using either water (r = 0.977, P<0.001) or EBC (r = 0.993, P<0.001) as matrix for the preparation of
standard curves (Fig. 5.3). This also revealed that LC-FLD gave slightly higher and LC-MS/MS slightly lower concentrations compared with the spiked H$_2$O$_2$ concentration. These observations suggest that either suppression occurred in the MS/MS or that another species fluoresced or possibly due to oxidation of 4-HPAA [20]. The latter is supported by work using FIA-FLD in a previous study (unpublished data), which demonstrated an increase in overall signal for blanks and standard solutions when the concentration of 4-HPAA in the working solution was increased (Appendix A). Comparison of the spiked H$_2$O$_2$ concentrations with the predicted concentrations determined from the standard curves did not reveal a significant difference between the actual and predicted value, determined by LC-MS/MS and LC-FLD (all P-values>0.05). Additionally, the LoD and LLoQ of the LC-MS/MS method was lower than that of both the LC-FLD and FIA-FLD method, demonstrating that LC-MS/MS was more sensitive and provides a LLoQ that is 1.5 times lower than other reported fluorescence methods, whilst providing greater specificity than fluorescence detection.

**Figure 5.3**
Comparison of H$_2$O$_2$ concentration when analysed by LC-MS/MS and LC-FLD using instrument grade water and pooled EBC.
3.4. LC-MS/MS method development

During MS/MS method development, mobile phase compositions were compared including different concentrations of FA (0.025, 0.05 and 0.1%) in water, as low concentrations of FA have been shown to improve the LoD of HPAA [21]. Water containing 0.1% FA resulted in the most symmetrical and sharpest peak shape, and a flow rate of 0.3 mL/min resulted in less band broadening than 0.2 mL/min. Due to the short retention time of DHPA, gradient elution showed no advantage over isocratic elution, so isocratic elution was chosen to enhance sample throughput water (0.1% FA):methanol (30:70, v/v). Peak broadening resulting from injection volumes greater than 6 μL were unacceptable, and a 35 °C column temperature was the best compromise between peak shape and retention. These optimised conditions resulted in a retention time of 0.8 min and overall analytical runtime of less than 2 min.

3.5. LC-MS/MS detection limits and repeatability

The LLoQ using the LC-MS/MS method (Table 5.1) was notably lower than the LLoQ (0.13 μM) previously reported for FIA-FLD [9]. Comparison of LLoQ with studies claiming superior LoD is not possible, as the LLoQ was not declared [14, 22, 23]. The LoD for LC-MS/MS was very similar to previous reports utilising flow injection with fluorescence detection [9, 22] and lower than that reported for bench-top fluorescence [24] and a commercially available biosensor method [10]. However, the LC-MS/MS was superior to both these aforementioned methods as it offered greater selectivity and better certainty. Interference when measuring H₂O₂ by FLD utilising HRP has been reported due to the presence of other oxidative substances, resulting in suppression of the observed fluorescence [13]. As such, the LC-MS/MS method in the current study is less susceptible to the influencing factors that affect fluorescence and allows direct quantitation of DHPA, and is therefore a more reliable and specific method for quantitation of low concentrations of H₂O₂ in EBC.

Repeatability of the LC-MS/MS method was determined by analysis of standards containing 0.5, 1 and 3 μM H₂O₂. Injection of four replicates of each concentration on the same day allowed assessment of intra-day repeatability. This process was repeated on two additional days to allow assessment of inter-day repeatability. The intra-day repeatability was 1.0-3.2% CV while the inter-day repeatability was 1.7-2.3% CV,
demonstrating that the LC-MS/MS method has negligible variation. Additionally, the inter-day CV was notably improved over that reported in a previous study using FIA-FLD [9]. Furthermore, both the intra- and inter-day repeatability was obviously improved when compared with the variation reported for bench-top fluorescence and biosensor methods [10, 24].

4. Conclusions

Fluorescence detection can suffer from spectral interferences, such as reabsorption of fluoresced light, as well as quenching of fluorescence by species such as chloride. MS/MS detection is not subject to these limitations and therefore provides superior selectivity and specificity. The results in the current study demonstrate a method for H₂O₂ quantification in EBC that is more sensitive and repeatable than existing methods, while maintaining the rapid sample throughput associated with methods using FIA-FLD and bench-top biosensors. Moreover, this is the first report demonstrating a significant matrix effect, emphasising the importance of matrix evaluation and consideration prior to analysis of incurred samples, to ensure accurate determination of H₂O₂ concentrations in EBC.

Funding

This study was supported by funds received from a professional doctorate scholarship from Charles Sturt University. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.
Supplementary material

Supplementary item 5.1

Figure 5.4
Chromatograms demonstrating the increasing peak height and peak area of a blank with the use of a working solution with increasing concentration of substrate (4-HPAA). a) blank prepared in instrument grade water; the added working solution had a 4-HPAA concentration of 1.5 mM, b) blank prepared in instrument grade water; the added working solution had a 4-HPAA concentration of 3 mM, c) blank prepared in instrument grade water; the added working solution had a 4-HPAA concentration of 4.5 mM.
References


The results of the studies included in this thesis demonstrate that the intra- and inter-day consistency of EBC $\text{H}_2\text{O}_2$ and pH is adequate to high, while the consistency of LTB$_4$ is poor. The concentration of $\text{H}_2\text{O}_2$ and pH measurements of EBC is higher in horses with lower airway inflammatory conditions. Both these biomarkers and EBC LTB$_4$ concentrations are associated with various BALF and TA leukocyte percentages, suggesting that analysis of EBC provides information relevant to the diagnosis and pathogenesis of certain cytological phenotypes of lower airway inflammatory conditions. Furthermore, EBC pH measurements and $\text{H}_2\text{O}_2$ and LTB$_4$ concentrations may be influenced by several environmental, animal and methodological factors that should be considered in experimental design of future studies of biomarkers in EBC. Measurement of pH and $\text{H}_2\text{O}_2$ concentrations in EBC has the potential to be useful diagnostic parameters in the investigation of lower airway inflammatory conditions, such as equine asthma (including IAD and RAO), STI and possibly EIPH. However, the poor intra-animal consistency of EBC LTB$_4$ suggests that this biomarker may not be worth pursuing, despite the positive association between LTB$_4$ concentrations and BALF eosinophil percentages.

1. **Overall contributions of the study to the equine literature**

Whilst there is a considerable body of knowledge regarding the evaluation of EBC in humans with various respiratory diseases, as evidenced by the establishment of task forces [1] and publication of meta-analyses [2,3], little understanding of EBC biomarkers in veterinary medicine is available. While comparative approaches to advances in certain areas of veterinary medicine have been achieved through consideration and integration of human research findings or approaches, it cannot be assumed that human EBC research findings can be translated directly to horses. In comparison to humans, horses have multiple physiological housing, management and dietary differences and are affected by different respiratory disorders. As such, it is of critical importance for the development of EBC analysis in horses that fundamental research is undertaken, to serve as the foundation for development of EBC methods, biomarker analysis and investigation of these biomarkers as potential clinical tools.

The results of the studies presented in this thesis provide important and valuable information to the small body of existing knowledge regarding the collection and
The variability (or lack thereof) of a biomarker can be expressed in many ways, including agreement, repeatability, consistency, reliability, and variability, each with a slightly different statistical definition and interpretation. Defining the level of agreement or alternatively the level of variance of a biomarker is essential for the process of developing a diagnostic tool. In this study, consistency was used to express the absence of variance of EBC biomarkers within a subject. The consistency study (Chapter 3) provides fundamental and more robust information regarding the variability associated with EBC pH, H₂O₂ and LTB₄ concentrations in horses. Information regarding the variability of pH and H₂O₂ concentrations in EBC collected from horses has only been reported once and twice, previously [4,5]. Duz et al. [5] reported minimal intra- and inter-day variation for EBC pH measurements but high levels of variation for concentrations of H₂O₂ in EBC. An earlier study including only 5 horses reported similarly large levels of variation for EBC H₂O₂ concentrations [4]. However, both these studies only expressed the level of EBC biomarker variation in terms of coefficient of variation (CV).

In the current study, determination of the consistency of each EBC biomarker was achieved by calculation of the intraclass correlation coefficient (ICC) using special case 3A[C,1] (consistency definition using single measurements) and confidence intervals were estimated as defined by McGraw and Wong [6]. This analysis of consistency also considered environmental and animal factor influences, by using two-way mixed effects model ANOVA to calculate variance components used in the ICC calculations. This method is a more robust method of assessing variability than determination of the CV, which expresses the extent of variability in relation to the mean of the sample or population [7]. According to McGraw and Wong [6] the most fundamental interpretation of ICC is that this statistical parameter is a measure of the proportion of variance attributable to the objects of measurements [6]. In the current study, the objects are each horse and the corresponding measurements are each of the specific EBC biomarkers. The consistency definition of ICC was used in this study, rather than the more commonly used absolute agreement definition, as it allows consideration of systematic, repeatable changes in EBC biomarker measurements rather than requiring absolute uniformity. A simplified example would be to consider changes in H₂O₂ concentration in relation to ambient temperature. When determining the level of
agreement, such as when measuring EBC H$_2$O$_2$ concentration in horses in the morning compared with the afternoon with a 10°C ambient temperature difference, the absolute H$_2$O$_2$ concentration will not be the same for both measurements for each horse. However, there will be a systematic/repeatable change in H$_2$O$_2$ concentration corresponding with the change in ambient temperature for each horse; this corresponds to a high degree of consistency but poorer absolute agreement. Therefore, the adequate to high consistency demonstrated in the current study for EBC pH and H$_2$O$_2$ measurements indicate that although biomarkers may be influenced by various factors, resulting in alterations in the absolute value of the biomarker, this variation is consistent and predictable allowing for statistical adjustment. Practically, standardisation of influencing factors will result in a reduction of the associated variation, thus improving absolute agreement. This in turn may allow application of the measurement of EBC pH and H$_2$O$_2$ in the investigation of lower airways in horses.

In the current study, multiple factors that may influence EBC pH, H$_2$O$_2$ and LTB$_4$ concentrations in horses were identified. Some of the identified factors, such as the influence of ambient temperature (T$_A$) and relative ambient humidity (RH) on EBC pH are consistent with previous findings of ambient influences on this biomarker in human studies [8]. Whereas others, such as the influence of respiratory rate (RR) during EBC collection on EBC pH are in contrast to other studies that reported no effect of RR on EBC pH [9,10]. Additionally, new influencing factors have been identified, such as the influence of T$_A$ and RH on EBC H$_2$O$_2$ and LTB$_4$ concentrations, the matrix and batch effects when measuring EBC H$_2$O$_2$ and the influence of vial type used during deaeration, on pH measurement in EBC. As such, this project corroborates some of the findings of previous human studies, but also identified previously unknown factors that may influence EBC biomarkers. These findings emphasise that standardisation of EBC sample collection and analysis is needed. However, widespread standardisation of EBC methodology and biomarker measurement between veterinary studies is likely to be challenging, as has been shown in human medicine despite various task-force [1] and review article recommendations [11-13].

Furthermore, interactions between some of these influencing factors were identified. The time of day at which EBC was collected interacted with ambient temperature and
collection location (whether EBC was collected with the horse in an indoor crush or outdoor stable/yard), while influencing EBC $\text{H}_2\text{O}_2$. Additionally, an interaction between the time of day of EBC collection and collection location as well as ambient temperature and collection location were identified to influence pH measured in neat and deaerated EBC, respectively. Though the current study lacked the statistical power to fully explore these relationships, these interaction terms demonstrate that the effect of influencing factors cannot be considered in isolation.

A limitation of the current study is that other influences that were not evaluated may have influenced EBC biomarkers and/or respiratory health of the horses included in this study. These include, but are not limited to atmospheric pressure, pollen, fungal spore and particle counts at the time of EBC collection and in the days preceding EBC and respiratory sample collection [14]. Future studies evaluating EBC biomarkers in horses should consider evaluation of these parameters in conjunction with the factors identified in this study.

Factors that should be standardised in future studies in an attempt to reduce variability of EBC biomarkers include ambient temperature and humidity, which may be achieved by performing EBC collections in an environmentally controlled room, which will also eliminate the effect of location on EBC collection. Further, the effect of time of day may be overcome in a clinical situation by collecting consecutive EBC samples at approximately the same time, although this may not be a practical solution.

