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Cytokine mRNA expression in the bronchoalveolar lavage cells from horses affected by different equine asthma subtypes

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ABSTRACT

Equine asthma (EA) is a respiratory syndrome associated with the increase of different leukocyte populations in the bronchoalveolar lavage fluid (BALF). Its pathogenetic mechanisms remain unclear. This study aimed to evaluate the associations between the mRNA expression of different cytokines in the BALF, different EA subtypes and lung function. Fifteen horses underwent physical examination, airway endoscopy, BALF cytology and lung function testing (8/15). One horse did not have evidence of EA and was used as healthy reference, while the others were classified as affected by neutrophilic or mixed granulocytic EA. Cells isolated from the residual BALF were used for IL-1β, IL-2, IFN-γ, IL-4, IL-17A genes expression by quantitative RT-PCR., Cytokine expression was compared between groups, and their correlations with BALF leukocyte and lung function were evaluated. IL-1β expression was positively correlated with BALF neutrophils count (p=0.038, r=0.56) and with increased expiratory resistance (p=0.047, r=0.76). IFN-γ was correlated with BALF mast cells (p=0.029, r=0.58). IL-4 was higher in horses with mixed granulocytic EA than neutrophilic (p=0.008), positively correlated with BALF mast cells (p=0.028, r=0.59) and inversely with whole-breath (p=0.046, r=-0.76) and expiratory reactance (p=0.003, r=-0.93). Finally, IL-17A was inversely correlated with expiratory reactance (p=0.009, r=-0.92). These results support that multiple immune responses are involved in EA pathogenesis; innate, Th2, and Th17 responses. Innate immunity appeared associated with neutrophilic inflammation, and Th2 response with increased mast cells. The role of Th1 response in EA remains questionable.

1. Introduction

Equine asthma (EA) is a non-infectious, inflammatory, recurrent, and chronic condition of horses, clinically characterized by airflow obstruction, mucus hypersecretion and airway hyperreactivity [1]. Based on the severity and recurrence of the condition, it can be classified as mild-moderate (MEA) or severe equine asthma (SEA). Clinical EA severity is reflected by impaired respiratory function. In particular, when measured by forced oscillometry, it is characterized by increased pulmonary resistance and decreased pulmonary reactance, indicating structural obstruction and loss of tissue elasticity [2]. Based on the relatively increased cell populations in the bronchoalveolar lavage fluid (BALF), EA can be classified into different cytological subtypes, including neutrophilic EA, mastocytic EA, eosinophilic EA, and mixed granulocytic EA [3,4]. However, etiopathogenetic, clinical and prognostic differences between different EA subtypes are still loosely defined [5].

In EA-affected horses, clinical signs are exacerbated by the exposure to environmental antigens, determining a hypersensitivity reaction in genetically susceptible patients [1,6,7]. However, pathogenetic mechanisms of EA are not fully understood, and the current literature shows inconsistency regarding the different types of immune responses involved. Indeed, most previous studies reported predominantly a Th2 immune response in asthmatic horses [8–12], while other authors also

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observed the involvement of innate [13,14], Th1 [15], Th17 [16–19], and mixed responses [4,20–22]. The heterogeneity of these findings suggests that multiple molecular endotypes of EA may exist, characterized by different pathogenetic pathways, which may be associated with different EA subtypes or disease stages [21–27]. Recently, it has been hypothesized that, similar to human asthma [28], EA may be distinguished into non-allergic, allergic, and late-onset phenotypes, differing in their pathogenetic mechanisms. Differences may involve the released cytokines, the type of recruited inflammatory cells, the responsiveness or resistance to standard treatments, chronicity features, and the reversibility of airway smooth muscle remodeling [29]. The recognition of different underlying immunological signatures may lead to a more accurate subcategorization of EA, allowing the development of novel therapeutic approaches based on novel immunomodulatory agents [30] or precision medicine [31].

