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METABOLOMICS OF TRACHEAL WASH SAMPLES AND EXHALED BREATH CONDENSATES IN HEALTHY HORSES AND HORSES AFFECTED BY EQUINE ASTHMA

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Abstract

The present work characterized the metabolomic profile of tracheal wash (TW) and exhaled breath condensate (EBC) in healthy horses and horses with respiratory disease. Six asthma-affected horses (Group A) and six healthy controls (Group H) underwent clinical, endoscopic and cytologic examinations of upper airways to confirm the active phase of asthma. TW and EBC samples were collected from each animal and investigated by Proton Nuclear magnetic resonance (^{1}H-NMR) metabolomic analysis. A total of 10 out of 38 metabolites found in TW were significantly different between groups ($p < 0.05$). Higher concentrations of histamine and oxidant agents like glutamate, valine, leucine and isoleucine, as well as lower levels of ascorbate, methylamine, dimethylamine and O-phosphocholine were found in Group A compared to Group H. Eight metabolites were found in equine EBC, namely methanol, ethanol, formate, trimethylamine, acetone, acetate, lactate and butanone, previously observed also in human EBC. Despite this was a pilot study, the results showed that metabolomic analysis of TW and EBC has the potentiality to serve as a basis for diagnostic tools in horses with asthma.

1. Introduction

Equine asthma, or heaves, is an environmental asthma-like disease of adult horses characterized by a recurring bronchoconstriction and airway inflammation [1], with alternating periods of remission and crises [2]. Clinical signs are not evident in low-grade airway obstruction cases, and exercise intolerance may be the only symptom. In severe cases cough, nasal discharge, increased respiratory effort, and weight loss can be observed [2]. When exposed to organic dust particulates in hay, horses affected by equine asthma show disease exacerbation [1] with a rapid development of lower airway inflammation, bronchoconstriction and mucus secretion. These three features of the disease have been extensively studied, however this cascade of events is not well understood yet [2], so that the pathogenetic and immunological basis of the disease are still controversially discussed [3]. Suggestion for equine asthma etiology and pathogenesis include Type I and III hypersensitivity reaction, inhalation of endotoxin, mold components, spores of fungi and actinomycetes such as *Aspergillus fumigatus, Faenia rectivirgula* and *Thermoactinomyces vulgaris*, noxious gases, bacterial and viral infections [4,5]. Despite differences in the predominant cell population of bronchoalveolar lavage fluid (BALF), that is neutrophil in horses and eosinophil in men, equine and human asthma share some features, like the occurrence of airways obstruction, bronchial hyperresponsiveness and airways inflammation, making horse a recognized model for human disease [6-8].

Several recent researches have been focused on the study of immune parameters such as cellular basis of inflammation or cytokine expression in respiratory cells [9-14]. Equine asthma, as well as human asthma, may be driven by an excessive innate immune response as well as by specific T-helper lymphocyte-mediated reactions [15].

Over the last few years, metabolomics gained an increasing interest in biomedical research. This new and rapidly expanding field of systems biology allows researchers to have an overall view of hundreds of small organic molecules that can be found in a given sample. By analyzing small metabolites (e.g. amino acids, organic acids and alcohols), metabolomics represents a viable alternative to transcriptomics, genomics, and proteomics, but it also completes the information provided by these *omics* studies [16]. Metabolomics consists of the analysis of all metabolites that are present within an organism or a specific compartment of the body. The detection and quantification of these metabolites provide unique insights into metabolic changes occurring in tangent to alterations in gene and protein activity associated with disease [16]. Metabolites represent the end products of complex interactions occurring inside the cell and all the events occurring outside of the cell or organism. Therefore, the comprehensive measurement of metabolites allows to determine the interactions between genes and the environment [17].

Proton Nuclear magnetic resonance (^{1}H-NMR) spectroscopy is a primary analytical technique used for metabolites detection, able to characterize and quantify different kinds of small molecules from biofluids [18-20].

In recent years, this approach has been found useful to evidence consequences of several respiratory diseases in humans by analyzing biofluids collected from the respiratory tract, such as BALF [21] or exhaled breath condensate (EBC) [22-25]. In equine species, several aspects of BALF and tracheal wash (TW) have been extensively investigated [26], while only few researches dealt with EBC [27-29]. However, to our knowledge no metabolomic study has been published on equine respiratory system.

The aim of the present work was to investigate whether it is possible to characterize the metabolomic profiles of TW and EBC fluids, to discriminate horses affected by equine asthma from healthy animals. For the purpose, we applied ^{1}H-NMR spectroscopy to the untargeted detection and quantification of low weight metabolites. The present work may grant an overall picture of the reaction of horse body to a chronic airways inflammatory condition like equine asthma.

2. *Animals, materials and methods*

2.1 Animals

Six clinically healthy horses (1 male, 3 geldings, 2 females, median age 13 years, body condition score (BCS) 3.0), with no history of respiratory disease in the last 6 years, were included in Group H. Six horses (1 male, 2 geldings, 3 females, median age 18 years, BCS 2.5-3.0) affected by asthma were included in Group A, as referred to the Veterinary Teaching Hospital Large Animal Department of Camerino University with a history of showing symptoms of asthma when stabled and exposed to dusty hay. In these animals, the diagnosis of equine asthma was previously confirmed by endoscopic examination and BALF cytology. Typical respiratory symptoms [30] were present at physical examination (e.g. exercise intolerance, crackles and wheezes, increased respiratory effort) and all horses have been shown respiratory signs for at least 1 month before enrollment. No drugs had been administered for at least 2 months.
All horses were stabled in boxes and fed the same polyphyte hay (7±1 kg/horse/day) for one week prior to samples collection. Samples of EBC and TW were collected in the morning (09.00 AM), before feeding.
All experimental procedures were approved by the Animal Care Committee of Camerino University (Registration number: E81AC.8.B, March 1st, 2018) and were in accordance with the standards recommended by the EU Directive 2010/63/EU for experiments on animals.

The study has been conducted in the month of April 2018.

2.2 Endoscopy, tracheal wash sampling and cytology.

A 140 cm long endoscope, with outer diameter of 0.9 cm (Mercury Endoscopia Italiana) was passed in the ventral meatus of the nasal cavity to reach the trachea. To confirm active inflammation, the amount of tracheal mucus in trachea was scored according to Gerber *et al.* by using the following 0 to 5 scales: grade 0, clean (no mucus); grade 1, little (multiple small drops); grade 2, moderate (large drops); grade 3, marked (stream-forming); grade 4, large (pool-forming); grade 5, extreme (abundant amount) [31,32].

During endoscopy, TW samples were collected as described by Hodgson and Hodgson [33]. Briefly, 10 mL of sterile saline solution was instilled through a polyethylene catheter passed through the working channel of the endoscope. Fluids accumulated in the “tracheal puddle” were aspirated and collected into sterile tubes. A minimum of 8 mL of instilled fluid was recovered from all horses. One 1.5 mL aliquot was stored at -80 °C for metabolomic analysis, while one 5 mL aliquot was used for cytological evaluation immediately after collection.

According to cytological appearance of TW smears and cytospin specimens, a grading of airways inflammation from 0 to 2 was made, based on the presence of neutrophils, with score= 0 indicating occasional neutrophils; score= 1 presence of moderate number of neutrophils (≤20%); score= 2 predominant number of neutrophils (>20%) [26,30,33].