This is the first study to evaluate EBC biomarkers obtained from horses with mild forms of lower airway inflammation (LAI), including IAD, STI/trIAD and EIPH. Previous studies evaluating EBC biomarkers in horses included animals with severe equine asthma (RAO) [4,5,15,16], a condition known to be associated with more severe pulmonary inflammation than IAD [17]. Statistically significant associations between EBC pH and LAI in horses have not been identified previously. A single previous study identified increased EBC $\text{H}_2\text{O}_2$ concentrations in horses with LAI; however, the subjects in that study had RAO [15]. Subsequent studies were unable to identify a difference in EBC pH and $\text{H}_2\text{O}_2$ in horses with and without LAI, despite the inclusion of horses with more severe pulmonary inflammation [4,5,18]. The ability of the current study to identify
relationships with disease factors may have been due to the nature of the multivariable analysis, allowing for inclusion of, and adjustment for influencing factors and perhaps due to a larger sample size. Additionally, earlier studies used a spectrophotometric H\textsubscript{2}O\textsubscript{2} analysis method. The usefulness of spectrophotometry for determination of H\textsubscript{2}O\textsubscript{2} concentration determination in EBC has been questioned, due to the high level of variation of measurements at the low range detected in EBC [19-21]. Therefore, use of spectrophotometric analysis may have contributed to the high degree of variation in EBC H\textsubscript{2}O\textsubscript{2} reported in previous equine studies [4,5] and to the inability to identify a difference between horses with and without LAI. The results of the current study suggest that measurement of EBC H\textsubscript{2}O\textsubscript{2} concentration can be used to detect differences between horses with LAI and horses without lung disease. However, variation due to influencing factors should be minimised and a sensitive analysis method, such as fluorescence detection (FLD) or the liquid chromatography tandem mass-spectrometry method (LC-MS/MS) developed during the third study (Chapter 5), should be used. Further clarification of the influence of airway inflammation on H\textsubscript{2}O\textsubscript{2} is needed to better determine the pathophysiology of these conditions and value of H\textsubscript{2}O\textsubscript{2} as a biomarker of respiratory tract disease.

Overall these studies provide new and invaluable information regarding the use of EBC pH, H\textsubscript{2}O\textsubscript{2} and LTB\textsubscript{4} measurements in the evaluation of lower airway health in horses. The purpose of this project was not to establish robust diagnostic criteria but rather to explore these EBC biomarkers in horses and expand the limited body of knowledge, which may be used to direct future research for the application of EBC biomarkers in horses.

2. The future of EBC pH analysis

Establishing the variability of a biomarker is a fundamental requirement in the process of evaluating the diagnostic applicability of the biomarker. The intra- and inter-day consistency of pH was adequate, with ICC >0.8 for both pH measured in neat EBC and after deaeration. This is consistent with the low level of variation reported by Duz et al. [5] where intra-day and inter-day CV of 0.05 ± 0.03 and 0.04 ± 0.02, respectively, was reported. Similarly, multiple human studies [10,22,23] have reported little variability of EBC pH measurements. These findings suggest that measurement of pH in EBC of
horses has sufficient repeatability to advocate future use of pH to evaluate lower airway health in horses.

**Measurement considerations**

Although it is traditionally accepted that the process of pH measurement is simple, there are a few considerations. Firstly, it is of utmost importance to ensure that the selected pH probe is suitable for small volumes and low ionic strength, as EBC is a dilute, low ionic strength fluid and some pH probes will not function appropriately in this matrix [24]. The pH probe used in the current studies conformed to these criteria, allowing pH reading stabilisation within 5 min, which is notably quicker than previously reported in equine studies (up to 20 min) [25] and was calibrated prior to use. Multiple other technical factors may also influence the pH of EBC, such as collection temperature, sampler design [24,26] and measurement temperature [27]. The current study identified vial type within which EBC was deaerated and subsequently measured in, as an additional influencing factor. The volume of some samples were not large enough to allow the use of 5 mL vials for all measurements due to the required minimum sample depth, necessitating the use of 1.5mL conical centrifuge tubes. Therefore, it is our recommendation to use the same type of vial for all pH measurements to exclude vial type as a potential confounding variable to the interpretation of deaerated EBC pH measurements.

The method of EBC sample preparation prior to measurement of pH is a matter of debate. Gas standardisation of EBC with argon or other inert gases [10] or CO₂ partial pressure [28] is considered necessary by some authors to obtain reliable pH measurements. Other authors argue that non-deaerated or neat samples reflect the “biological” pH of the airway surface, as CO₂ is a relevant component of EBC [29] and the influence of deaeration on other components of EBC, such as ammonia, are not understood [30]. In the current study, superior consistency in deaerated samples was demonstrated as the ICC for inter-day measurements were higher (Chapter 3), which is supported by a previous study [26]. However, rather than increasing measurement precision, deaeration might artificially reduce true biologic variation and hence decrease the number of associations between EBC pH and LAI categories, as found in
Measurement of pH in neat EBC is likely preferable considering that the consistency of pH measurements in deaerated EBC was only marginally better than that measured in neat EBC and the associations of pH measured in neat EBC with multiple categories of LAI and BALF neutrophil percentage. This is in agreement with the findings of previous studies in horses and humans [5,23,31]. In light of the conflicting evidence in the current studies and the available literature, it is recommended that pH values measured in both neat and deaerated EBC are reported, until more information regarding the utilisation of EBC pH measurements becomes available.

**Associations of pH with environmental, animal and method factors**

Various associations of EBC pH with environmental, animal and method factors were identified in the current studies. Multivariable analysis during the first study (Chapter 3) identified that pH measured in both neat EBC and after deaeration was significantly influenced by $T_A$ and RR during collection, whereas the second study (Chapter 4) identified RH as a confounder when interpreting pH measured in neat EBC and after deaeration. Vial type was identified as an additional confounder of the interpretation of pH in deaerated EBC. This disparity in terms of the dominant influencing environmental factors, likely reflects the inverse relationship between $T_A$ and RH in our climatic region. Univariate analysis in both studies revealed a significant negative correlation between RH and EBC pH as well as a significant positive correlation between $T_A$ and EBC pH, however only one of the factors were retained in the final multivariable models. Similarly, Kullmann *et al.* [8] reported an influence of environmental temperature and humidity on EBC pH in humans with increased EBC pH during periods of dry, warm weather and reductions in EBC pH during cold, humid weather. However, previous studies in horses [31] and calves [29] reported that EBC pH was not influenced by environmental factors, perhaps because the range of ambient temperatures and humidity was not as diverse in these studies as in the current studies.

Interactions between the time of day of EBC collection and collection location (whether EBC was collected in a crush in a well ventilated examination room or in an
outside stable/concreted yard) as well as ambient temperature and collection location were identified to influence pH measured in neat and deaerated EBC during the first study (Chapter 3), respectively. Although the sample size limited adequate assessment of these interactions terms, it is possible that the identified influence of $T_A$ and RH may have contributed to the influence of time of day and collection location, as the ambient conditions indoors demonstrated less variation than in the outside yards. Additionally, other factors that were not evaluated in the current study may also have contributed, such as ambient and exhaled ammonia concentrations [32]. Nevertheless, further research will be required to evaluate the full extent of interactions between environmental and animal factors.

In contrast to the findings of the consistency study (Chapter 3), EBC pH has previously been demonstrated to be independent of variables of spontaneous breathing in both humans and animals [10,29,31]. This disparity may be due to a larger sample size in the current study than that used in the previous study of horses [31]. The discordance of the current study findings with those of human studies may be due to species variation and differences in EBC collection method. EBC collection in humans is done through oral breathing, whereas horses are obligate nasal breathers, therefore airflow through the nasopharynx and nasal passages may influence EBC pH in horses. However, the study by Vaughan et al. [10] demonstrated that EBC pH was similar when collected either by oral breathing or through endotracheal intubation, suggesting that control of EBC pH is likely at the lower airway level. Although an association with RR was not consistently demonstrated in the current studies, it may be preferable that an expiratory flow meter is used in future studies to allow more accurate assessment of associations between EBC pH and recorded breathing variables. In addition, the comparison of EBC pH in samples collected by facemask with those collected during tracheal intubation, may be helpful to identify the principle region responsible for EBC pH control in horses. From the results of the current studies, it is evident that EBC pH should not be measured without consideration of environmental and animal factors.
Relationship of EBC pH with lower airway inflammation

The current study demonstrated that EBC pH was significantly higher in horses with LAI and airway neutrophilia than in those without, indicating that EBC pH is associated with, and may be used as a marker of inflammation of the distal airways in horses. This relationship with airway inflammation has not been identified in horses previously. However, we hypothesised that EBC acidification would be detected in association with LAI as has been demonstrated in humans [22,33,34] and calves [35] with pulmonary disease. Determination of the cause of this disparity was outside the scope of this study; however, EBC pH in horses with LAI has been evaluated only once [5] and an association with respiratory fluid leukocyte percentages in horses has not been reported previously. Therefore, it is not known whether airway acid-base dysregulation occurs with respiratory disease in horses. It is possible that acid-base dysregulation is dissimilar in horses with mild LAI than in humans with inflammatory conditions such as asthma and COPD. The influence of race on EBC pH in human studies have been demonstrated [36], and it is possible that differences in the response of EBC pH to LAI could be due to species or breed variation. Glutaminase activity of respiratory epithelium may be upregulated in horses with mild LAI resulting in increased ammonia production from glutamine. During an in vitro study Hunt et al. [37] demonstrated that certain cell cultures responded to low pH stress by upregulation of glutaminase activity, which resulted in increased ammonia production. Additionally, ambient ammonia concentrations in equine housing environments are likely to be substantially higher than most human housing environments [32,38]. Furthermore, increased concentrations of environmental ammonia have been demonstrated to be associated with increased exhaled ammonia and EBC pH [39], which may have contributed to the overall increase in EBC pH in horses in this study and may have played a role in the difference between pH in horses with and without LAI. However, ambient and exhaled ammonia were not measured in the current study. Although the mean RR did not change during collection in the horses in the first study (Chapter 3), tidal volume was not measured. Therefore, it remains possible that increased alveolar ventilation (associated with larger tidal volumes (V\text{T})), longer inspiratory times (T\text{I}) and lower respiratory frequencies (F\text{R}) in horses with LAI [40,41], resulting in an overall increase in systemic pH, secondary to relative hypocapnoea, and by extension EBC pH, could have contributed to the increased EBC pH seen in horses.
with LAI. Further research is required to investigate this hypothesis, as variables of respiration were not measured in the current study.

Additionally, the influences of RH and $T_A$ on pH may have added to the unexpected change in EBC pH in horses with LAI in the current study compared to human studies [22,33] and the previous study in horses [5]. EBC pH demonstrated a positive association with $T_A$ and a negative association with RH. Summer in the geographical region (Central southern NSW) in which the study was conducted is associated with high $T_A$ and low RH and univariate analysis revealed that various IAD categories demonstrated a positive association with $T_A$ and a negative association with RH (Appendix 2). Therefore, it is possible that the influence of ambient factors may have contributed to the increased pH in association with LAI in the current study. Additionally, the $T_A$ and RH range in the current study was likely much larger compared with that in the location (Scotland) in which the study by Duz et al. [5] was performed, resulting in greater environmental influence [42,43].

Methodological influences on EBC pH including collection temperature and sample storage while frozen, may have contributed to differences in EBC pH between the current study and previous studies in humans [22,33,34] and calves [35]. Condenser temperatures low enough to cause EBC sample freezing during collection has been proposed to result in reduced capture of exhaled volatile acids and higher EBC pH [24]. However, Whittaker et al. [26] found that pH of EBC collected from horses, was higher when collected as a liquid compared with the frozen state. As such, the effect of condenser temperature on equine EBC requires further clarification and standardization for comparison between studies. When a frozen EBC sample container is opened before thawing and agitation, the volatile acids that have sublimated from the solid EBC may be lost to the environment, resulting in a falsely elevated pH measurement [24]. This however, should not have influenced the results of the current study, as all samples were agitated after thawing to allow resuspension of the volatile acids. Additionally, condenser characteristics are unlikely to have contributed to the discrepancy with the study in horses [5], as the EBC collection method used in the current study was based on that described by Duz et al. [5]. As such, the cause of increased EBC pH seen in horses with LAI remains unknown.
The different directions of the relationships of EBC pH with BALF neutrophil and mast cell percentage may reflect the difference in pathophysiology of different subtypes of IAD, as has been suggested previously [44,45]. Inflammatory leukocytes such as neutrophils and eosinophils contain a variety of substances associated with their lysosomes, such as hypochlorous and hypohalous acids. The release of substances such as these, would be expected to result in a reduction in local pH [46] and an increased number of these leukocytes in BALF are expected to be associated with a decrease in EBC pH. The unexpected increase in EBC pH, may have been due, in part, to the limitation of cytological evaluation of BALF and the reliance on cellular percentages to diagnose LAI, even though BALF cell percentages do not necessarily equate to cellular activity [47,48]. In addition, leukocyte changes observed in the current study may not have been of sufficient magnitude to cause airway acidification.