To date, most studies investigating the pathogenetic pathways of EA focused on one or two immune response types and evaluated their associations with EA clinical severity or cytological subtypes. However, to the authors' knowledge, no studies considered the contribution of each immune response type in a single population of EA-affected horses, and no associations with lung function have been investigated. Therefore, in the present study, we decided to select at least one representative cytokine for every immune response type, including IL-1β (innate), IL-2 and IFN-γ (Th1), IL-4 (Th2), and IL-17A (Th17). We hypothesized that different EA subtypes may be associated with the involvement of different cytokines, reflecting different immunological pathways. Hence, the aim of the present study was to evaluate the associations of the mRNA expression of the afore-mentioned cytokines in the BALF of EA-affected horses with the cytological profile and the lung function measurements.

2. Materials and methods

2.1. Ethics statement

Procedures performed on horses were approved by the Animal Welfare Organization of the University of Milan (Protocol Numbers OPBA_122_2021 and OPBA_136_2022), and samples were collected as part of a routine diagnostic protocol for lower airway evaluation. Written informed consent for the use of the data was obtained by all owners or delegated holders.

2.2. Study population

Among a population of equine patients referred to the Equine Unit of the Veterinary Teaching Hospital (University of Milan, Italy) for lower airway investigation or for poor athletic performance evaluation, fifteen client-owned horses were selected. To be included in the study, horses had to undergo at least physical examination, airway endoscopy and collection of the BALF for cytological examination. Exclusion criteria were the presence of signs of systemic illness, a diagnosis of respiratory diseases other than EA, or having received steroidal or non-steroidal anti-inflammatory, immune-modulating or bronchodilating medications during the previous 30 days. Based on the clinical and laboratory findings, horses were considered as asthmatic according to the following criteria: history and/or detection at physical examination of cough, nasal discharge, and/or dyspnea, presence of mucus in the trachea $>$ 1/5, and BALF cytology consisting of neutrophils $>$ 10 % and/or eosinophils $>$ 1 % and/or mast cells $>$ 2 % [32,33]. One horse did not have history of respiratory clinical signs, was normal at physical examination, had no mucus in the trachea, and showed a normal BALF cytological profile: therefore, this horse was used as healthy reference. Asthmatic horses were classified as affected by neutrophilic EA subtype, when only increase in BALF neutrophils percentage was observed, or mixed EA subtype, when increase of more than one leukocyte population in the BALF was detected [34].

2.3. Lung function testing

The severity of EA has been associated with different grades of lung function impairment, due to airflow obstruction, airway remodeling and progressive loss of tissue elasticity [2,35]. Based on this rationale, lung function was tested in 8/15 included horses, due to the unavailability of the necessary equipment during some periods of the study. The test was performed after clinical examination, and before airway endoscopy. During the test, horses were not sedated, and their head was maintained in a neutral position to avoid any interference with spontaneous breathing. A recently developed oscillometry portable device, based on the forced oscillation technique, was used [36]. The measurements were performed by applying at the horse airway opening small amplitude sinusoidal pressure waveforms at frequencies ranging from 2 to 6 Hz, with each frequency being applied for 30 seconds. Lower frequencies reflect the most distal portion of the airways, while higher frequencies the central part of the bronchial tree. Airflow and pressure were sampled and stored on a laptop for subsequent analysis. After data recording, the respiratory input impedance was computed and reported as resistance (Rrs) and reactance (Xrs) by a least squares algorithm using Matlab software framework (Matlab, MathWorks, USA). Briefly, Rrs is defined as the resistance to flow in the airways, which depends on the caliber and architecture of the airways; Xrs, instead, represents their capacitive and inertive properties, and reflects lungs' stiffness and distensibility [37]. Therefore, horses with lower airway inflammation and obstruction commonly show higher values of respiratory Rrs, and lower values of respiratory Xrs [2]. From within-breath impedance tracings, the whole breath, inspiratory and expiratory Rrs and Xrs were calculated. Data were filtered following visual inspection and the values of the flow-shape index, excluding breaths showing the presence of artifacts [36].