2.3 Exhaled breath condensate collection

Before starting EBC sampling, all horses were accustomed to the EBC collection system that was well tolerated by animals. EBC samples were collected indoors (16±2°C), before feeding, between

9.00 – 09.30 am, without any sedation and prior to TW collection. A condensation system consisting of a face mask connected via tubing to a condensation chamber was used (Figure 1).

Because of the lack of commercially available EBC collection equipment for horses, we created a custom condensation system adapted from Whittaker *et al.* [28]. The modified aerosol face mask (SM Trade&Technology SRL) had a tight fitting rubber shroud and three unidirectional valves, with the first valve, positioned ventrally, allowing the air to enter into the mask during inspiration. The other two valves, positioned over the nares, were connected via thermally-insulated tubing to a condensation device that allowed expired air to unidirectionally pass through the system. The flexible plastic tubes (length: 280 cm; radius: 2.1 cm) were coated with thermal insulating tubes to maintain the temperature of expired air, thus preventing air condensation inside the tubing system. The condensation chamber consisted of a 500 mL glass becker inserted into an ice block, having a one-way valve on the top to prevent EBC contamination by retrograde flow of environmental air. During EBC collection the temperature inside the condensation chamber was monitored by means of a suitable thermometer (-20°C).

Considering the materials and dimensions of the system (total volume estimated about 5 l) and the use of thermal insulation tubes, the maximal thermal dispersion of exhaled air throughout the tubing system was calculated <3 °C during sampling.

From each subject EBC was collected over 15 min, allowing to obtain 1.5-3 ml samples that were immediately cooled on ice and stored at -80°C within 30 minutes from collection. Respiratory rate and respiratory pattern of each horse were monitored at rest, 15 min before, and 15 min after the collection, as well as continuously throughout the sampling period.

2.4 Metabolomic analysis by ^{1}H-NMR

TW and EBC samples collected from each horse were centrifuged at 18640 g and 14 °C for 15 min [34]. After centrifugation, 0.7 mL of supernatant were added to 0.1 mL of a D_2O solution of 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP) 10 mM, used as NMR chemical-shift reference, and NaN_3 2 mM, to avoid microorganisms proliferation, buffered at pH 7.00±0.02 by means of 1M phosphate buffer. Afterwards, each sample was centrifuged again at the above conditions.

^{1}H-NMR spectra were recorded at 298 K with an AVANCE III spectrometer (Bruker, Milan, Italy) operating at a frequency of 600.13 MHz. According to Laghi *et al.* [19], the signals from broad resonances originating from large molecules were suppressed by a CPMG-filter composed by 400 echoes separated by 0.400 ms and created with a 180° pulse of 0.024 ms, for a total filter of 330 ms. The residual water signal was suppressed by presaturation. This was done by employing the cpmgpr1d sequence, part of the standard pulse sequence library. Each spectrum was acquired by summing up 256 transients using 32 K data points over a 7184 Hz spectral window, with acquisition time 2.28 sec. To apply NMR as quantitative technique, the recycle delay was set to 5s, keeping into consideration the relaxation time of the protons under investigation. ^{1}H-NMR spectra baseline-adjusted by means of the peak detection according to the “rolling ball” principle [35] implemented in the baseline R package [36]. A linear correction was then applied to each spectrum, so to make the points pertaining to the baseline randomly spread around zero. Differences in water content among

samples were taken into consideration by probabilistic quotient normalization [37] applied to the entire spectra array.

The signals were assigned by comparing their chemical shift and multiplicity with Chenomx software library (Chenomx Inc., Canada, ver 8.3).

2.5 Statistical analysis

Parameters and molecules whose concentration varied between Groups H and A were looked for by means of Wilcoxon test. For the purpose, a significance limit $p < 0.05$ was accepted.

To highlight the underlying trends characterizing the samples, principal component analysis models in their robust version (rPCA) were built on the molecules concentrations, centered and scaled to unity variance [38].

For each rPCA model, we calculated the scoreplot, the projection of the samples in the PC space, tailored to highlight the underlying structure of the data. Besides, we calculated the correlation plot, relating the concentration of each variable to the components of the rPCA model, therefore tailored to highlight the most important molecules in determining the trends highlighted by the scoreplot.

3. Results

Tracheal mucus was found to differ significantly ($p<0.01$) between groups, with healthy horses having a mean score of 0.3, and asthma affected horses characterized by a mean score of 2.2. The proportion of neutrophils differed as well, with Group H and Group A characterized by mean scores of 0.2 and 1.2, respectively ($p< 0.001$).

The metabolomics investigation of TW samples allowed the quantification of 38 molecules (Figure 2). Ten metabolites showed significantly different concentrations between Groups H and A (Table 1).

Table 1. Metabolites concentrations (mmol/L), expressed as median (interquartile range), quantified by ^{1}H-NMR in tracheal wash (TW) samples of healthy horses (Group H) and horses with asthma (Group A).

Metabolites	Group H (n=6)	Group A (n=6)	Trend
Formate	$2.04x10^{-2}$ ($1.74x10^{-2}$)	$1.40x10^{-2}$ ($5.69x10^{-3}$)	=
3-Methylxanthine	$1.41x10^{-2}$ ($2.97x10^{-3}$)	$1.40x10^{-2}$ ($1.57x10^{-2}$)	=
Phenylalanine	$9.23x10^{-3}$ ($5.97x10^{-3}$)	$1.47x10^{-2}$ ($1.96x10^{-2}$)	=
Histamine*	$1.56x10^{-2}$ ($1.93x10^{-3}$)	$2.21x10^{-2}$ ($1.07x10^{-2}$)	↑
Tyrosine	$1.80x10^{-2}$ ($7.67x10^{-3}$)	$2.90x10^{-2}$ ($2.23x10^{-2}$)	=
1,3-Dihydroxyacetone	$1.29x10^{-2}$ ($2.43x10^{-3}$)	$7.40x10^{-3}$ ($2.10x10^{-2}$)	=
Lactate	$1.13x10^{-1}$ ($8.47x10^{-2}$)	$2.26x10^{-1}$ ($4.77x10^{-2}$)	=
Ascorbate*	$4.91x10^{-1}$ ($5.77x10^{-1}$)	$1.10x10^{-1}$ ($2.34x10^{-1}$)	↓
Serine	$2.97x10^{-2}$ ($2.52x10^{-2}$)	$1.68x10^{-2}$ ($1.35x10^{-2}$)	=
Threonine	$7.50x10^{-2}$ ($4.04x10^{-2}$)	$8.03x10^{-2}$ ($4.65x10^{-2}$)	=
Glycine	$4.85x10^{-2}$ ($6.00x10^{-2}$)	$1.04x10^{-1}$ ($1.69x10^{-2}$)	=
Methanol	$2.49x10^{-2}$ ($1.01x10^{-2}$)	$2.70x10^{-2}$ ($4.72x10^{-2}$)	=
Proline	$2.02x10^{-2}$ ($1.73x10^{-2}$)	$2.93x10^{-2}$ ($1.04x10^{-2}$)	=
myo-Inositol	$2.10x10^{-2}$ ($6.92x10^{-3}$)	$2.44x10^{-2}$ ($1.16x10^{-2}$)	=
Taurine	$7.92x10^{-2}$ ($1.05x10^{-1}$)	$1.47x10^{-1}$ ($4.69x10^{-2}$)	=
Glucose	$1.63x10^{-2}$ ($1.85x10^{-2}$)	$3.16x10^{-2}$ ($2.81x10^{-2}$)	=
Carnitine	$3.46x10^{-2}$ ($2.21x10^{-2}$)	$2.61x10^{-2}$ ($2.65x10^{-2}$)	=
O-Phosphocholine*	$3.42x10^{-2}$ ($1.26x10^{-2}$)	$1.18x10^{-2}$ ($1.77x10^{-2}$)	↓
Choline	$8.50x10^{-2}$ ($6.86x10^{-2}$)	$1.20x10^{-1}$ ($9.97x10^{-2}$)	=
Dimethyl sulfone	$9.48x10^{-2}$ ($6.64x10^{-2}$)	$1.65x10^{-1}$ ($1.11x10^{-1}$)	=
Creatine	$1.52x10^{-2}$ ($6.87x10^{-3}$)	$1.85x10^{-2}$ ($2.42x10^{-3}$)	=
Dimethylamine*	$6.41x10^{-3}$ ($1.19x10^{-3}$)	$3.29x10^{-3}$ ($2.41x10^{-3}$)	↓
Aspartate	$3.80x10^{-2}$ ($1.61x10^{-2}$)	$5.47x10^{-2}$ ($2.81x10^{-2}$)	=
Methionine	$5.89x10^{-3}$ ($2.42x10^{-3}$)	$5.67x10^{-3}$ ($6.58x10^{-3}$)	=
Methylamine*	$9.97x10^{-3}$ ($2.96x10^{-3}$)	$4.94x10^{-3}$ ($2.07x10^{-3}$)	↓
Glutamine	$1.80x10^{-2}$ ($7.04x10^{-3}$)	$2.03x10^{-2}$ ($1.06x10^{-2}$)	=
Succinate	$1.32x10^{-2}$ ($6.16x10^{-3}$)	$1.15x10^{-2}$ ($3.09x10^{-3}$)	=
Pyruvate	$7.87x10^{-3}$ ($3.49x10^{-3}$)	$1.32x10^{-2}$ ($9.22x10^{-3}$)	=
Glutamate*	$3.45x10^{-2}$ ($2.36x10^{-2}$)	$7.19x10^{-2}$ ($3.80x10^{-2}$)	↑
Acetone	$1.79x10^{-2}$ ($3.22x10^{-3}$)	$1.12x10^{-2}$ ($8.45x10^{-3}$)	=
Acetate	$2.71x10^{-2}$ ($1.48x10^{-2}$)	$4.63x10^{-2}$ ($2.47x10^{-2}$)	=
Alanine	$2.28x10^{-2}$ ($1.45x10^{-2}$)	$5.20x10^{-2}$ ($5.85x10^{-2}$)	=
Ethanol	$1.38x10^{-2}$ ($9.35x10^{-4}$)	$9.08x10^{-3}$ ($4.13x10^{-3}$)	=
Propylene glycol*	$3.88x10^{-3}$ ($8.71x10^{-3}$)	$1.92x10^{-3}$ ($4.88x10^{-4}$)	↓