A negative association was observed between EBC pH and mast cell percentage in TA and BALF. This association has not previously been reported in any species and may reflect increased volatile acid liberation secondary to inflammatory mediator release associated with metachromatic inflammation. However, cellular pathways mediating the observed changes in EBC pH remain to be determined. Additionally, the effect of TA and RH on the pH response in association with airway fluid leukocyte counts, as discussed for LAI categories, cannot be discounted and further research is required to clarify the relationship. Nonetheless, these findings indicate that there is a significant relationship between EBC pH and the degree of LAI in horses. These findings highlight that it is possible that acid base regulation and EBC pH alterations in response to pulmonary inflammation in horses, and perhaps even humans, is not as simple as inevitable acidification of EBC in inflammatory disease. While measurement of EBC pH is unlikely to replace BALF analysis in the diagnosis of IAD, this biomarker will likely provide valuable information regarding leukocyte activity and contribute to the overall assessment of the inflammatory process associated with LAI conditions.

Following the strong statistical associations of EBC pH with LAI in the current studies, preliminary evaluation of EBC pH performance as a diagnostic test was undertaken. Receiver operator characteristic curves in horses with IAD with or without EIPH (LAD
group) (Appendix 3) demonstrated a sensitivity of 72-78% and specificity of 67-80% at a cut-point of 6.67 for pH measured in neat EBC without statistical adjustment for influencing factors. In human medical research, measurement of EBC pH has repeatedly been associated with pulmonary inflammatory conditions and response to treatment, including asthma, COPD and bronchiectasis [33,49,50]. Additionally, Guillen-Del Castillo et al. [34] demonstrated an association of EBC pH with decreased pulmonary function parameters and increased likelihood for non-survival during follow-up in humans with interstitial lung disease. Therefore, EBC pH may prove to be even more robust and diagnostically useful than demonstrated to date with additional adjustments made for the identified influencing factors.

The results of this study indicate that EBC pH may prove to be a valuable complimentary or standalone diagnostic tool for the evaluation of LAI conditions in horses. Additionally, it may be a simple method to monitoring the response to treatment in horses with IAD. However, further research is required before the application of EBC pH as a clinical tool can be recommended. Future directions identified in this study, are to determine how to adjust for environmental factors using statistical models or to evaluate EBC pH in subjects with all EBC collections done in a temperature-controlled environment, standardising Tₐ and RH for each collection. Additionally, a larger study including horses with mild LAI to strengthen or disprove our findings regarding the directionality of EBC pH changes in horses due to mild LAI, is required. Furthermore, evaluation of EBC pH alterations in response to more severe non-infectious pulmonary inflammation, such as RAO and comparison with EBC pH alterations in response to mild LAI, are also warranted.

3. The future of EBC H₂O₂ analysis

As with the measurement of pH in EBC, the first step in determining whether a biomarker may be diagnostically useful is to evaluate a measure of variability, in this case, the consistency of EBC H₂O₂ concentration. The intra-day consistency of H₂O₂ expressed as measured (µmol/L) or standardised according to total volume of exhaled breath (EBTV, pmol/100L EB) was high, whilst the inter-day consistency was adequate, with ICC >0.9 and >0.8 respectively. Similarly, acceptable and good repeatability of EBC H₂O₂ measurements have been reported in cats and humans respectively [51,52].
However, an earlier study in horses demonstrated high levels of intra- and inter-day intra-subject variation in horses with LAI, with CV ranging from 0.6 to 1.03 [5]. Similarly, a study evaluating horses with RAO, subjected to environmental challenges, demonstrated large intra-subject CV (0.8) for H$_2$O$_2$ measurements [4]. The authors of that study speculated that the high levels of variation were due to a probable wide range of severity of LAI in the horses and the small number of study subjects [5](Duz, personal communication). Although the cause of the discrepancy in variation in equine EBC H$_2$O$_2$ measurements between these studies and the current study is unknown, the reduced level of variability/good consistency in the current study, was likely due to consideration of influencing/confounding factors during determination of the variance components used to calculate the ICC. Additionally, the use of an inspiratory filter in the EBC collection apparatus likely added to the reduction in variation by reducing the inspired fraction of ambient H$_2$O$_2$, which has been demonstrated previously to reduce variation [29,53]. Additionally, the demonstrated influences of environmental factors on EBC H$_2$O$_2$ [54] and the difference between ambient conditions in Australia (where the current studies were conducted) compared with Scotland [4,5,42,43], may have contributed to the disparity in consistency between the current study and previous studies in horses [4,5]. Therefore, ongoing consideration of EBC H$_2$O$_2$ measurement as a biomarker for LAI in horses is warranted.

**Measurement considerations**

Multiple factors have been identified that should be considered when collecting and analysing EBC for H$_2$O$_2$ concentration determination. These include the environmental H$_2$O$_2$ concentrations [29] and the total expiratory volume during EBC collection [9]. Ambient H$_2$O$_2$ has been suggested to play an important role in the variation of EBC H$_2$O$_2$ [53] and has been associated with the concentration of H$_2$O$_2$ in EBC in calves [29]. Therefore, the collection of environmental air and EBC concurrently has been recommended, to allow H$_2$O$_2$ concentration determination in environmental air with subsequent subtraction from that measured in EBC [29,55]. Alternatively, inspiratory filters can be used to limit the effect of environmental H$_2$O$_2$ on EBC H$_2$O$_2$ measurement [29,53]. Knobloch et al. [29] demonstrated that the use of PALL and nitric oxide (NO) filters markedly reduced the concentration of H$_2$O$_2$ when collecting ambient air
condensate compared to measurements of H$_2$O$_2$ in samples collected without use of these filters. PALL inspiratory filters were used in the current study and although the reduction of inhaled H$_2$O$_2$ was not quantitated, the reduced variation of H$_2$O$_2$ concentrations in the current study compared to previous studies [4,5], suggest a reduction in the influence of ambient H$_2$O$_2$ on EBC H$_2$O$_2$ measurements. Furthermore, a recent study demonstrated that the use of a P3-type inspiratory filter effected an 81% reduction in inhaled H$_2$O$_2$ [53]. Additionally, this study demonstrated a reduction in the between subject variation of EBC H$_2$O$_2$, suggesting that the well-known high levels of variation between subjects are due to variations in inhaled ambient H$_2$O$_2$ [53]. As such, future studies investigating the use of EBC H$_2$O$_2$ in horses could incorporate the use of P3-type filters and/or determination of ambient air H$_2$O$_2$ concentration at the time of EBC collection.

In the current study, all statistical analysis were done on both as measured ($\mu$mol/L) and standardised concentration of H$_2$O$_2$ (pmol/100L EB), as the volume of EBC collected per volume of EB is not constant in spontaneously breathing animals [9,56]. Therefore, to minimise the confounding effects of various biological factors associated with respiration, it is recommended to express EBC biomarkers in relation to both volume of condensate and 100L of exhaled breath [9,29]. The ICC values in the consistency study (Chapter 3) was higher when expressed as EB$_{TV}$ in agreement with previous studies [9]. However, a limitation of our study was the lack of accurate measurement of EB$_{TV}$ by spirometry. Instead, EB$_{TV}$ was estimated from RR, throughout EBC collection, and published tidal volumes at rest [57]. Although this method of estimation will not have been as accurate as direct measurement of EB$_{TV}$, it is unlikely to have altered our results as all subjects (and data) were treated equally, resulting at most, in a systematic bias. Additionally, resting tidal volumes of horses of various breeds with similar body weights, demonstrate little variation between studies [57,58]. Nevertheless, the findings of the current studies support the recommendation to standardise EBC H$_2$O$_2$ expression to EB$_{TV}$ and the use of an expiratory spirometer or alternatively respiratory impedance plethysmography, to allow accurate measurement of EB$_{TV}$, RR during EBC collection and changes in respiratory character [9,56].
A measurement consideration identified in this study was the batch of H\textsubscript{2}O\textsubscript{2} analysis and therefore the batch of reagents used. Samples were not stored for longer than 40 days, as recommended by van Beurden \textit{et al.} [59] and analysed in batches as dictated by sampling dates. The stock solutions were prepared every three months and stored at -80°C between batches while the working solution was prepared fresh for each batch as recommended by van Beurden \textit{et al.} [59]. Batches with sample sizes of three or less were excluded from statistical analysis, which revealed that one of the batches included in the analysis resulted in significantly lower H\textsubscript{2}O\textsubscript{2} concentrations. The cause of these low H\textsubscript{2}O\textsubscript{2} concentrations remains unknown but is likely due to methodological issues. It may be due to the indirect nature of H\textsubscript{2}O\textsubscript{2} concentration determination by FLD, rather than EBC H\textsubscript{2}O\textsubscript{2} concentration being truly lower. This analysis method relies on the conversion of a substrate (HPAA) to a fluorescent dimer in the presence of both H\textsubscript{2}O\textsubscript{2} and an enzyme (HRP) [60]. Such a reaction is dependent on multiple factors being identical between reagent batches to provide similar results. HRP stock is a crystalline, anhydrous powder that is not 100% pure and therefore may result in variable activity within solutions of the same concentration. As enzyme activity is responsible for utilisation of H\textsubscript{2}O\textsubscript{2} and creation of the fluorescent dimer, any variation in enzyme purity or activity may affect measured H\textsubscript{2}O\textsubscript{2} concentrations. Additionally, fluorescence detection can suffer from spectral interferences, such as reabsorption of fluoresced light, as well as quenching of fluorescence by molecules such as chloride and sulphide [61]. Any of these methodological factors may have contributed to the differences in EBC H\textsubscript{2}O\textsubscript{2} demonstrated between batches. Regardless of the cause of this effect, it is important to consider the effect of batch in all H\textsubscript{2}O\textsubscript{2} analyses when using FLD. The novel method developed using LC-MS/MS (Chapter 5) will minimise this methodological limitation. Even though the LC-MS/MS still measures the concentration of the dimer (DHPA), it does this directly and is not subject to spectral interferences and therefore provides superior selectivity and specificity.

The novel method developed using LC-MS/MS was more sensitive (LLOQ of 0.093 μM) than previously published routine methods [60]. Comparison of the lower limit of quantitation (LLOQ) with studies claiming superior limits of detection (LoD) is not possible, as the LLOQ was not declared [59,62,63]. The LoD for LC-MS/MS was very similar to previous reports utilising flow injection with fluorescence detection [59,60]
and lower than that reported for bench-top fluorescence [53] and a commercially available biosensor method [64]. The intra- and inter-day variability, expressed as CV, was negligible (0.01-0.03 and 0.02, respectively). Several LC columns were compared as the use of LC for the separation of the dimer (DHPA) from HPAA, has not been reported previously, even though it has the potential to improve sensitivity of analysis. Inclusion of a LC column improved peak shape and sensitivity, without unduly slowing analysis. This method was 1.5-3.7 times more sensitive than published methods and provided superior selectivity and repeatability compared with both fluorescence and biosensor detection methods while providing high sample throughput. Therefore, this method is superior to FLD methods [53,60] and a validated biosensor method [64]. It may provide improved determination of H2O2 concentration in EBC while eliminating potential confounding influences associated with FLD. Additionally, this method could be considered to validate commercially available or new bench-top biosensor analysis methods.

In a previous study, foals with *Rhodococcus equi* pneumonia were reported to have significantly higher concentrations of EBC H2O2 concentrations compared with control foals on the same farm [65]. However, the reported mean EBC H2O2 concentrations, including the control foal group, were substantially higher than previous reports in veterinary [4,5,15,51,66] and human studies [3,49,59]. The H2O2 analysis method used in that study [65] is a commercial, biosensor method based on spectrophotometric technology. However, this biosensor system has not been subject to independent validation. Considering the discrepancy in magnitude of detected H2O2 concentrations and the use of a non-validated method, comparison of the results of the current study with those reported by Crowley et al. [65] is not possible. Given the limited knowledge available on EBC biomarker analysis in horses, it is important to use validated analysis methods to quantitate concentrations of EBC biomarkers. The LC-MS/MS method developed in the third study (Chapter 5) could be used to validate commercial biosensor methods that have the advantage of greater availability and ease of use.