2.4. Airway endoscopy and BALF examination

Airway endoscopy and BALF collection were performed as described elsewhere [38]. In brief, after horse sedation with detomidine hydrochloride (0.01 mg/kg IV; Domosedan; Vetoquinol, Italy) and restriction with a twitch, a flexible videoendoscope (EC-530WL-P, Fujifilm, Tokyo, Japan) was passed through the left nasal passages and the upper and lower tracts of the respiratory system were examined. A 0-5 score was assigned to tracheal mucus accumulation by the same operator (L.S.) [39]. Once the tracheal bifurcation was visualized, 60 mL of a 0.5 % lidocaine hydrochloride solution was sprayed to inhibit coughing reflex; then, the endoscope was passed into the right bronchial tree until it was wedged firmly within a segmental bronchus. Here, a 300 mL pre-warmed sterile saline 0.9 % was instilled through a sterile catheter inserted into the biopsy channel of the endoscope, and immediately re-aspirated. The BALF sample was then divided into aliquots: 10 mL were stored in sterile ethylenediaminetetraacetic acid (EDTA) tubes for cytological examination, while the residual BALF was stored in one or more 50 mL sterile plain tubes for cytokine expression analysis. For the cytological examination, within 90 min from the BALF collection, 300 μL of pooled BALF were cytocentrifugated (Rotofix 32, Hettich Cyto System, Germany) at 25 *g* for 5 min. The slides were air-dried, stained with May-Grünwald Giemsa and observed under a light microscope at 400x and 1000x for 400-cell leukocyte differential count [40].

2.5. Cells isolation

To allow cytokine expression analysis, cells were isolated from the residual BALF within 1 h from collection. Samples were centrifugated at 250 *g* for 10 min at 20°C, supernatants were removed and cells were re-suspended in 2 mL Dulbecco modified Eagle's medium (DMEM) high glucose supplemented with 2 mM L-glutamine, 0.1 mg/mL streptomycin, 100 IU/mL penicillin, and 5 μg/mL gentamicin, and counted using a Burker chamber with the Trypan Blue dye exclusion assay. A

suspension of 2 million cells was then transferred into a sterile plain 2 mL tube, centrifugated at 250 *g* for 10 min at 20°C and the pellet was re-suspended in a 350 µL lysis buffer included in a commercial kit for RNA isolation (RNeasy Mini Kit, (Qiagen, Hilden, Germany) and supplemented with 1/100 vol of β-mercaptoethanol. The obtained suspension was stored in a -80°C freezer for subsequent analysis.

2.6. RNA extraction and cDNA synthesis

Total RNA was extracted from each sample using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and eluted in a final volume of 30 µl of Rnase-free water. In order to remove any genomic DNA contamination, an on-column DNase enzyme treatment was performed according to the manufacturer's instructions. Five hundred nanograms of RNA were retro-transcribed to cDNA using the Quantitect Reverse Transcription Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. An additional reaction without retrotranscriptase enzyme was performed to verify the complete genomic DNA removal. Finally, cDNAs were stored at -80°C until use.

2.7. Target genes selection and gene expression profile

Genes involved in immune response (IL-1β, IL-4, IL-2, IFN-γ, and IL-17A) were selected for subsequent molecular analyses, based on previous literature. In particular, for each immune response type previously implicated in the pathogenesis of EA, one or two specific cytokines were chosen as representatives: IL-1β for innate immunity [4,12,20,23,26], IL-4 for Th2 type immunity [4,8–10,20,21], IL-2 and IFN-γ for Th1 type immunity [4,15,20,21,24,33], and IL-17A for Th17 type immunity [12,16,18,27,29]. The β-actin gene was used as reference gene as described by previous studies [14,20,33,41,42]. The primer sequences and the amplification size of each fragment are described in Table 1.