Methylsuccinate	$9.83x10^{-3}$ ($8.19x10^{-3}$)	$8.18x10^{-3}$ ($6.44x10^{-3}$)	=
Valine*	$7.91x10^{-3}$ ($2.17x10^{-3}$)	$2.24x10^{-2}$ ($2.82x10^{-2}$)	↑
Leucine*	$1.58x10^{-2}$ ($1.13x10^{-2}$)	$5.74x10^{-2}$ ($7.62x10^{-2}$)	↑
Isoleucine*	$1.17x10^{-2}$ ($2.35x10^{-3}$)	$3.41x10^{-2}$ ($3.50x10^{-2}$)	↑

*Significantly different metabolite concentrations ($p<0.05$) between Group H and Group A, assessed by Wilcoxon test for unpaired samples.

To gain an insight of the underlying trends induced by equine asthma in TW metabolome, metabolites concentrations were employed as a basis for a rPCA model, as showed in figure 3.

Along PC 1 of its scoreplot (**a**), representing as much as 87.6% of the samples variability explained by the rPCA model, Group H and Group A are significantly separated ($p<0.01$).

Although minute ventilation was not recorded, the EBC collection system used in the present study was well tolerated by the horses that showed no obvious changes in respiratory rate or respiratory pattern during sampling.

The metabolomic analysis applied to TW samples was extended to EBC samples (Figure 4). The use of ^{1}H-NMR technique allowed to quantify eight different molecules in EBC samples (Table 2), however no significant differences were found between groups.

Table 2. Metabolites concentration (mmol/L), expressed as median (interquartile range), quantified by ^{1}H-NMR in exhaled breath condensate (EBC) samples of healthy horses (Group H) and horses with asthma (Group A).

Metabolites	**Group H (n=6)**	**Group R (n=6)**	**Trend**
Formate	$2.56x10^{-2}$ ($9.64x10^{-3}$)	$2.47x10^{-2}$ ($1.83x10^{-2}$)	=
Methanol	$5.41x10^{-1}$ ($1.81x10^{-1}$)	1.15 ($9.98x10^{-1}$)	=
Trimethylamine	$3.64x10^{-2}$ ($3.77x10^{-2}$)	$6.74x10^{-2}$ ($1.44x10^{-2}$)	=
Acetone	$2.34x10^{-1}$ ($5.03x10^{-2}$)	$2.78x10^{-1}$ ($1.64x10^{-2}$)	=
Acetate	$1.01x10^{-1}$ ($5.81x10^{-2}$)	$1.00x10^{-1}$ ($1.03x10^{-1}$)	=
Lactate	$9.96x10^{-2}$ ($1.20x10^{-1}$)	$9.16x10^{-2}$ ($9.68x10^{-2}$)	=
Ethanol	1.19 (4.15)	$8.65x10^{-1}$ (8.30)	=
Butanone	$1.46x10^{-2}$ ($1.13x10^{-1}$)	$2.03x10^{-2}$ ($1.94x10^{-2}$)	=

4. Discussion

To the best of the authors' knowledge, this is the first metabolomics approach attempted by ^{1}H-NMR on both TW and EBC samples in equine species.

Despite considered less sensitive than BALF to diagnose equine asthma, TW allows to obtain a better representation of the whole lung condition [26]. Furthermore, TW collection can be easily performed without using sedatives that might interfere with horse metabolomic profile. The significant ($p<0.01$) different degree of tracheal mucus and percentage of neutrophils in TW samples confirmed the presence of active inflammation in horses from Group A. Twenty-four of the 38 metabolites we found by NMR analysis had been previously found in humans with respiratory inflammation by Ciaramelli *et al.* [21]. This metabolomic analysis also allowed the detection in EBC samples of metabolites like ethanol, formate, acetate and ethanol that were found previously in EBC of humans with lung disfunction [25]. Equine and human asthma are different diseases but they share some characteristics such as the so called remodeling of the pulmonary tissue, which includes reduction of bronchial luminal caliber, smooth muscle hypertrophy, peribronchiolar fibrosis formation and airway epithelial cell hyperplasia, all impeding gas exchange [39-40]. These findings could support the hypothesis that studies on naturally occurring equine asthma could provide information on human respiratory inflammatory diseases [6, 8].

It is known that saliva reflects blood composition, but it varies in its molecular profile as a consequence of several *stimuli* like specific diets [41]. It is safe to postulate that a similar mechanism involves respiratory glands as well, when they produce higher quantities of mucus in connection to asthma. This mechanism, together with changes in microbiota, is likely to lead to major modifications of mucus molecular profile.