The aims of Chapters 3 and 4 were to evaluate EBC biomarkers to determine whether further assessment of these biomarkers for investigation of lower airway health in horses is warranted. To achieve this objective, a validated analysis method, flow
injection with fluorescence detection, was chosen to conform with and allow comparison with, both human and veterinary literature, whilst reducing the risk associated with the use of a new method. Given the paucity of information and the contradictory findings of the association of EBC H$_2$O$_2$ with lower airway inflammatory conditions in horses [4,5,15,18], development of the novel, superior LC-MS/MS method was reserved until evidence of an association of EBC H$_2$O$_2$ and lower airway inflammation in our population, was found.

The study (Chapter 5) comparing a novel H$_2$O$_2$ analysis method (LC-MS/MS) with the more traditionally used FLD (as used in Chapters 3 and 4) revealed that the matrix used to prepare the standard curve had a significant effect when using both FLD and MS/MS. Previous studies, evaluating the use of water or pooled EBC as a matrix for standard curve preparation, did not detect a difference [59,60], whereas the current study identified a significant difference in the slopes of the standard curves when prepared using either instrument grade water or pooled EBC. As such, the matrix used to prepare the standard curve should be considered whenever developing an H$_2$O$_2$ analysis method. It is recommended to use pooled EBC for preparation of the standard/calibration curve, that has been heated to 40°C for at least 5 h, based on the decomposition rate of H$_2$O$_2$ [67], to eliminate native H$_2$O$_2$ and allow spiking with H$_2$O$_2$ standards.

**Associations of H$_2$O$_2$ with environmental, animal and method factors**

In this study, a variety of factors influencing the final concentration of H$_2$O$_2$ in EBC were identified. The first study (Chapter 3) identified $T_a$, RH, collection location and time of day as influences on H$_2$O$_2$ concentration. Additionally, these terms were identified to interact with each other. The time of day at which EBC was collected interacted with both ambient temperature and collection location (independently), while influencing EBC H$_2$O$_2$. However, the sample size was not large enough to thoroughly explore interaction terms during multivariable analysis. No associations with environmental or animal factors were evident in the second study (Chapter 4). This is likely because EBC was collected from most clinical cases while restrained in a crush, in a temperature-controlled environment, with only a few collected in an alternate location (yard or stable), as dictated by their temperaments. As such there
was less variation between environmental factors in this data set, which likely precluded the identification of an influencing effect.

Diurnal variation in H$_2$O$_2$ has been reported for humans [52,68] and calves [29] however, the influences of T$_A$ or RH have not been reported previously. In this study, the observed effects likely reflect the inverse relationship between T$_A$ and RH in our climatic region [42]. Interactions between time of day and T$_A$ and time of day and collection location (crush or yard) were observed and may be due, at least in part, to the positive correlation between UV-radiation and atmospheric H$_2$O$_2$, resulting in an increase in ambient H$_2$O$_2$ in the afternoon [55,69]. Correlations between ambient and EBC H$_2$O$_2$ concentrations have been demonstrated [29,55], suggesting that ambient H$_2$O$_2$ affects EBC H$_2$O$_2$. In this study, collection of EBC in an outside yard was associated with a significantly higher concentration compared with indoors. While this finding may reflect a greater influence of UV-radiation on ambient and therefore EBC H$_2$O$_2$ in the yards, it remains speculative, as ambient air samples were not collected. However, these findings indicate that EBC should not be collected outdoors or in common housing environments for horses (stable, yard or paddock), which may limit the clinical applicability of EBC H$_2$O$_2$ measurements in future.

EBC collection standardization for H$_2$O$_2$ analyses can be achieved by collection of EBC in a temperature-controlled room. However, recommendations to minimize the effect of time of day on EBC H$_2$O$_2$ concentrations in horses cannot be made at this time, as greater elucidation of the potential influence on measurements is required. However, the influence of time of day is likely related to T$_A$ and RH, and standardization of these factors will likely eliminate the influence of time of day, as was the case in the second study (Chapter 4). Additionally, in line with human recommendations [1] and the univariate analysis findings in the first study (Chapter 3); horses should not be fed within a couple of hours of EBC collection. Considering the rate of gastric emptying in horses, a period of food withholding of at least 4 hours would likely be sufficient [70,71] however, further research is required to establish more precise guidelines.

The results of the current studies indicate that concentrations of H$_2$O$_2$ in EBC cannot simply be measured to determine whether lower airway inflammatory conditions are
present, without consideration of environmental and animal level factors. However, standardization of these influencing factors and consideration of methodological influences may result in the development of EBC H$_2$O$_2$ concentration as a reliable biomarker for airway inflammation.

**Relationship of EBC H$_2$O$_2$ with lower airway inflammation**

EBC H$_2$O$_2$ concentrations were significantly higher in horses with LAI. Specifically, concentrations were higher in horses with IAD or EIPH or both, and in horses with IAD due to increased relative percentages of more than one leukocytes type in BALF (mixed IAD). Additionally, a positive association between H$_2$O$_2$ and BALF neutrophil percentage was demonstrated. These differences were only identified when EBC H$_2$O$_2$ was standardised according to EB$_{TV}$. These findings suggest that standardisation of H$_2$O$_2$ concentrations according to the total volume of EB during EBC collection, not only results in improved consistency, but also allows the identification of LAI in horses. As such, expression of EBC H$_2$O$_2$ concentration in terms of EB$_{TV}$ may be preferable in the development of protocols for measurement of this biomarker.

The current study is the first to identify an association of EBC H$_2$O$_2$ concentrations in horses with mild LAI. Multiple human studies have identified increased EBC H$_2$O$_2$ concentrations in subjects with lower airway inflammatory conditions, including asthma and COPD [3,49]. However to date, the association between EBC H$_2$O$_2$ concentration and LAI in horses has been unclear. Deaton *et al.* [15] found that EBC H$_2$O$_2$ was increased in horses with RAO, with both active inflammation and those in remission, compared with control horses. Similarly EBC H$_2$O$_2$ concentration was demonstrated to be greater in foals with *R. equi* pneumonia than in control foals [65]. Conversely, multiple other studies could not demonstrate an association between EBC H$_2$O$_2$ concentration and LAI [4,5,18]. The demonstrated influences of environmental factors on EBC H$_2$O$_2$ [54] and the difference between ambient conditions in Australia (where the current studies were conducted) compared with Scotland (where many of the previous studies were conducted) [4,5,42,43], may have contributed to the disparity between the current study and previous findings in horses [4,5]. Based on the results of the current study, further investigation of the relationships between EBC
H₂O₂ and specific cytological phenotypes of LAI are justified. The association with mixed IAD in the current study, should be considered in light of the sample size limitation, as there were only 3 horses with mixed IAD, limiting the value of this specific finding.

A positive association between H₂O₂ concentration and BALF neutrophil percentage was demonstrated. This corroborates the identification of a positive correlation with BALF neutrophil percentage in horses with RAO [15] and in healthy cats [51]. A relationship between the number of neutrophils in sputum and increased EBC H₂O₂ concentrations has also been demonstrated in humans with asthma and bronchiectasis [72,73]. These findings support the association of EBC H₂O₂ with the level of inflammation in the lower airways of horses. Additionally, our study demonstrated a significant positive correlation with BAL macrophage morphology but a negative association with BALF macrophage percentage. This may be suggestive of an association of EBC H₂O₂ with macrophage activity rather than number of cells present or more simply, may be due to the inverse relationship of different cell types in a differential cell count.

H₂O₂ is produced by the conversion of superoxide anions O₂⁻ to H₂O₂ by superoxide dismutase [74] during the respiratory burst of activated inflammatory cells, such as neutrophils, eosinophils and macrophages in response to oxidative stress [75]. As such, increased concentrations of EBC H₂O₂ are indicative of increased reactive oxygen species (ROS) production and/or impaired mechanism of removal. Therefore, EBC H₂O₂ concentration may depend, in part, on pulmonary leukocyte activity, which cannot be determined from cell counts. As such, EBC H₂O₂ concentration may be a useful indicator of airway inflammation and provide information about the respiratory tract inflammatory activity that cannot be obtained by BAL and TA fluid cytology and respiratory tract endoscopy. It is unlikely that measurement of EBC H₂O₂ concentrations will replace BALF cytological examination; however, the findings of the current study have revealed potential application for lower airway assessment. Therefore, EBC H₂O₂ may prove to be a valuable tool in the diagnosis and monitoring of lower airway inflammatory conditions.
Future research into EBC H$_2$O$_2$ analysis should include the use of an expiratory spirometer that does not increase resistance to breathing or alternatively respiratory impedance plethysmography, to more accurately measure parameters of spontaneous breathing and allow more accurate expression of EBC H$_2$O$_2$ concentration per EB$_{TV}$. Additionally, EBC should be collected under standardised environmental conditions or a correction factor should be applied during statistical analysis to account for the known influences of environmental factors. Future use of LC-MS/MS, a more sensitive and specific analysis method, may eliminate further confounding variables associated with existing analysis methods, thus providing more reliable measures of EBC H$_2$O$_2$ concentration. Once these factors are addressed, it may be possible to establish ‘reference values’ for horses without evidence of LAI on TA and BALF cytology. Such foundation information will assist in the development of the use of EBC H$_2$O$_2$ measurements as a diagnostic and monitoring tool. However, it must be acknowledged that the measurement of EBC H$_2$O$_2$ concentrations may be impractical in a field setting, where control of the influencing factors may not be possible. Despite these possible limitations, it is likely that the evaluation of EBC H$_2$O$_2$ concentrations will add to our knowledge and understanding of the pathophysiology of non-infectious LAI conditions.

4. The future of EBC LTB$_4$ analysis

The concentration of LTB$_4$ in EBC was expressed using as measured concentrations in EBC (pg/mL) and concentrations standardised to EB$_{TV}$ per collection, pg/100L EB. The first study (Chapter 3) revealed poor consistency for EBC LTB$_4$ concentrations in horses. Even though there was no significant difference between the mean intra-day and inter-day EBC LTB$_4$ concentrations, the large standard deviation (SD), the poor intra-day consistency and erroneous inter-day ICC, indicate poor suitability of EBC LTB$_4$ concentration as a biomarker to evaluate the lower airway of horses. Similarly, a study in human COPD patients revealed substantial within- (CV 0.48) and between-day (CV 0.76) variation with wide limits of agreement, demonstrating little reproducibility [76]. However, previous studies in calves demonstrated large inter-subject variation (CV up to 0.8) [77,78] but very little intra-subject inter-day variation (CV 0.03 to 0.26) [79]. These disparities may be due to the use of enzyme linked immunoassay (EIA) methods,
with known suboptimal sensitivity, to determine LTB₄ concentrations in these studies and/or species variation [13].

The cause of high levels of variation in the EBC concentrations of LTB₄ in the current study may be due to the inclusion of horses with no or mild LAI. Human studies have repeatedly demonstrated that healthy individuals have very low concentrations of LTB₄ in EBC [13]. Additionally, the mean ± SD (7.44 ± 5.88 pg/mL) LTB₄ concentration in the consistency study (Chapter 3) was very close to the LoD (3.22 pg/mL) of our analysis method, which likely contributed to the low levels of consistency.

As such, even though our findings do not necessarily support the use of LTB₄ for lower airway assessment in horses, studies with subjects with more severe LAI and the use of a LC-MS/MS with improved sensitivity, might be more suitable to assess the consistency and therefore utility of this analyte.