Gene expression was evaluated by quantitative PCR (qPCR) using an iQ5 Real-Time PCR instrument (Bio-Rad, California, USA) and Universal SYBR Green Supermix (Bio-Rad, California, USA) as fluorescent molecule. The amplification conditions were: 150 nM (final concentration) of forward and reverse primers for β-actin gene fragment; 250 nM (final concentration) of forward and reverse primers for the other analysed genes. Annealing temperatures were 60°C for β-actin, IL-1β and IL-4 and 58°C for IL-2, INF-γ, and IL-17A fragment genes respectively. A melting profile was also included. Cycle threshold (Ct) values were determined for each sample and normalized using β-actin as reference. The relative gene expressions of samples collected from EA horses were calculated using the ΔΔCt method and were compared to the healthy sample, considered as calibrator.

Table 1
Primers sequences, amplification size and literature.

Target gene	Sequence (5′→ 3′)	Amplification fragment size (bp)	Refs.
IL-1β	F: TGGCAGAGGGGAATAGAAGGGTTTG R: ATAGGGAAGGCAGCTGGGCATTGA	86	[13]
IL-4	F: TCGTGCATGGAGCTGACTGTA R: GCCCTGCAGATTTCCTTTCC	75	[43]
IL-2	F: TGCATCGCACTAACTCTTGC R: CAATTCTGTGGCCTTCTTGG	195	[44]
INF-γ	F: TGGACACCATCAAGGAGGAC R: GGACCTTCAGATCATTTACCG	108	[44]
IL-17A	F: GGCCTCAGATTACCACAACC R: ATCTCTCAGGGTCCTCGTTG	68	[23]
β-actin	F: CAAGGCCAACCGCGAGAAGATGAC R: GCCAGAGGCGTACAGGGACAGCA	103	[13]

2.8. Statistical analysis

Data were collected on an electronic sheet (Microsoft Excel, Redmond, WA, USA) and analyzed using two commercial statistical software packages (GraphPad Prism 10.0.0 for MacOs, GraphPad Software, San Diego, CA; JASP 0.17.2.1 Intel, University of Amsterdam, The Netherlands). Data normality was assessed by Shapiro-Wilk test, and descriptive statistics were performed. Normally distributed data are presented as mean ± standard deviation, while non-normally distributed data are reported as median and interquartile range (IQR). As the distribution of the data of BALF cell percentages and cytokine mRNA expressions was non-normal, non-parametric tests were used. The percentages of different leukocyte populations and cytokine mRNA expressions in the BALF were compared between horses with neutrophilic and mixed EA by Mann-Whitney test. Then, the associations between cytokine mRNA expressions and BALF leukocyte populations were evaluated by Spearman's partial correlations, conditioned on the variable "age". The same test was used to assess the associations between cytokine mRNA expressions and lung function measures, including total, inspiratory, and expiratory Rrs and Xrs at each frequency. To exclude the possible role of sex as confounding variable, cytokine expressions were compared between horses of different sex by Kruskal-Wallis test. Significance was set at $p < 0.05$. Correlation was considered as negligible if the Spearman *r* coefficient was 0.00 - 0.10, weak if 0.10-0.39, moderate if 0.40 - 0.69, strong if 0.70 - 0.89 and very strong if 0.90 - 1.00 [45].

3. Results

3.1. Horses

Fifteen horses were enrolled in the present study, including two mares, seven geldings and six stallions, aged between 2 and 20 years old (median 5, IQR 2-12 years old). The population included eight Thoroughbreds, three Warmbloods, two ponies, an Arabian, and a Quarter Horse. Based on inclusion criteria, six horses showed neutrophilic EA, eight showed mixed EA, and one horse was considered as healthy control.

3.2. Lung function

Lung function was tested by oscillometry in eight out of 5 horses (one healthy horse, four affected by mixed asthma, and three by neutrophilic asthma). Mean results and SDs are displayed in Table 2.

3.3. Airway endoscopy and BALF cytology

Tracheal mucus accumulation scores and results of BALF cytology in the whole population and within different EA subtype groups are shown

Table 2
Oscillometry results in 8 horses (1 healthy horse, 4 affected by mixed asthma, and 3 by neutrophilic asthma) from the study population. Data are presented as mean ± standard deviation.