One of greatest modifications in TW metabolites involved histamine, which showed higher levels in horses with asthma compared to healthy animals (Figure 4B). Histamine is a prominent contributor to allergic diseases [42] but its involvement in etiology of equine asthma, as well as the role of IgE, is still controversial [4]. McGorum *et al.* found a higher level of histamine in pulmonary epithelial lining fluid (PELF) of asthma affected horses after natural challenge compared to normal horses [43]. This difference was observed in the late phase of disease, as the horses included in the present study, but not in early stage. These findings could therefore support the hypothesis that equine asthma is connected to a late phase IgE mediated hypersensitivity reaction.

In the complex pathophysiology of equine asthma, also oxidative stress might play a role as the active phase of asthma results in an infiltration of neutrophils into the tracheobronchial lumen and thereby leads to a greater oxidative load [44-45].

In our study, Group A showed significantly higher levels of glutamate and of the branched-chain amino acids (BCAAs) valine, leucine and isoleucine. Each of them can be found in several mammalian tissues [46] and is considered to have an oxidant activity, by inducing lipid peroxidation *in vitro* [47-48]. Moreover, BCAAs have been found involved in stress and systemic inflammation [17,49].

Metabolomic results on horses with asthma also showed a decrease in some amines that appear in biosynthetic pathways of amino acids, such as methylamine and dimethylamine (DMA) [50]. These amines are normally present in blood, and, from blood, in saliva [51], in different concentrations

according to glands stimulation [41]. Their different concentration could be therefore connected to abnormal mucus production by respiratory gland occurring in equine asthma disease. Recently, nevertheless, it was established that DMA is also a metabolic product of asymmetric dimethylarginine (ADMA), which is an endogenous competitive inhibitor of nitric oxide synthase (NOS) [52-54].

Another antioxidant metabolite like ascorbate was found at lower concentration in horses with asthma compared to healthy animals. The principal non-enzymatic antioxidant identified in BALF of horses is ascorbic acid [45]. This agent is 50 times more concentrated in healthy horses than people, probably because horses are able to synthetize it, unlike humans [45,55]. Ascorbic acid is the first antioxidant to be oxidised in the airway during the active phase of equine asthma [45,56] by transition metal ions occurring at the site of airways inflammation [57-58] and its repletion follows the same course of the inflammation resolution [45,56]. The role of oxidative stress in the pathogenesis of equine asthma is also linked to airway smooth muscle contraction, as observed in other species [45,59]. Level of lung dysfunction in asthma affected horses correlated positively with depletion of ascorbic acid in airways implicating oxidative stress and antioxidant consumption in the modulation of airway smooth muscle tone [56,60]. Further studies could confirm if ascorbate evaluation by metabolomics analysis could be an evaluable tool to monitor the disease progression and evaluate smooth muscle condition.

The decrease of DMA and ascorbate in Group A, together with the significant increase in the oxidant agents, glutamate, valine, leucine and isoleucine noticed above, could support the hypothesis that oxidative stress is associated to lower airway disorders occurring in horses affected by asthma [8,44,61].

Lower levels of O-phosphocholine were found in horses with respiratory disease compared to healthy controls. A marked decreased in O-phosphocholine levels was also observed by Jung and colleagues in the serum of patients with asthma [62]. This decrease might reflect a reduced protection of the alveolar region and of the conducting airways [63], as phosphocholine is a component of the endothelial cell barrier and a pulmonary surfactant.

Finally, NMR analysis revealed the presence of propylene glycol both in healthy horses and in asthma affected animals. The presence of this molecule in mucus was not unexpected. In fact, the polysaccharides constituting horse mucus are largely composed by fucose, which can be transformed into propylene glycol by microorganisms due to an upregulation of lactaldehyde reductase [64]. In the present work, we found that propylene glycol was significantly decreased by equine asthma. Interestingly, several reports underline the antibacterial properties of propylene glycol [65-66], so that it can be speculated that this molecule might be at the base of a natural defense mechanism of respiratory tract in healthy horses.

EBC is a biofluid of respiratory origin that can be collected in horses in a totally noninvasive way by cooling and condensing the exhaled breath. Anyway, a larger number of animals should be sampled to obtain information about reproducibility of our results. The obtained liquid contains soluble exhaled gases and metabolites of the extracellular lining fluid. Little is known about the genesis of these exhaled breath volatile organic compounds (VOCs), some are thought to be endogenous end-products of metabolic pathways [67]. According to recent studies, one of the origins of exhaled VOCs is airway inflammation after reactive oxygen species react with cell membranes [68-70]. Notwithstanding the differences between human and equine physiology, we found some of the most common molecules observed in human EBC [23-25]. Recently, NMR-based metabolomics of EBC

has been effective in recognizing biomarkers and predict asthma exacerbation in children [22,70], chronic obstructive pulmonary disease (COPD) in adults [71], or stable or unstable cystic fibrosis [72]. Even if no significant differences were found in EBC metabolites between groups, the increase of methanol in Group A was found in agreement with the observations by Maniscalco *et al*. [25] in humans with chronic obstructive pulmonary disease (COPD). In this respect, it is interesting to notice that methanol is metabolized to formaldehyde, which shows a pro-inflammatory action and exacerbates airways inflammation in alveolar and bronchial cells and in animal models [25].

5. Conclusions

Despite equine asthma have been extensively studied in veterinary medicine, this is the first work describing the metabolomic profile of TW and EBC in healthy and asthma affected horses. Among biofluids of respiratory origin, EBC and TW are the most accessible samples as no or minimally invasive, and they can be obtained without sedation and adverse effects. The increase in histamine and oxidant agents, together with the decrease in antioxidant metabolites confirm that oxidative stress is strongly involved in asthma pathogenesis and showed that metabolomic analysis of TW by ^{1}H-NMR could represent a potential diagnostic tool to differentiate horses with asthma from healthy animals. Furthermore, equine TW metabolomic profile might provide suitable information for some human diseases sharing some features with equine asthma, like human asthma and COPD.
Although a pilot study, the metabolomic profile of EBC showed interesting results as well. We could identify molecules already found in humans with chronic respiratory disease, despite a better standardization of sampling collection is needed in order to identify a recognized EBC metabolomic profile of horses affected by equine asthma. Further study involving a larger number of horses could confirm the results obtained in this report, considering that, as usually happen in similar studies, the high number of measured variables could lead to coincidental correlations [73].

The results herein reported not only emphasize the value of ^{1}H-NMR as a diagnostic tool, but also demonstrate the potential of this approach to identify established and novel biomarkers to be used for further pathogenetic investigations, differential diagnosis or therapeutic targets in veterinary medicine.