**Measurement considerations**

Various methods have been described for the determination of LTB₄ concentrations, including commercially available EIA [16,80], enzyme-linked immunosorbent assay (ELISA) [79], gas chromatography/mass spectrometry (GC/MS) [81], LC-M/S [82] and LC with spectrophotometry (LC-UV) [83]. EIA has been the most commonly used technique as it was previously recommended as the technique of choice [1] based on a study that demonstrated reasonable specificity of EIA, in human EBC, when coupled with reverse-phase high performance liquid chromatographic separation of different leukotrienes prior to concentrations determination by EIA [84]. However, most studies that have used EIA have not included this step. Additionally, the manufacturer reported LoD of 13 pg/mL [85] is above the reported lower range of concentrations for healthy humans (10 pg/mL) [1,86,87] and the equine population in the consistency study (Chapter 3), whilst being close to the reported lower range of concentrations in other veterinary species (15-20 pg/mL) [80,88]. Moreover, these EIA methods are not designed for EBC samples, increasing the risk of cross reactivity and false positive results [89]. In fact, the high levels of within-assay variation reported for LTB₄ (CV 0.18) [76], has been attributed to the low sensitivity of EIA [13]. As such, it is now
recommended to use the more selective GC/MS or LC/MS methods for determination of LTB₄ concentrations in EBC [13,81,90]. These methods have comparable or better sensitivity to EIA and provide additional structural information, removing the confounding effect of false positive results [13]. Additionally, LC-MS is not influenced by the matrix of EBC [91], unlike the EIA methods [89]. The more selective LC-MS/MS method was used in the current study in preference to EIA methods [13]. However, the method developed to determine EBC LTB₄ in the current study was less sensitive than reported for a similar LC-ESI-MS/MS method [92], which reported a LLoQ of 50 pg/mL compared to the LoD and LLoQ in the current study of 3.22 pg/ml and 93.23 pg/mL respectively. This disparity was likely due to individual variation of each MS and analysis method. As such we conclude that determination of the LoD and LLoQ for each method should be done before embarking on EBC sample collection for the purpose of measuring LTB₄.

**Association of LTB₄ with environmental, animal and method factors**

This is the first study to demonstrate that environmental factors can influence the concentrations of LTB₄ in EBC. Both Tₐ and RH were significant influencing factors of EBC LTB₄ in the multivariable analysis of the clinical study (Chapter 4). The measured EBC LTB₄ demonstrated a negative association with both Tₐ and RH. Although the cause of this association was not identified during the current study, it is speculated that the weak to moderate positive correlations seen during the univariate analysis (Appendix 4) between RR and Tₐ, Tₐ and EBC volume and RR during EBC collection and EBC volume, in conjunction with the negative association of EBC LTB₄ and Tₐ, may indicate that with increasing Tₐ, LTB₄ concentrations decreased due to increased water vapour content of collected EBC [79,93] associated with increased RR or increased airflow. However, other studies have demonstrated that even though increased minute ventilation results in greater EBC volume, the conductivity does not decrease, nor the concentration of cys-leukotrienes, indicating that increasing EBC volumes due to increased expired volume does not necessarily result in dilution by water vapour [94]. Further, both univariate and multivariable analysis did not reveal RR during collection as a significant covariate of LTB₄ concentration in EBC. Therefore, further
research is indicated to clarify the association between LTB₄ concentrations and Tₐ and RH.

**Relationship of EBC LTB₄ with lower airway inflammation**

This study did not demonstrate a difference of EBC LTB₄ concentrations when comparing horses with various subcategories of LAI with those horses without. This is in contrast to the study by Fey *et al.* [16] who reported that ‘elevated’ levels of EBC LTB₄ in horses with RAO were found however, in that study, horses without LAI were not included. As such, the true relationship of EBC LTB₄ with categories of LAI in horses has not yet been identified and further research is required.

In the current study, a significant positive association with BALF eosinophil percentage was demonstrated during the multivariable analysis (Chapter 4), indicating that EBC LTB₄ concentration is associated with LAI and may be influenced by cell type. Such an association has not been demonstrated previously [95]. Further, the ability to quantitate inflammatory and pro-resolving eicosanoids have allowed advances in the understanding of inflammatory pathways in human asthma. A study of humans with asthma demonstrated that both the inflammatory and pro-resolving eicosanoid pathways were upregulated and that a relative underproduction of lipoxins (a pro-resolving eicosanoid) occurs with increasing asthma severity [96]. Macrophages, activated neutrophils, mast cells, eosinophils and epithelial cells [97] are known to produce LTB₄ and contribute to local concentrations of this eicosanoid. Therefore, even though EBC LTB₄ concentration was highly variable in the current study, it is likely that there are associations between certain cytological phenotypes of airway inflammation and EBC LTB₄ in horses. Furthermore, quantitation of LTB₄ in EBC in conjunction with BALF cytology and/or pulmonary function in horses with more severe LAI conditions, such as RAO, may result in improved understanding of inflammatory pathways associated with these conditions.

Multiple human studies have reported associations between the concentration of LTB₄ in EBC and inflammatory lower airway conditions [96,98]. Studies have demonstrated that LTB₄ concentrations were significantly higher in subjects with asthma compared with healthy controls [96,99]. Additionally, a cut-point of 11 pg/mL EBC LTB₄ has been
demonstrated to provide a sensitivity and specificity of 100% in adult human patients with asthma [96].

Even though it seems unlikely that the measurement of EBC LTB₄ will be of use in horses with mild LAI conditions, it is possible that the development and use of more sensitive analysis methods and inclusion of horses with more severe forms of lower airway inflammation, such as severe equine asthma, may result in findings more concordant with human literature.

5. Lower airway inflammation: a continued diagnostic challenge

Mild to moderate equine asthma or IAD is a challenging syndrome to diagnose, as the exact pathophysiology and the defining characteristics of the condition remain uncertain. The current study purposefully evaluated horses with milder forms of lower airway inflammation, including IAD, STI/trIAD and EIPH, rather than including horses with overt RAO, as it is these milder forms of LAI, specifically IAD that has proven difficult to define and monitor. Continued uncertainties regarding the aetiopathogenesis of IAD, the most appropriate diagnostic modalities for the condition and the associated criteria that should be used to define healthy versus diseased, are aspects requiring clarification. One possibility is the analysis of EBC may become a non-invasive diagnostic method used to screen horses for the presence of LAI with additional diagnostic tests performed if indicated. Additionally, EBC collection and analysis may provide more information regarding the pathophysiology of IAD and the proposed subtypes, as the EBC biomarkers do not merely provide information regarding leukocyte numbers, but rather the activity and degree of active inflammation within the respiratory tract.

A major limitation of any project evaluating horses with IAD is the lack of a true ‘gold standard’ diagnostic test. Wichtel et al. [100] demonstrated that horses can have pulmonary function aberrations, consistent with IAD but have BALF cytology within normal limits and vice versa, thereby identifying the weaknesses of both methods. This
study demonstrated that as many as a third of their population would have been misclassified as free of IAD, on the basis of BALF cytology only [100]. Therefore, the use of BALF evaluation to classify horses into categories of IAD, as was done in the current studies, may have resulted in the incorrect classification of subjects, thereby altering all on-going analyses. However, this is a clinical difficulty for which a solution has yet to be revealed. Furthermore, an experimental model of LAI is not available. The issue of airway inflammation diagnosed by TA (often identified concurrently with IAD) remains a confounding factor in research and clinical settings. Recently, the use of TA was considered not suitable to identify IAD due to the discordance of TA and BALF cytology and the paucity of data relating TA cytological findings and performance [17,101]. Additionally, EIPH and IAD commonly coexist. An increased risk of EIPH has been associated with IAD [102] and instillation of autologous blood into the airways elicits an inflammatory response [103]. Additionally, increased concentrations of inflammatory biomarkers have been demonstrated in BALF and blood from horses with EIPH compared the control horses [104,105]. However, the potential relationship between IAD and EIPH remains controversial [17]. Although the role of inflammation in the aetiopathogenesis of EIPH is uncertain [106], it remains probable that EIPH is associated with inflammation of the distal airways [107]. As such, lower airway pathology and the inflammation associated with STI/trIAD and EIPH cannot be separated practically, from that identified by BALF cytology in IAD and therefore remain confounding factors when evaluating a new diagnostic method to assess the lower airways in horses. For this reason, the current studies included IAD, but also EIPH and STI/trIAD in accordance with the aforementioned clinical situation, although the inclusion of horses affected only by EIPH may have influenced our results. Because there is no true ‘gold standard’ method whereby to identify the overall inflammatory status of lower airways in horses, it is difficult to truly evaluate the performance of a new test.

The certainty of the diagnosis of IAD in the horses in this study, could have been improved by the use of a pulmonary function testing (PFT) method as part of the lower airway assessment. Various PFT methods have been described to evaluate the pulmonary function of horses, including conventional PFT [108], forced expiratory manoeuvres [109], impulse oscillatory mechanics [110], flowmetric plethysmography
and spirometric PFT [112,113]. However, each PFT method has limitations, including issues regarding interpretation and repeatability, invasiveness and validation [114]. Additionally, most PFT methods are not sensitive enough to detect small alterations of pulmonary function or mild airway obstruction, as frequently occurs in IAD. Therefore, these methods require the use of hyperventilation by chemical stimulation of ventilation using either hypercapnoeic rebreathing techniques [40,115] or intravenous administration of lobeline [111], and/or bronchoprovocation [44,114,116] for detection of IAD-associated pulmonary dysfunction. As such, an ideal PFT method has not yet been developed for use in horses to identify mild LAI. Although the addition of a PFT modality may have resulted in improved classification of horses with IAD, a validated PFT method was not available for the current study.

The frequent co-existence of EIPH and IAD [17,102,117], precluded the exclusion of EIPH from this study. Increased risk of EIPH has been associated with LAI as diagnosed by TA cytology [102]. Experimentally, LAI was associated with a significantly increased risk of EIPH [118] and instillation of autologous blood into the airways elicited a neutrophilic inflammatory response [103], suggesting inter-relationships of these two conditions. Additionally, increased concentrations of inflammatory biomarkers have been demonstrated in BALF from horses with EIPH [104]. However, other studies did not find an association between haemosiderophage and neutrophil percentages in BALF from horses with IAD [119,120], while in another study, pulmonary fibrosis did not occur after prolonged intra-alveolar administration of autologous blood [106]. As such, the association between IAD and EIPH remains to be clarified. Based on experimental study findings, it is probable that EIPH results in localised inflammation which forms an integral part of the propagation of EIPH [118]. Currently, the most accepted hypothesis for the pathogenesis of EIPH is alveolar capillary wall stress failure, due to repeated regional (caudo-dorsal) pulmonary venous hypertension during strenuous exercise, followed by regional pulmonary vein remodelling [107,121,122]. These structural changes results in further pulmonary capillary hypertension, stress failure and repeated bouts of bleeding with accumulation of erythrocytes and other blood components within the alveoli and interstitial tissues [107,123,124]. Erythrocytes are degraded to haemosiderin and other heme pigments that cause oxidative damage and have pro-inflammatory effects, culminating in a
localised inflammatory response [125]. This is supported histologically by the presence of bronchiolitis and interstitial fibrosis [123,126].

It remains possible that additional relationships between airway disease and EBC biomarkers would have been identified if horses with more severe LAI, such as RAO, were included. However, horses with clinically-apparent RAO are readily identified using the ‘HOARSI’ grading system [127] with or without the need for additional diagnostic tests, such as BALF cytology. Therefore, the greatest need for additional diagnostic tests lies not with clinical detection of RAO or SPA-RAO, but rather with milder, more subtle disorders causing diagnostic and monitoring difficulties. However, EBC may also be a useful method to monitor the response to treatment and management protocols in horses with RAO and SPA-RAO.

Monitoring the response to treatment in horses with LAI, specifically equine asthma, is difficult with the available diagnostic modalities, as it has been demonstrated that the use of inhalation corticosteroids, without appropriate environmental management, does not result in a detectable decrease in BALF neutrophilia [128,129]. These findings may suggest that either the LAI in the horses in these studies remained unresolved despite treatment or, more likely, that the BALF cytological characteristics may not reflect the level of active airway inflammation [128,129]. A recent study evaluating the response to treatment in a small number of horses with IAD, demonstrated a reduction in airway hyper-responsiveness in response to treatment with corticosteroids; however, BALF cellular percentages and total nucleated cell counts remained unchanged after 16 days of treatment [47]. The findings of this study [47] suggests that evaluation of BALF cytology is not specific or sensitive enough to monitor the response to treatment in horses with IAD and absolute leukocyte numbers or percentages do not necessarily equate to cellular activity and the level of active airway inflammation [47,48] nor correlate with pulmonary function derangements [100,130]. Furthermore, BAL is moderately invasive, requires sedation and induces a transient local neutrophilia for at least 48-72 hours [131,132]. The collection and analysis of EBC however, may provide information regarding the activity of inflammatory leukocytes and could be a more sensitive tool to monitor treatment response in horses with LAI.
without induction of additional pulmonary inflammation or the need for rest from high intensity exercise.

6. In conclusion

In conclusion, the findings of this study add substantially to the available body of knowledge regarding EBC analysis in horses. The intra- and inter-day consistency of EBC H$_2$O$_2$ and pH measurements are adequate to high, suggesting that these biomarkers may be useful for evaluation of the lower airways in horses. However, environmental factors including $T_a$, humidity, time of day, collection location, RR during EBC collection, vial used during the measurement of EBC pH after argon gas deaeration and H$_2$O$_2$ method factors may influence these biomarkers, necessitating standardisation when collecting samples longitudinally. Additionally, EBC H$_2$O$_2$ concentrations and pH are higher in horses with LAI. Both these biomarkers had a positive association with BALF neutrophil percentage, while EBC LTB$_4$ demonstrated a positive association with BALF eosinophil percentage, and mast cell percentage in BALF was negatively associated with EBC pH. Thus, these EBC biomarkers may be useful indicators of airway inflammation and more accurate measures of respiratory tract inflammatory activity than conventional diagnostic modalities. Moreover, EBC biomarker analysis, specifically H$_2$O$_2$ and pH, may provide more information regarding the pathogenesis of LAI and allow more certain diagnosis of IAD when combined with BALF analysis. EBC pH measurement has shown the most promise as a potential diagnostic tool for the evaluation of LAI conditions in horses and ongoing investigation is certainly warranted.

This study has improved our knowledge and understanding of EBC biomarker analysis in equine medicine to support further research and development of EBC biomarkers as clinical tools. In addition to the value provided to the field of equine medicine, our study identified new associations of EBC biomarkers with environmental and subject factors, which may improve future human and non-equine veterinary studies. Additionally, it has raised questions regarding acid-base alterations in response to lower airway inflammation, perhaps prompting re-evaluation of current theories.
References


Appendix 1

Full length article – Chapter 3

The consistency and influence of environmental and animal factors on exhaled breath condensate hydrogen peroxide, pH and leukotriene B4 in horses.

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Abstract

Exhaled breath condensate (EBC) analysis is a non-invasive method to assess the lower respiratory tract. In humans, EBC hydrogen peroxide (H₂O₂), pH and leukotriene B₄ (LTB₄) are useful for detection and monitoring of asthma and chronic obstructive pulmonary disease. While EBC analysis could offer a non-invasive alternative to conventional methods for the diagnosis of equine asthma, little information about the use of EBC biomarkers in horses is available. The aims of this study were to determine the intra- and inter-day consistency of EBC H₂O₂ and LTB₄ concentrations and pH in horses and to determine whether these biomarkers are influenced by environmental and animal factors. Intra-day and inter-day consistency were assessed by collecting EBC samples from 10 horses on three occasions on day one, and at midday on the following two days. To evaluate the influence of environmental and animal factors on EBC biomarkers, EBC was collected from 20 horses (pH and LTB₄) and 10 horses (H₂O₂).

Intra- and inter-day consistency for both H₂O₂ and pH were adequate, with intraclass correlation coefficients ≥0.8. The intra- and inter-day consistency for LTB₄ was poor. H₂O₂ was influenced by ambient temperature (Tₐ, \( P=0.004 \)), humidity (\( P=0.009 \)), time of day (\( P=0.009 \)) and collection location (\( P=0.007 \)), while EBC pH was influenced by respiratory rate during EBC collection (\( P<0.001 \)) and Tₐ (\( P<0.001 \)). The study demonstrated that the consistency of both H₂O₂ and pH was sufficient for potential use as diagnostic biomarkers in horses; however, the influences of environmental and animal factors need to be considered.

Keywords: EBC biomarker; Equine; H₂O₂; LTB₄; Respiratory
Introduction

Analysis of biomarkers in exhaled breath condensate (EBC) is a non-invasive, widely used method for direct assessment of the lower respiratory tract in human medical research. Alterations in concentrations of EBC biomarkers occur in inflammatory lung diseases, including asthma [1] and chronic obstructive pulmonary disease (COPD) [2]. EBC is collected by condensing expired breath to a liquid or frozen state [3]. EBC constituents are likely present in concentrations that reflect those within the epithelial lining fluid (ELF) of the lower airways [4] and contain many non-volatile and water-soluble volatile molecules [3]. However, biomarker concentrations may be influenced by methodological, physiological and environmental factors [5-7].

The concentration of hydrogen peroxide (H$_2$O$_2$) in human EBC has been reported to be correlated with the severity and treatment response of several diseases, including asthma [8]. H$_2$O$_2$ concentrations may be affected by diurnal variation [5], age [9], respiratory rate (RR) [7] and methodological factors [10]. Associations between EBC H$_2$O$_2$ and lower airway inflammation (LAI) in horses with recurrent airway obstruction (RAO) [11] are reported, while no association was found in other studies [12].

EBC acidification has been reported in humans with asthma [13] and COPD [2] and may be a useful biomarker for LAI. Although more recently EBC pH was not correlated with disease severity in subjects with asthma or COPD [14,15]. LAI has been associated with EBC acidification in calves and horses [16,17]. In horses, EBC pH has been reported to be independent of breathing pattern and ambient temperature (T$_A$) [18] with good within-horse repeatability [16]. However, pH may be affected by collection and measurement temperatures [19,20], emphasising the need for further characterisation of influencing factors.

Leukotriene B$_4$ (LTB$_4$), an arachidonic acid metabolite, is a non-specific pro-inflammatory mediator [21]. Increased concentrations in EBC from humans with COPD and asthma has been reported [22,23]. LTB$_4$ has been associated with airway inflammation in calves and pigs with pneumonia [24,25] and horses with RAO [26]; however, physiological and environmental influences have not been determined.
Non-infectious LAI conditions (collectively termed equine asthma) are common in horses and result in compromise to animal welfare and wastage [27]. Diagnosis of equine asthma is often based on endoscopy and bronchoalveolar lavage fluid (BALF) cytology [27], but these methods are invasive, often require sedation, and do not provide information about the level of active inflammation. Measurement of specific biomarkers in EBC could offer a non-invasive alternative to conventional diagnostic methods and allow more frequent monitoring in horses with equine asthma. To date, there are few reports of EBC biomarkers in horses and further investigation of EBC analysis in this species is warranted. The aims of this study were to assess (1) intra- and inter-day consistency of EBC H$_2$O$_2$, pH and LTB$_4$ measurements and (2) the influence of animal and environmental factors on these biomarkers.

**Materials and methods**

**Animals**

Ten horses (Group-1) from the Charles Sturt University (CSU) research herd were used to determine the consistency of EBC pH, H$_2$O$_2$ and LTB$_4$ and for investigation of animal and environmental factors influencing H$_2$O$_2$. Investigation of animal and environmental factor influences on pH and LTB$_4$ were determined using 20 horses (Group-2). Sample size calculations ($\alpha = 0.05$, power of 0.8) were based on previously reported findings [16,18]. Group-1 consisted of seven mares and three geldings; six Thoroughbreds, three Standardbreds and one Quarter Horse; mean age was 7.6 years (range: 4-15). Group-2 consisted of 14 mares and six geldings; 10 Standardbreds, nine Thoroughbreds and one Quarter Horse; mean age was 6.7 years (range: 4-15). Prior to the study, the horses were at pasture, and housed in yards with access to Lucerne hay during the study. All horses were considered free of respiratory disease (asymptomatic) based solely on history and clinical examination. The study was approved by the CSU Animal Care and Ethics Committee (protocol number: 14/082, 14 October 2014).

**EBC collection and handling**

The EBC collection system (Fig. A1.1) was adapted from previously described methods [16,19]. Collection time was standardised to 15 min. All samples were frozen during collection and stored at -80 °C until analysis. Relative humidity (RH), $T_A$, food
consumption within one hour of EBC collection and RR during collection were recorded. During EBC collection, horses were restrained in a crush in a well-ventilated building or in an outdoor, roofed yard with rubber flooring, depending on temperament. Between collections, the facemask and tubing were rinsed with deionised water and dried.

Figure A1.1
Photographs of equipment used for collection of exhaled breath condensate (EBC) from horses in this study. EBC was collected by condensation of expired breath.
A custom built facemask (A) was connected via flexible corrugated tubing with an internal diameter of 35 mm (B), to an airtight EBC condensation chamber (C). The facemask, constructed of a modified grazing muzzle, included two one-way non-rebreathing valves (D; Salford Valve, Cranlea) with an internal diameter of 35 mm, positioned over the nares with inspiratory PALL filters (E; Protec PF30S, PALL) attached. The insert provides a close-up image of the valves and inspiratory filters.
An elastic rubber diaphragm (F) was attached to the facemask to create and airtight seal around the horse’s muzzle. The condensation chamber (C) was constructed of three 50 mL polypropylene tubes arranged in parallel (insert), immersed in an ethanol-liquid nitrogen slurry, cooling the condensation surface to less than -50 °C. Two one-way valves (G; Aeromask valve, Trudell Medical) at the outlet of the condensation chamber prevented contamination of the EBC sample by environmental air.

Intra-day biomarker consistency was assessed by collection of EBC samples from each horse on three occasions on day one at 08:00, 12:00 and 16:00. To determine inter-day consistency, a single sample obtained at 12:00 for three days was
used. A randomised crossover design was used to assess the influence of environmental factors.

*Endoscopy and respiratory fluid collection*

After EBC collection on day 3, each horse was sedated with xylazine (0.3 to 0.5 mg/kg BW IV, Ilium Xylazine, Troy Laboratories). Video-endoscopy was used to examine the upper airways and grade tracheal mucus [28]. BALF was then obtained by blind placement of a commercial BAL catheter (BAL300, SurgiVet), instillation of 300 mL of sterile isotonic saline and retrieval by aspiration. Pooled BALF was analysed within three hours of collection. Results were recorded as the relative percentages of neutrophils, mast cells and eosinophils in BALF.

*EBC \( \text{H}_2\text{O}_2 \) measurement*

Flow injection analysis with fluorescence detection allowed indirect determination of \( \text{H}_2\text{O}_2 \) concentrations. In brief, EBC was incubated with horseradish peroxidase (Sigma Aldrich), causing the non-fluorescing 4-hydroxyphenyl acetic acid (HPAA, Sigma Aldrich) to produce a fluorescent dimer as a result of \( \text{H}_2\text{O}_2 \) in EBC [29]. A detailed description is supplied in Supplementary item 3.1.

*EBC pH measurement*

After thawing, samples were mixed to resuspend sublimated volatile acids [30]. The pH of neat EBC was measured using a bench top pH meter (HI 2211 pH/ORP meter, Hanna Instruments) and a micro bulb pH-electrode (HI 1083B, Hanna Instruments), suited to small volume samples and calibrated prior to use. Subsequently, EBC was deaerated using argon gas and pH was recorded when the reading stabilised. Each sample was measured in triplicate at room temperature (21.23 ± 0.57 °C).

*EBC LTB\(_4\) measurement*

LTB\(_4\) concentrations were determined using liquid chromatography and triple quadrupole tandem mass-spectrometry (LC-MS/MS) adapted from Montuschi *et al.* [31]. In brief, LTB\(_4\)-d\(_4\) (Sapphire Bioscience) was added to EBC as an internal standard.
before sample concentration under vacuum and analysis by LC-MS/MS. A detailed description is supplied in Supplementary item 3.1.

Statistical methods

Correlations between EBC biomarkers were assessed using Pearson’s correlation coefficients. For each biomarker, differences between intra- and inter-day means were assessed using linear mixed models. Variance components used in the consistency analysis were estimated using two-way mixed effects model ANOVA with horses as the random term and collection period as a fixed term. The intra- and inter-day consistency for each EBC biomarker was calculated with the intraclass correlation coefficient (ICC) using special case 3A[C,1] (consistency definition using single measurements) and confidence intervals were estimated [32]. Thresholds of $\geq 0.9$ as high consistency and $\geq 0.8$ as adequate consistency were adopted for interpretation of ICC results [33].

For assessment of environmental and animal factors on biomarkers, univariate analyses, using linear mixed models, were performed and variables with $P<0.2$ were included in multivariable models. The model with the lowest AIC/BIC [34], was analysed using a linear mixed model with restricted maximum likelihood ANOVA. Multivariable models including two-way interaction terms were also explored with a backward elimination method, with the highest P-value term ($P>0.05$) removed at each step. Assumptions were that residuals in the model were normally distributed, had constant variance and were independent. Factor level variances for categorical variables were tested using the Brown-Forsythe test and weighted least squares analysis was used to account for the observed differences in variance. Predicted means and 95% confidence intervals (CIs) were calculated for variables retained in the multivariable analyses (Supplementary item 3.3). Data were analysed using ASReml-R [35]; with significance set at $P<0.05$.