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Conflict of interest

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

REFERENCES

1. Leclere M, Lavoie-Lamoureux A and Lavoie JP 2011 Heaves, an asthma-like disease of horses *Respirology* **16** 1027-1046

2. Léguillette R 2003 Recurrent airway obstruction-heaves *Vet. Clin. Equine* **19** 63-86

3. Korn A, Miller D, Dong L, Buckles EL, Wagner B and Ainsworth DM 2015 Differential Gene Expression Profiles and Selected Cytokine Protein Analysis of Mediastinal Lymph Nodes of Horses with Chronic Recurrent Airway Obstruction (RAO) Support an Interleukin-17 Immune Response *PLoS ONE* **10**(11) e0142622

4. Pirie RS 2014 Recurrent airway obstruction: a review *Equine Vet. J.* **46** 276-88

5. Lavoie JP 2007 Recurrent airway obstruction (heaves) and summer-pasture-associated obstructive pulmonary disease In: McGorum BC, Dixon PM, Robinson NE, Schumacher J (eds) *Equine Respiratory Medicine and Surgery* (Saunders-Elsevier) 565-589

6. Bullone M and Lavoie JP 2015 Asthma "of horses and men"-How can equine heaves help us better understand human asthma immunopathology and its functional consequences? *Mol. Immunol.* **66** 97–105

7. Kirschvink N and Reinhold P 2008 Use of alternative animals as asthma models. *Curr. Drug Targets* **9** 470-484.

8. Bullone M and Lavoie JP 2017 The Contribution of Oxidative Stress and Inflamm-Aging in Human and Equine Asthma. *Int. J. Mol. Sci.* **5** 18(12)

9. Ainsworth DM, Grünig G, Matychak MB, Young J, Wagner B, Erb HN and Antczak DF 2003 Recurrent airway obstruction (RAO) in horses is characterized by IFN-gamma and IL-8 production in bronchoalveolar lavage cells *Vet. Immunol. Immunopathol.* **96** 83-91

10. Ainsworth DM, Wagner B, Franchini M, Grünig G, Erb HN and Tan JY 2006 Time-dependent alterations in gene expression of interleukin-8 in the bronchial epithelium of horses with recurrent airway obstruction *Am. J. Vet. Res.* **67** 669–677

11. Cordeau ME, Joubert P, Dewachi O, Hamid Q and Lavoie JP 2004 IL-4, IL-5 and IFN-gamma mRNA expression in pulmonary lymphocytes in equine heaves *Vet. Immunol. Immunopathol*. **97** 87-96

12. Kleiber C, McGorum BC, Horohov DW, Pirie RS, Zurbriggen A and Straub R 2005 Cytokine profiles of peripheral blood and airway CD4 and CD8 T lymphocytes in horses with recurrent airway obstruction *Vet. Immunol. Immunopathol.* **104** 91-97

13. Debrue M, Hamilton E, Joubert P, Lajoie-Kadoch S and Lavoie JP 2005 Chronic exacerbation of equine heaves is associated with an increased expression of interleukin-17 mRNA in bronchoalveolar lavage *Vet. Immunol. Immunopathol.* **105** 25-31

14. Padoan E, Ferraresso S, Pegolo S, Castagnaro M, Barnini C and Bargelloni L 2013 Real time RT-PCR analysis of inflammatory mediator expression in recurrent airway obstruction-affected horses *Vet. Immunol. Immunopathol.* **156** 190-199

15. Pirie RS, Dixon PM, Collie DD and McGorum BC 2001 Pulmonary and systemic effects of inhaled endotoxin in control and heaves horses *Equine Vet. J.* **33** 311-318

16. Stringer KA, McKay RT, Karnovsky A, Quémerais B and Lacy P 2016 Metabolomics and its application to acute lung diseases *Front. Immunol.* **7** 44

17. Goldansaz SA, Guo AC, Sajed T, Steele MA, Plastow GS and Wishart DS 2017 Livestock metabolomics and the livestock metabolome: A systematic review. *PLoS ONE* **12**(5) e0177675

18. Foschi C, Laghi L, D'Antuono A, Gaspari V, Zhu C, Dellarosa N, Salvo M and Marangoni A 2018 Urine metabolome in women with Chlamydia trachomatis infection *PLoS ONE* **13**(3) e0194827

19. Laghi L, Zhu C, Campagna G, Rossi G, Bazzano M, Laus F 2018 Probiotic Supplementation in Trained Trotter Horses: Effect on Blood Clinical Pathology Data and Urine Metabolomic Assessed in Field *J. Appl. Phys.* japplphysiol.01131.2017

20. Parolin C, Foschi C, Laghi L, Zhu C, Banzola N, Gaspari V, D'Antuono A, Giordani B, Severgnini M, Consolandi C, Salvo M, Cevenini R, Vitali B and Marangoni A 2018 Insights into vaginal bacterial communities and metabolic profiles of Chlamydia trachomatis infection: positioning between eubiosis and dysbiosis *Front. Microbiol.* **9** 600

21. Ciaramelli C, Fumagalli M, Viglio S, Bardoni AM, Piloni D, Meloni F, Iadorola P and Airoldi C 2017 1H NMR To Evaluate the Metabolome of Bronchoalveolar Lavage Fluid (BALf) in Bronchiolitis Obliterans Syndrome (BOS): Toward the Development of a New Approach for Biomarker Identification *J. Proteome Res.* **16**(4) 1669-1682

22. Carraro S, Giordano G, Reniero F, Carpi D, Stocchero M, Sterk PJ and Baraldi E 2013 Asthma severity in childhood and metabolomic profiling of breath condensate *Allergy* **68** 110-117

23. de Laurentiis G, Paris D, Melck D, Maniscalco M, Marsico S, Corso G, Motta A and Sofia M. 2008 Metabonomic analysis of exhaled breath condensate in adults by nuclear magnetic resonance spectroscopy *Eur. Respir. J.* **32** 1175-1183

24. Dent AG, Sutedja TG and Zimmerman PV 2013 Exhaled breath analysis for lung cancer *J. Thorac. Dis.* **5** S540-S550

25. Maniscalco M, Paris D, Melck DJ, Molino A, Carone M, Ruggeri P, Caramori G and Motta A 2018 Differential diagnosis between newly diagnosed asthma and chronic obstructive pulmonary disease using exhaled breath condensate metabolomics: a pilot study. *Eur. Respir. J.* **8** 51

26. Rossi H, Virtala AM, Raekallio M, Rahkonen E, Rajamäki MM and Mykkänen A 2018 Comparison of Tracheal Wash and Bronchoalveolar Lavage Cytology in 154 Horses With and Without Respiratory Signs in a Referral Hospital Over 2009−2015 *Front. Vet. Sci.* **5** 61

27. Duz M, Whittaker AG, Love S, Parkin TDH and Hughes KJ 2009 Exhaled breath condensate hydrogen peroxide and pH for the assessment of lower airway inflammation in the horse *Res. Vet. Sci.* **87** 307-312

28. Whittaker A G, Love S, Parkin TDH, Duz M, Cathcart M and Hughes KJ 2012 Assessment of the impact of collection temperature and sampler design on the measurement of exhaled breath condensate pH in healthy horses *Vet. J.* **191** 208-212

29. du Preez S, Raidal SL, Doran GS, Nielsen SG, Hughes KJ 2017 The consistency and influence of environmental and a[nimal factors on exhaled breath condensate hydrogen peroxide, pH and leukotriene B4 in horses *Vet. J.* **226** 46-50

30. Couëtil LL, Cardwell JM, Gerber V, Lavoie JP, Léguillette R and Richard EA 2016 Inflammatory Airway Disease of Horses—Revised Consensus Statement *J.Vet. Intern. Med.* **30** 503-515

31. Gerber V, Straub R, Marti E, Hauptman J, Herholz C, King M, Imhof A, Tahon L and Robinson NE 2004 Endoscopic scoring of mucus quantity and quality: observer and horse variance and relationship to inflammation, mucus viscoelasticity and volume *Equine Vet. J.* **36** 576-582

32. Laus F, Attili AR, Cerquetella M, Spaterna A, Tesei B and Cuteri V 2009 Endoscopic findings, microbiological and cytological evaluation of tracheal aspirates in a population of Standardbred horses with poor performances *Vet. Med. (Praha)* **54** 444-50

33. Hodgson JL and Hodgson DR 2007 Collection and analysis of respiratory tract samples. In: McGorum BC, Dixon PM, Robinson NE, Schumacher J (eds) *Equine Respiratory Medicine and Surgery* (Saunders-Elsevier) 19-150