Results

The mean $T_A$ and RH during EBC collection for Group-1 was $29.36 \pm 3.76$ °C and $41.1 \pm 13.3\%$, respectively and $19.23 \pm 10.64$ °C and $51.63 \pm 15.66\%$ for Group-2, respectively. Changes in respiratory effort and depth were not observed during sample
collection; however, RR (15.96 breaths/min) was lower than prior to sampling (17.17 breaths/min) \( (P=0.072) \). Cytological findings from all horses are provided in Table A1.1; six horses in Group-1, and 11 in Group-2, had cytological evidence of airway inflammation [27].

**Table A1.1**

The bronchoalveolar lavage fluid (BALF) cytological findings of horses in Groups-1 and 2. The presence (Y) or absence (N) of mild equine asthma as classified by BALF cytology [27] is indicated. Group-2 consists of horses from Group-1 and 10 additional horses.

<table>
<thead>
<tr>
<th>Horse ID</th>
<th>Mild equine asthma</th>
<th>BALF cellular percentages (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Neutrophil</td>
</tr>
<tr>
<td>Group-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>N</td>
<td>4.0</td>
</tr>
<tr>
<td>2</td>
<td>N</td>
<td>4.0</td>
</tr>
<tr>
<td>3</td>
<td>N</td>
<td>7.0</td>
</tr>
<tr>
<td>4</td>
<td>Y</td>
<td>5.0</td>
</tr>
<tr>
<td>5</td>
<td>Y</td>
<td>14.0</td>
</tr>
<tr>
<td>6</td>
<td>N</td>
<td>8.3</td>
</tr>
<tr>
<td>7</td>
<td>Y</td>
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</tr>
<tr>
<td>8</td>
<td>Y</td>
<td>3.3</td>
</tr>
<tr>
<td>9</td>
<td>Y</td>
<td>7.3</td>
</tr>
<tr>
<td>10</td>
<td>Y</td>
<td>6.3</td>
</tr>
</tbody>
</table>

| Group-2  |                    |            |            |           |
| 1        | N                  | 4.0        | 0.3        | 4.7       |
| 2        | N                  | 4.0        | 3.0        | 3.3       |
| 3        | N                  | 7.0        | 3.7        | 2.0       |
| 4        | Y                  | 5.0        | 8.0        | 2.3       |
| 5        | Y                  | 14.0       | 0.0        | 2.0       |
| 6        | N                  | 8.3        | 0.3        | 0.7       |
| 7        | Y                  | 2.7        | 10.0       | 4.3       |
| 8        | Y                  | 3.3        | 0.3        | 6.7       |
| 9        | Y                  | 7.3        | 5.7        | 3.3       |
| 10       | Y                  | 6.3        | 18.3       | 6.3       |
| 11       | N                  | 4.0        | 0.7        | 2.3       |
| 12       | Y                  | 3.0        | 0.0        | 6.7       |
| 13       | Y                  | 1.0        | 0.0        | 5.0       |
| 14       | Y                  | 3.0        | 0.0        | 8.7       |
| 15       | N                  | 4.0        | 0.0        | 2.3       |
| 16       | N                  | 3.0        | 0.0        | 3.3       |
| 17       | Y                  | 3.3        | 0.7        | 5.3       |
| 18       | N                  | 1.0        | 0.0        | 4.0       |
| 19       | N                  | 4.7        | 0.0        | 1.0       |
| 20       | Y                  | 1.0        | 0.0        | 10.7      |
**Intra-day and inter-day consistency**

$H_2O_2$ and LTB$_4$ were expressed using direct concentrations in EBC ($\mu$mol/L and pg/mL, respectively) and concentrations standardised to the average total volume of exhaled breath (EB$_{TV}$) per collection: pmol/100 L EB and pg/100L EB, respectively (Supplementary item 3.1 and 3.2). Concentrations of biomarkers for each collection period are shown in Fig. A1.2. There were weak, but significant, correlations between pH and $H_2O_2$ and pH and LTB$_4$ (Table A1.2).

![Figures A1.2](image)

**Figure A1.2.**

EBC $H_2O_2$ (A and B), pH (C and D) and LTB$_4$ (E and F) for Group 1 horses. EBC was collected from each horse on three occasions on day one at 08:00, 12:00 and 16:00 to assess intra-day consistency and at 12:00 for three days to assess inter-day consistency. Results are shown as median and mean (cross), quartiles (box), with whiskers calculated using the Tukey method.
Table A1.2
Correlations between EBC biomarkers as determined by Pearson’s correlation coefficient.

<table>
<thead>
<tr>
<th></th>
<th>$H_2O_2$ $\mu$mol/L</th>
<th>$H_2O_2$ pmol/100L EB</th>
<th>LTB$_4$ pg/ml</th>
<th>LTB$_4$ pg/100L EB</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pH neat</strong></td>
<td>$r = 0.32$</td>
<td>$r = 0.32$</td>
<td>$r = 0.47$</td>
<td>$r = 0.28$</td>
</tr>
<tr>
<td></td>
<td>$P = 0.022$</td>
<td>$P = 0.025$</td>
<td>$P &lt; 0.001$</td>
<td>$P = 0.008$</td>
</tr>
<tr>
<td><strong>pH deaerated</strong></td>
<td>$r = 0.32$</td>
<td>$r = 0.36$</td>
<td>$r = 0.47$</td>
<td>$r = 0.27$</td>
</tr>
<tr>
<td></td>
<td>$P &lt; 0.026$</td>
<td>$P = 0.011$</td>
<td>$P &lt; 0.001$</td>
<td>$P = 0.007$</td>
</tr>
</tbody>
</table>

The mean intra-day concentration of $H_2O_2$ was significantly higher than the inter-day concentration (Table A1.3). The intra-day ICCs were ≥0.9, while inter-day ICCs were ≥0.8 and consistency was greater for EB TV (Table A1.4). The mean intra-day and inter-day pH for both neat and deaerated EBC were not significantly different (Table A1.3). Intra- and inter-day ICCs for pH of neat and deaerated EBC were ≥0.8: consistency was greater for deaerated EBC (Table A1.4). No significant differences between mean intra- and inter-day LTB$_4$ were present (Table A1.3). The intra-day ICC for both measures of LTB$_4$ was ≈0.6 with wide CIs. The inter-day ICCs were not within the CIs (Table A1.4), as the variance was large, relative to the mean for each collection period, preventing determination of ICC across multiple days.

Table A1.3
Means and standard deviations (SD) of intra-day and inter-day EBC $H_2O_2$, and LTB$_4$ concentrations and pH.

<table>
<thead>
<tr>
<th></th>
<th>Intra-day</th>
<th>Inter-day</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td>$H_2O_2$ $\mu$mol/L</td>
<td>1.80</td>
<td>1.10</td>
<td>1.19</td>
</tr>
<tr>
<td>$H_2O_2$ pmol/100L EB</td>
<td>659.00</td>
<td>419.30</td>
<td>456.26</td>
</tr>
<tr>
<td>pH neat</td>
<td>6.85</td>
<td>0.38</td>
<td>6.99</td>
</tr>
<tr>
<td>pH deaerated</td>
<td>7.89</td>
<td>0.53</td>
<td>8.02</td>
</tr>
<tr>
<td>LTB$_4$ pg/mL</td>
<td>6.47</td>
<td>5.55</td>
<td>8.40</td>
</tr>
<tr>
<td>LTB$_4$ pg/100L EB</td>
<td>2.42</td>
<td>2.15</td>
<td>3.29</td>
</tr>
</tbody>
</table>

Effect of environmental and animal factors on EBC $H_2O_2$, pH and LTB$_4$

Results of the multivariable analyses for $H_2O_2$ and pH are provided in Table A1.5 and predicted effects of factors significantly associated with EBC $H_2O_2$ and pH are presented in Supplementary item 3.3. There were no significant associations between
environmental or animal factors and LTB₄. Several interaction terms for H₂O₂ and pH were significant (Supplementary item 3.4).

**Table A1.4**

The intra- and inter-day consistency, expressed as intraclass correlation coefficient (ICC) and 95% confidence interval (CI), of EBC H₂O₂, pH and LTB₄ as measured per volume of condensate and standardised according to the total estimated volume of exhaled breath during EBC collection.

<table>
<thead>
<tr>
<th>EBC biomarker</th>
<th>Intra-day consistency</th>
<th>Inter-day consistency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ICC</td>
<td>95% CI</td>
</tr>
<tr>
<td>H₂O₂ μmol/L</td>
<td>0.969</td>
<td>0.765 to 0.975</td>
</tr>
<tr>
<td>H₂O₂ pmol/100 L EB</td>
<td>0.976</td>
<td>0.810 to 0.980</td>
</tr>
<tr>
<td>pH neat</td>
<td>0.861</td>
<td>0.271 to 0.88</td>
</tr>
<tr>
<td>pH deaerated</td>
<td>0.860</td>
<td>0.268 to 0.879</td>
</tr>
<tr>
<td>LTB₄ pg/ml</td>
<td>0.599</td>
<td>-0.196 to 0.601</td>
</tr>
<tr>
<td>LTB₄ pg/100L EB</td>
<td>0.608</td>
<td>-0.186 to 0.613</td>
</tr>
</tbody>
</table>

**Discussion**

The results of this study indicate that the intra- and inter-day consistency of EBC H₂O₂ and pH is adequate to high, while the consistency of LTB₄ is poor. Additionally, H₂O₂ and pH may be influenced by several environmental and animal factors. These findings extend the understanding of EBC analysis in horses and the suitability of these biomarkers for respiratory assessment.

Concentrations of H₂O₂ in EBC were similar to previous reports [11,12,16]. In our study, H₂O₂ was also standardised to EB volume, as the volume of EBC collected per volume of EB is not constant in spontaneously breathing animals [36]; this method may avoid the confounding effects of respiration on biomarker concentrations [5,36]. A limitation of our study was the lack of accurate measurement of EBTᵥ by spirometry. Instead, EBTᵥ was estimated from RR, throughout EBC collection, and published tidal volumes at rest [37]. The high intra- and inter-day consistency of H₂O₂ suggests it may be a suitable biomarker in horses. There is limited information regarding the consistency of EBC H₂O₂ in veterinary species. Substantial intra- and inter-day variations in H₂O₂ in horses has been reported previously [16], while acceptable intra- and inter-day repeatability has been found in cats [38]. Similarly, good repeatability of
Table A1.5 Animal and environmental factors identified as influencing EBC biomarkers. Results of univariate and multivariable analyses and brief descriptions of observed effects are presented.

<table>
<thead>
<tr>
<th>Covariates</th>
<th>Univariate analysis</th>
<th>Multivariable analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P-value</td>
<td>Predicted effect</td>
</tr>
<tr>
<td>H₂O₂ μmol/L</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Ambient temperature               | < 0.001             | curvilinear increase with temperature | 0.004   | linear increase by 0.124 μmol/L for each 1 °C increase in ambient temperature  
|                                   |                     |                        |         |
| Ambient humidity                  | 0.018               | linear decrease with humidity | 0.052   |                                  |
| Feeding within 1 h of EBC collection | 0.069                 |                        | 0.007   | 0.6 μmol/L higher when collected in the yard, 1.6 (1.01-2.19) μmol/L vs crush, 1 (0.41-1.59) μmol/L  
| Collection location               | 0.142               |                        |         |
| Time of day                       | 0.006               | higher when collected in yard vs crush | < 0.001 | 318.7 pmol/100L EB higher when collected in the yard, 606.9 (371.9 - 841.9) pmol/100L EB vs the crush, 288.2 (32.03-544.37) pmol/100L EB  
| H₂O₂ pmol/100L EB                 |                     |                        |         |
| Collection location               | 0.006               |                        | < 0.001 |                                  |
| Ambient humidity                  | < 0.001             | linear decrease with humidity | 0.09    | linear decrease by 137.834 pmol/100L EB for each 5% increase in humidity  
| Ambient temperature               | < 0.001             | curvilinear increase with temperature | 0.009   |                                  |
| Time of day                       | 0.005               | linear increase from am to pm | 0.009   | changes in response to time of day; interaction with collection location influences the change  
| Feeding within 1 h of EBC collection | 0.047                  | higher in horses that have been fed |         |
| Metachromatic IAD pH neat         | 0.129               |                        |         |
| Average RR during collection      | 0.006               | linear increase with RR | < 0.001 | linear increase by 0.029 units for every 1 breath/min increase  
| Ambient temperature               | < 0.001             | linear increase with temperature | < 0.001 | linear increase by 0.031 units for each 1 °C increase in ambient temperature  
| Ambient humidity                  | < 0.001             | linear decrease with humidity |         |                                  |
| Collection location               | < 0.001             | higher when collected in yard vs crush |         |
| Time of day                       | 0.143               |                        |         |
| Eosinophilic IAD                  | 0.063               |                        |         |
| Metachromatic IAD                 | 0.142               |                        |         |
| BAL neutrophil %                  | 0.012               | linear increase with BAL neutrophil % | 0.089   |                                  |
| BAL eosinophil % pH deaerated     | 0.089               |                        |         |
| Average RR during collection      | 0.006               | linear increase with RR | < 0.001 | linear increase by 0.055 units for every 1 breath/min increase  
| Ambient temperature               | < 0.001             | curvilinear increase with temperature | < 0.001 | curvilinear increase as temperature increases  
| Ambient humidity                  | < 0.001             | linear decrease with humidity |         |                                  |
| Collection location               | < 0.001             | higher when collected in yard vs crush |         |
| Eosinophilic IAD                  | 0.058               |                        |         |
| BAL neutrophil %                  | 0.008               | linear increase with BAL neutrophil % |         |
| BAL eosinophil %                  | 0.057               |                        |         |

The effect of the covariate on the EBC biomarker is only provided for covariates with P<0.05

Refer to the supplementary item 3.3 for figures of the predicted effects

Mean (95% confidence intervals)

Refer to supplementary item 3.4 for more information regarding interaction terms
H$_2$O$_2$ in healthy humans and those with COPD has been reported [39]. The ICC for H$_2$O$_2$ in the current study was higher for EB$_{TV}$, suggesting that standardisation improves consistency of H$_2$O$_2$ for each horse between collections, as has been previously suggested for calves [5].