34. Beckonert O, Keun HC, Ebbels TMD, Bundy J, Holmes E, Lindon JC and Nicholson JK 2007 Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts *Nat. Protoc.* **2** 2692-703

35. Kneen MA and Annegarn HJ 1996 Algorithm for fitting XRF, SEM and PIXE X-ray spectra backgrounds *Nucl. Instrum. Methods Phys. Res. B* **109–110** 209–213

36. Liland KH, Almøy T and Mevik BH 2010 Optimal choice of baseline correction for multivariate calibration of spectra *Appl. Spectrosc.* **64** 1007–1016

37. Dieterle F, Ross A, Schlotterbeck G and Senn H 2006 Probabilistic quotient normalization as robust method to account for dilution of complex biological mixtures Application in1H NMR metabonomics. *Anal. Chem.* **78** 4281–4290

38. Hubert M, Rousseeuw PJ and Vanden Branden K 2005 ROBPCA: A New Approach to Robust Principal Component Analysis *Technometrics* **47** 64-79

39. Lugo J, Harkema J R, de Feijter-Rupp H, Bartner L, Boruta D and Robinson NE 2006 Airway inflammation is associate with mucous cell metaplasia and increased intraepithelial stored mucosubstances in horses *Vet. J.* **172** 293-301

40. Barton AK, Shety T, Bondzio A, Einspanier R and Gehlen H 2016 Metalloproteinases and their inhibitors are influenced by inhalative glucocorticoid therapy in combination with environmental dust reduction in equine recurrent airway obstruction *BMC Vet. Res.* **12** 282

41. De Filippis F, Vannini L, La Storia A, Laghi L, Piombino P, Stellato G, Serrazanetti DI, Gozzi G, Turroni S, Ferrocino I, Lazzi C, Di Cagno R, Gobbetti M, Ercolini D 2014 The same microbiota and a potentially discriminant metabolome in the saliva of omnivore, ovo-lacto-vegetarian and vegan individuals *PLoS ONE* **9** e112373

42. Bachert C 2002 The role of histamine in allergic disease: Re-appraisal of its inflammatory potential *Allergy* **57** 287-296

43. McGorum BC, Dixon PM and Halliwell RE 1993 Quantification of histamine in plasma and pulmonary fluids from horses with chronic obstructive pulmonary disease, before and after 'natural (hay and straw) challenges' *Vet. Immunol. Immunopathol.* **36**(3) 223-37

44. Niedzwiedz A and Jaworski Z 2014 Oxidant-Antioxidant Status in the Blood of Horses with Symptomatic Recurrent Airway Obstruction (RAO) *J. Vet. Intern. Med.* **28** 1845–1852
45. Deaton CM 2006 The role of oxidative stress in an equine model of human asthma *Redox Rep.***11** 46–52
46. Raj D, Langford M, Krueger S, Shelton M and Welbourne T 2001 Regulatory responses to an oral D-glutamate load: formation of D-pyrrolidone carboxylic acid in humans *Am. J. Physiol. Endocrinol. Metab.* **280** E214-E220

47. Herrera F, Sainz RM, Mayo JC, Martín V, Antolín I and Rodriguez C 2001 Glutamate induces oxidative stress not mediated by glutamate receptors or cystine transporters: protective effect of melatonin and other antioxidants *J. Pineal Res.* **31** 356-362

48. Barschak AG, Sitta A, Deon M, de Oliveira MH, Haeser A, Dutra-Filho CS, Wajner M and Vargas CR 2006 Evidence that oxidative stress is increased in plasma from patients with maple syrup urine disease *Metab. Brain Dis.* **21** 279-286

49. Ubhi BK, Riley JH, Shaw PA, Lomas DA, Tal-Singer R, MacNee W, Griffin JL, Connor SC 2012 Metabolic profiling detects biomarkers of protein degradation in COPD patients *Eur. Respir. J.* **40**(2) 345-55

50. Simenhoff ML, Burke JF, Saukkonen JJ, Ordinario AT and Doty R 1977 Biochemical profile or uremic breath *New England J. Med.* **297** 132–135

51. Takeda I, Stretch C, Barnaby P, Bhatnager K, Rankin K, Fu H, Weljie A, Jha N and Slupsky C 2009 Understanding the human salivary metabolome *NMR Biomed.* **22**(6) 577–584

52. Billecke SS, D'Alecy LG, Platel R, Whitesall SE, Jamerson KA, Perlman RL and Gadegbeku CA 2009 Blood ontent of asymmetric dimethylarginine: New insights into its dysregulation in renal disease *Nephrol. Dial. Transplant.* **24** 489–496

53. Vallance P, Leone A, Calver A, Collier J andMoncada S 1992 Endogenous dimethylarginine as an inhibitor of nitric oxide synthesis *J. Cardiovasc. Pharmacol.* **20** S60-62

54. Chobanyan K, Mitschke A, Gutzki FM, Stichtenoth DO and Tsikas D 2007 Accurate quantification of dimethylamine (DMA) in human plasma and serum by GC-MS and GC-tandem MS as pentafluorobenzamide derivative in the positive-ion chemical ionization mode *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **851** 240–249

55. Kelly FJ, Mudway I, Blomberg A, Frew A and Sandstrom T 1999 Altered lung antioxidant status in patients with mild asthma *Lancet* **354** 482–483
56. Deaton CM, Marlin DJ, Smith NC, Harris PA, Dagleish MP, Schroter RC and Kelly FJ 2005 Effect of acute airway inflammation on the pulmonary antioxidant status *Exp. Lung Res.* **31** 653–670
57. Ou P and Wolff S.P. 1994 Erythrocyte catalase inactivation (H2O2 production) by ascorbic acid and glucose in the presence of aminotriazole: role of transition metals and relevance to diabetes *Biochem. J.* **303** 935–939
58. Deaton CM, Marlin DJ, Smith NC, Smith KC, Newton RJ, Gower SM, Cade SM, Roberts CA, Harris PA, Schroter RC, Kelly FJ 2004 Breath condensate hydrogen peroxide correlates with both airway cytology and epithelial lining fluid ascorbic acid concentration in the horse *Free Radic. Res.* **38** 201-208.

59. Stewart RM, Weir EK, Montgomery MR and Niewoehner DE 1981 Hydrogen peroxide contracts airway smooth muscle: a possible endogenous mechanism *Respir. Physiol.* **45** 333–342
60. Kirschvink N, Smith N, Fievez L, Bougnet V, Art T, Degand G, Marlin D, Roberts C, Génicot B, Lindsey P and Lekeux P 2002 Effect of chronic airway inflammation and exercise on pulmonary and systemic antioxidant status of healthy and heaves-affected horses. *Equine Vet. J.* **34** 563–571
61. Art T, Kirschvink N, Smith N and Lekeux, P 1999 Indices of oxidative stress in blood and pulmonary epithelium lining fluid in horses suffering from recurrent airway obstruction. *Equine Vet. J.* **31** 397-401

62. Jung J, Kim SH, Lee HS, Choi GS, Jung YS, Ryu DH, Park HS and Hwang GS 2013 Serum metabolomics reveals pathways and biomarkers associated with asthma pathogenesis *Clin. Exp. Allergy.* **43** 425-433

63. Baritussio A 2004 Lung surfactant, asthma, and allergens: a story in evolution A*m. J. Respir. Crit. Care Med.* **169** 550-551

64. González R, Klaassens ES, Mahnen E, De Vos WM, Vaughan EE 2008 Differential transcriptional response of Bifidobacterium longum to human milk, formula milk, and galactooligosaccharide *Appl. Environ. Microbiol.* **74** 4686–4694