The mean pH of neat EBC was slightly higher than previous reports in horses [16], calves [5] and pigs [36]. Mean pH of deaerated EBC was comparable to previous reports in healthy horses, humans and dogs [3,19] and the average change in pH after deaeration was 1 unit, similar to a previous study [40]. The collection method was similar to that used by Duz et al. [16]; however, methodological differences, including higher collection temperatures, may influence results [19]. Change in pH due to storage at -80 °C was unlikely, as EBC pH remains stable for up to 2 years of storage at -20 °C [41]. In the current study, the intra- and inter-day consistency of pH was adequate, similar to an earlier study of horses where minimal variability in pH was found [16]. These findings support the use of EBC pH to monitor the lower airway of horses. In healthy humans, EBC pH is also highly repeatable [41], and is a useful biomarker for several inflammatory pulmonary diseases [2]. Our results indicate that both neat and deaerated EBC provided adequate consistency in pH, although deaerated EBC provided slightly superior inter-day consistency in pH, similar to a previous study [19].

The mean EBC LTB$_4$ was substantially lower than in horses with RAO [26], healthy calves [24] and humans [3]. These results may reflect species variation and a study population with little LAI. However, the mean concentration in our study was close to the limit of detection of the LC-QQQ method (3.22 pg/mL), despite sample concentration by nitrogen evaporation and improved assay specificity relative to enzyme immunoassays [21]. There was also poor consistency of LTB$_4$. Although our findings do not support the use of LTB$_4$ for lower airway assessment in horses, the poor consistency may reflect low levels of LTB$_4$ in clinically healthy, asymptomatic horses. Studies using horses with more severe asthma/LAI might be more suitable to assess the consistency of this analyte.
Horses were not categorised based on BALF results, as the study aim was to determine EBC biomarker consistency in horses without clinical signs of respiratory disease. Previous studies in horses and humans [16,39] demonstrated that biomarker variability was not affected by respiratory health status, similar to the results of this study. Weak but significant correlations between pH and H\textsubscript{2}O\textsubscript{2} and pH and LTB\textsubscript{4} were present. Previously, Duz et al. [16] observed that horses with increased H\textsubscript{2}O\textsubscript{2} concentrations tended to have lower pH, although associations were not significant.

Information on the impact of environmental factors on EBC biomarkers in horses is scarce. In our study, H\textsubscript{2}O\textsubscript{2} increased with increasing T\textsubscript{A} and decreased with increasing RH, while changes due to the time of day was dependent on interactions with other factors. However, our sample size limits the interpretation of these interactions, and further investigation to better define these interaction terms, is warranted. Diurnal variation in H\textsubscript{2}O\textsubscript{2} has been reported for humans [9,39] and calves [5], however, the influences of T\textsubscript{A} or RH have not been reported previously. The observed effects likely reflect the inverse relationship between T\textsubscript{A} and RH in our climatic region. Interactions between time of day and T\textsubscript{A} and, time of day and collection location (crush or yard) were observed and may be due, at least in part, to the positive correlation between UV-radiation and atmospheric H\textsubscript{2}O\textsubscript{2}, resulting in an increase in ambient H\textsubscript{2}O\textsubscript{2} in the afternoon [4,42]. Correlations between ambient and EBC H\textsubscript{2}O\textsubscript{2} concentrations have been demonstrated [4,5], suggesting that ambient H\textsubscript{2}O\textsubscript{2} affects EBC H\textsubscript{2}O\textsubscript{2}. In this study, collection of EBC in an outside yard was associated with a significantly higher concentration compared with indoors. While this finding may reflect a greater influence of UV-radiation on ambient and EBC H\textsubscript{2}O\textsubscript{2} in the yards, this remains speculative, as ambient air samples were not collected. However, PALL inhalation filters were used to minimise the amount of inspired H\textsubscript{2}O\textsubscript{2} [5], which may have limited the effect of ambient H\textsubscript{2}O\textsubscript{2} on EBC measurements and improved intra- and inter-day consistency. Caution is warranted with interpretation of the interaction terms (Supplementary item 3.4) as our study was not sufficiently powered to allow adequate evaluation of such interaction terms.

Considering the demonstrated influences of environmental factors on EBC H\textsubscript{2}O\textsubscript{2}, the difference between ambient conditions in Australia compared with Scotland
[12,16] may have contributed to the disparity in consistency between the current study and previous reports in horses [12,16]. The significant difference in intra-day and inter-day H2O2 concentrations in this study was likely due to the association with time of day and the influence of environmental factors, as there was a trend for lower RH and higher TA in the afternoon compared with morning.

In our study, increased RR and TA were both associated with increased pH. Similarly, Kullmann et al. [6] reported an influence of TA and RH on pH and suggested that these environmental factors may contribute to the observed variability. However, in other studies neither ambient conditions (TA, RH) nor ventilation parameters were associated with EBC pH in calves, humans [5,36,41] and horses following exercise [18]. To avoid the influence of sample temperature on pH readings [20], pH was measured at a constant temperature in the current study.

Conclusions

This study demonstrated that the intra- and inter-day consistency of EBC H2O2 and pH measurements are adequate to high, suggesting that these biomarkers may be useful for evaluation of the lower airways in horses. However, environmental factors including TA, humidity, time of day, collection location and RR during EBC collection may influence these biomarkers, necessitating standardisation when collecting samples longitudinally.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Acknowledgements

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receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

**Supplementary material**

Supplementary material associated with this article can be found in chapter 3.
References


### Appendix 2

**Table A2.1**

Various inflammatory airway disease (IAD) categories and bronchoalveolar lavage fluid (BALF) leukocyte percentages demonstrated a positive association with ambient temperature ($T_A$) and a negative association with relative humidity (RH).

<table>
<thead>
<tr>
<th>LAI category or BALF leukocyte %</th>
<th>Correlation coefficient or Association</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Neutrophilic IAD</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative humidity</td>
<td>RH for horses without neutrophilic IAD (57%) was a mean of 13.2% (95% CI: 2.8 to 23.7%) higher than for horses with neutrophilic IAD (43.8%)</td>
<td>0.014</td>
</tr>
<tr>
<td><strong>Eosinophilic IAD</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ambient temperature</td>
<td>Mean $T_A$ for horses with eosinophilic IAD (26.7°C) was a mean of 9.1°C (95% CI: 5.4 to 12.9°C) higher than for horses without eosinophilic IAD (17.5°C)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Relative humidity</td>
<td>Mean RH for horses without eosinophilic IAD (56.1%) was a mean of 13.1% (95% CI: 5.6 to 20.7%) higher than for horses with eosinophilic IAD (43%)</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>Mixed IAD</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ambient temperature</td>
<td>Mean $T_A$ for horses with mixed IAD (25.3°C) was a mean of 7.2°C (95% CI: 1.1 to 13.3°C) higher than for horses without mixed IAD (18.1°C)</td>
<td>0.029</td>
</tr>
<tr>
<td>Relative humidity</td>
<td>Mean RH for horses without mixed IAD (55.9%) was a mean of 17.5% (95% CI: 12.6 to 22.4%) higher than for horses with mixed IAD (38.5%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>BALF neutrophil %</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ambient temperature</td>
<td>$r = 0.38$</td>
<td>0.008</td>
</tr>
<tr>
<td>Relative humidity</td>
<td>$r = -0.43$</td>
<td>0.003</td>
</tr>
<tr>
<td><strong>BALF eosinophil %</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ambient temperature</td>
<td>$r = 0.43$</td>
<td>0.003</td>
</tr>
<tr>
<td>Relative humidity</td>
<td>$r = -0.38$</td>
<td>0.008</td>
</tr>
</tbody>
</table>

$r$, Pearson’s correlation coefficient  
CI, confidence intervals
Appendix 3

Table A3.1

Results of receiver operator characteristic curve analysis and cross-tabulation for all categories of lower airway inflammation (LAI) identified during the univariate analysis to be significantly associated with pH measured in neat exhaled breath condensate (EBC). Horses with inflammatory airway disease (IAD) were additionally categorised as neutrophilic and eosinophilic based on bronchoalveolar lavage cytology. The case definitions are provided below and more information can be found in Chapter 4. The pH cut-points chosen for analysis are provided in the table.

<table>
<thead>
<tr>
<th>LAI category</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH neat ≥6.67</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAD</td>
<td>72</td>
<td>80</td>
<td>88</td>
<td>57</td>
</tr>
<tr>
<td>Any IAD&lt;sup&gt;a&lt;/sup&gt;</td>
<td>78</td>
<td>67</td>
<td>69</td>
<td>76</td>
</tr>
<tr>
<td>pH neat ≥6.86</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophilic IAD</td>
<td>78</td>
<td>76</td>
<td>44</td>
<td>97</td>
</tr>
<tr>
<td>pH neat ≥6.96</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eosinophilic IAD</td>
<td>67</td>
<td>78</td>
<td>31</td>
<td>94</td>
</tr>
</tbody>
</table>

PPV, Positive predictive value; NPV, Negative predictive value
<sup>a</sup>Any IAD: any of subtypes of IAD
Case definitions

The following definitions were adopted:

- **Inflammatory airway disease (IAD):** BALF cytology with $\geq 10\%$ neutrophils or $\geq 5\%$ mast cells or $\geq 5\%$ eosinophils or any combination of these [1]. For statistical analysis, horses were further categorised as neutrophilic, metachromatic, eosinophilic ‘mixed’ (combination of $\geq 2$ subtypes) and ‘any’ (any subtype) IAD.

- **Exercise-induced pulmonary haemorrhage (EIPH):** tracheobronchoscopic blood grading $\geq 1$ [2], and/or BALF cytology with $\geq 20\%$ haemosiderophages [3] and/or total haemosiderin score (THS) $\geq 75$ in BALF [4].

- **Lower airway disease (LAD):** IAD and/or EIPH based on BALF cytology

- **Syndrome of tracheal inflammation (STI):** tracheal inflammation score (TIS) $\geq 6/9$; the 9-point inflammation score comprised the sum of tracheal mucus (0-3), smear cell density (0-3) and neutrophil proportion scores (0-3) [5].
References


### Table A4.1
Correlations identified between environmental factors, respiratory rate during exhaled breath condensate (EBC) collection and EBC volume.

<table>
<thead>
<tr>
<th>Environmental or methodological covariates</th>
<th>Pearson’s correlation coefficient</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EBC volume</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ambient temperature</td>
<td>$r = 0.68$</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Relative humidity</td>
<td>$r = -0.60$</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Respiratory rate during EBC collection</td>
<td>$r = 0.61$</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Exhaled breath volume</td>
<td>$r = 0.61$</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Respiratory rate during EBC collection</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ambient temperature</td>
<td>$r = 0.31$</td>
<td>0.032</td>
</tr>
<tr>
<td>EBC volume</td>
<td>$r = 0.61$</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>