65. Nalawade T, Sogi SP and Bhat K 2015 Bactericidal activity of propylene glycol, glycerine, polyethylene glycol 400, a polyethylene glycol 1000 against selected microorganisms *J. Int. Soc. Prev. Community Dent.* **5** 114

66. Thomas PA, Bhat KS and Kotian KM 1980 Antibacterial properties of dilute formocresol and eugenol and propylene glycol *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **49** 166–170

67. Phillips M, Gleeson K, Hughes JMB, Greenberg J, Cataneo RN, Baker L, McVay WP 1999 Volatile organic compounds in breath as markers of lung cancer: a cross-sectional study. *Lancet* **353** 1930-1933

68. van der Schee MP, Hashimoto S, Schuurman AC, van Driel JSR, Adriaens N, van Amelsfoort RM, et al. 2015 Altered exhaled biomarker profiles in children during and after rhinovirus-induced wheeze *Eur. Respir. J.* **45** 440-8

69. Boots AW, van Berkel JJ, Dallinga JW, Smolinska A, Wouters EF and van Schooten FJ 2012 The versatile use of exhaled volatile organic compounds in human health and disease *J. Breath Res.* **6** 027108.

70. van Vliet D, Smolinska A, Jöbsis Q, Rosias P, Muris J, Dallinga J, et al. 2017 Can exhaled volatile organic compounds predict asthma exacerbations in children? *J. Breath Res* **11** [016016]
71. De Laurentiis G, Paris D, Melck D, Montuschi P, Maniscalco M, Bianco A, Sofia M and Motta A 2013 Separating smoking-related diseases using NMR-based metabolomics of exhaled breath condensate *J. Proteome Res.* **12**1502–1511
72. Montuschi P, Paris D, Melck D, Lucidi V, Ciabattoni G, Raia V, Calabrese C, Bush A, Barnes PJ and Motta A 2011 NMR spectroscopy metabolomic profiling of exhaled breath condensate in patients with stable and unstable cystic fibrosis *Thorax* **67** 222-228

73. Miekisch W, Herbig J and Schubert JK 2012 Data interpretation in breath biomarker research: pitfalls and directions *J. Breath Res.* **6** 036007.

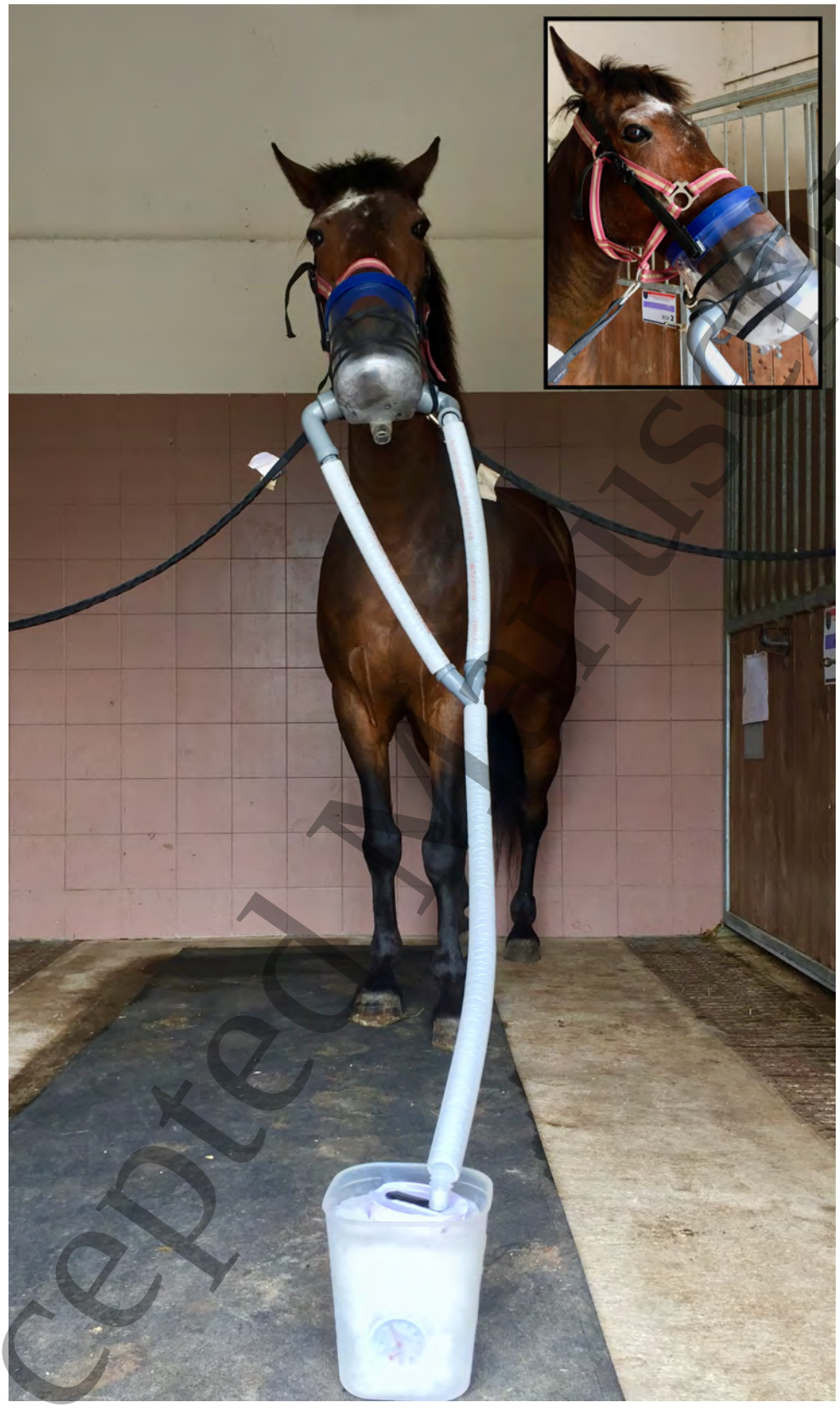

Figure 1. Condensation system used to obtain EBC in horses. The face mask is connected via thermally-insulated tubing to a condensation chamber. In the upper right corner, a detail of the face mask.

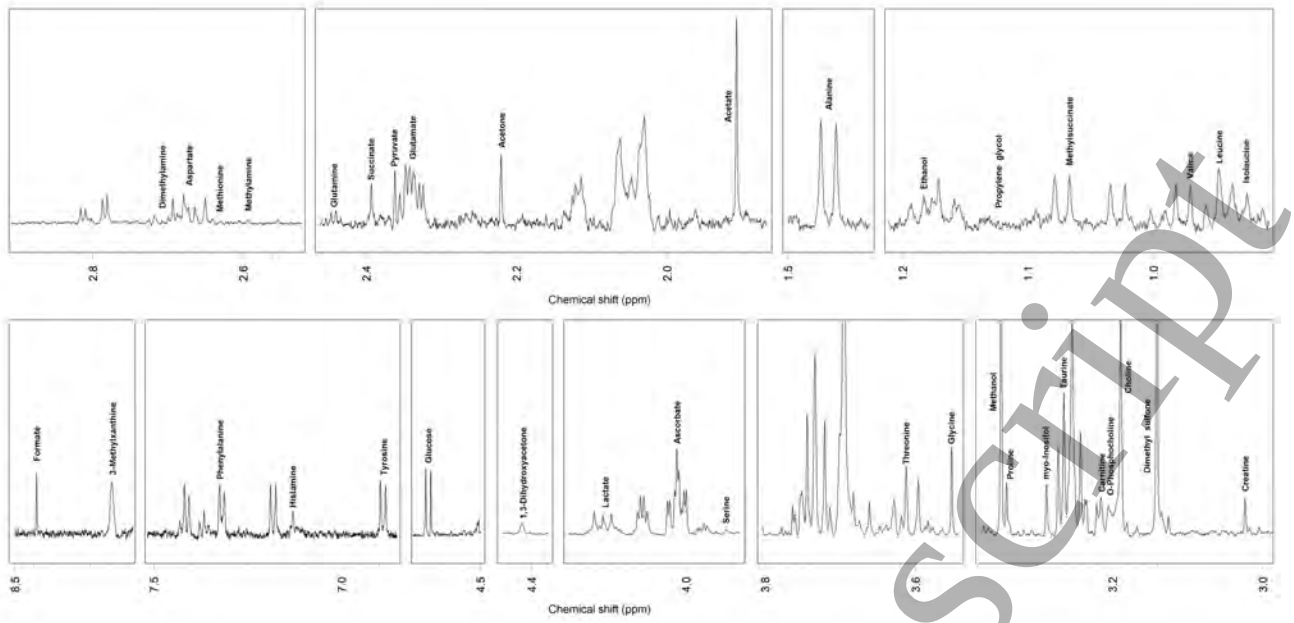

Figure 2 Portions of ^{1}H-NMR spectra from typical TW samples. Assignments appear on the signals used for molecules quantification. The vertical scale of each portion is conveniently set to ease the signals observation.

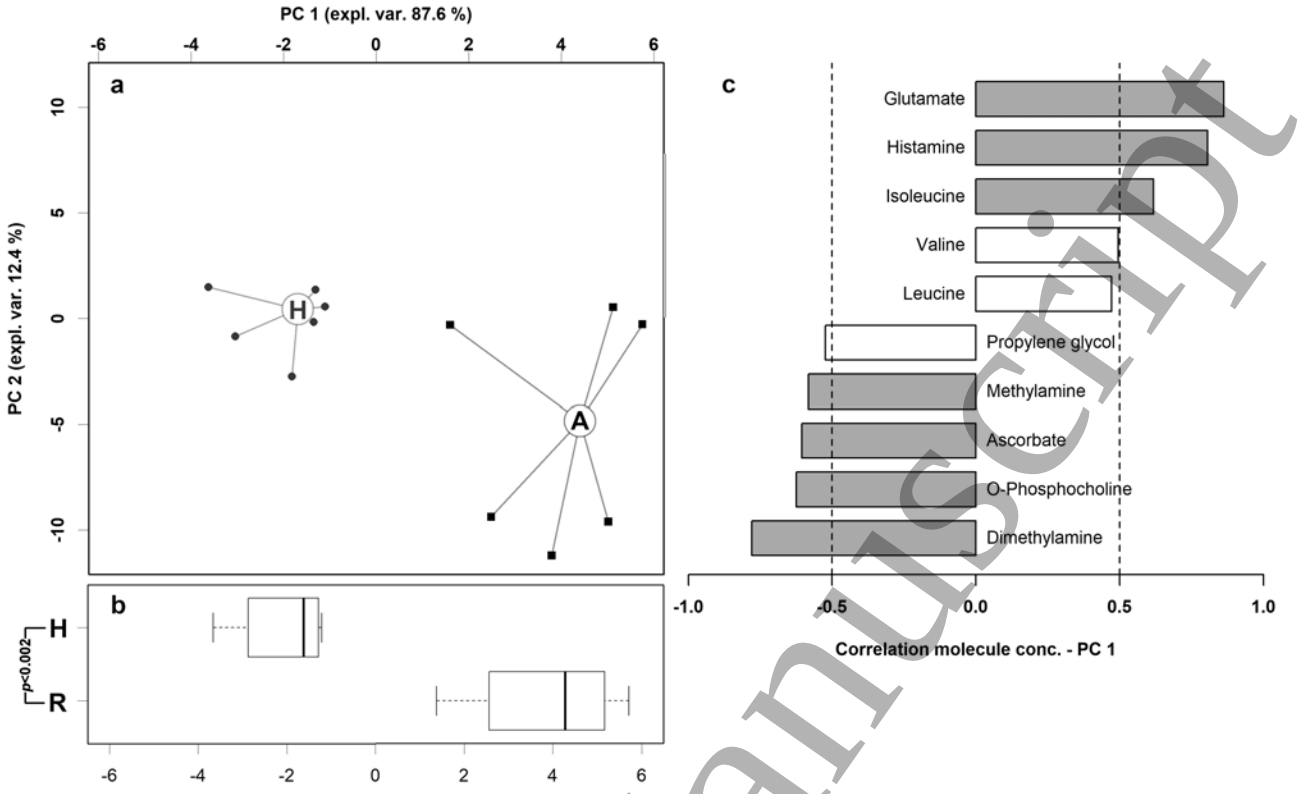

Figure 3. rPCA model built on the space constituted by the concentration of the molecules listed in table 1. In the scoreplot **a**, samples from healthy (H) and asthma affected (A) animals are represented with squares and circles respectively. The wide, empty circles represent the median of the samples. The position of the animals along PC 1 is summarized in boxplot **b**. The loadingplot (**c**) reports the correlation between the concentration of each substance and its importance over PC 1. Significant correlations (p<0.05) are highlighted with gray bars.

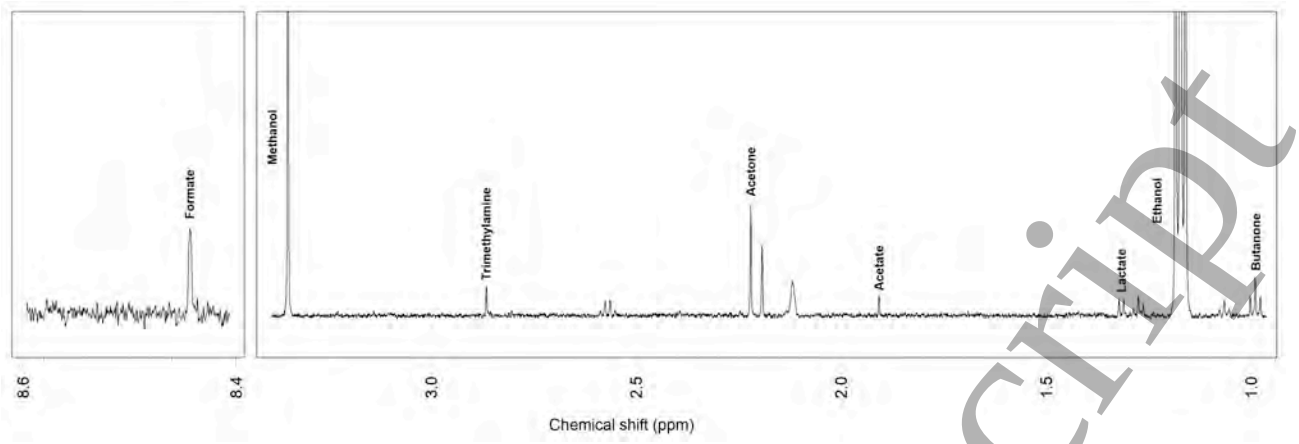

Figure 4. Portions of ^{1}H-NMR spectra from typical EBC samples. Assignments appear on the signals used for molecules quantification. The vertical scale of each portion is conveniently set to ease the signals observation.