	Whole breath	Inspiratory	Expiratory
Resistance ($cmH_2O \cdot s/L$)			
2 Hz	0.528 ± 0.229	0.445 ± 0.137	0.497 ± 0.255
3 Hz	0.613 ± 0.271	0.514 ± 0.204	0.680 ± 0.343
4 Hz	0.677 ± 0.248	0.546 ± 0.154	0.753 ± 0.359
5 Hz	0.747 ± 0.290	0.593 ± 0.154	0.866 ± 0.483
6 Hz	0.746 ± 0.255	0.599 ± 0.236	0.805 ± 0.349
Reactance ($cmH_2O \cdot s/L$)			
2 Hz	-0.048 ± 0.067	-0.050 ± 0.099	-0.074 ± 0.067
3 Hz	0.013 ± 0.055	-0.003 ± 0.053	0.005 ± 0.082
4 Hz	-0.006 ± 0.067	-0.013 ± 0.033	-0.057 ± 0.156
5 Hz	0.011 ± 0.073	0.009 ± 0.040	-0.011 ± 0.061
6 Hz	-0.033 ± 0.079	0.019 ± 0.030	-0.045 ± 0.040

in Table 3.

3.4. BALF cytokine mRNA expression

The median (IQR) BALF relative mRNA expression of IL-1β in EA-affected horses was 4.65 (1.29 – 11.19), IL-2 was 2.82 (0.59 – 6.90), IFN-γ was 2.70 (1.35 – 10.27), IL-4 was 20.41 (10.55 – 78.96), and IL-17A was 3.57 (2.27 – 25.71). Cytokines mRNA expressions did not differ between horses of different sex. Results in horses affected by neutrophilic or mixed asthma subtypes are displayed in Fig. 1. In horses with mixed EA, the mRNA expression of IL-4 was significantly higher compared to horses with neutrophilic EA ($p = 0.008$). The expression of the other cytokines did not differ between EA subtypes.

A positive moderate correlation was observed between the percentage of neutrophils in the BALF and the mRNA expression of IL-1β ($p = 0.038$, $r = 0.56$). The percentage of mast cells in the BALF moderately correlated with the mRNA expression of IL-4 ($p = 0.028$, $r = 0.59$) and IFN-γ ($p = 0.029$, $r = 0.58$). A trend of moderate correlation was also observed between the BALF eosinophils percentage and the mRNA expression of IL-17A ($p = 0.058$, $r = 0.54$).

Concerning the correlations between lung function results and cytokine mRNA expressions, the mRNA expression of IL-1β strongly correlated with increased expiratory Rrs at 5 Hz ($p = 0.047$, $r = 0.76$). The mRNA expression of IL-4 inversely correlated strongly with the whole-breath Xrs at 5 Hz ($p = 0.046$, $r = -0.76$), and very strongly with the expiratory Xrs at 5 Hz ($p = 0.003$, $r = -0.93$). Finally, the mRNA expression of IL-17A inversely correlated very strongly with the expiratory Xrs at 5 Hz ($p = 0.009$, $r = -0.92$). The mRNA expressions of IL-2 and IFN-γ were not associated with lung function.

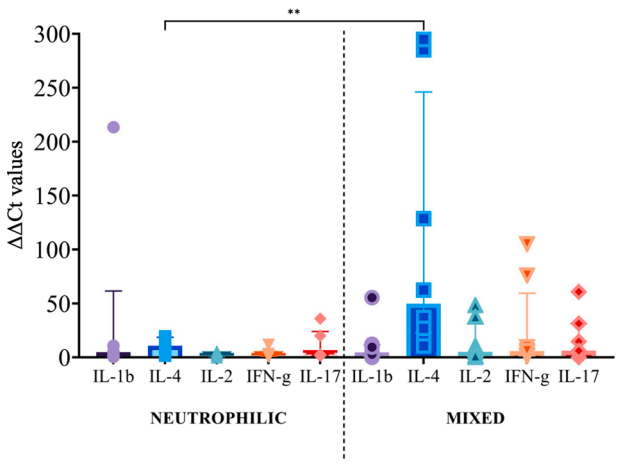

Fig. 1. Aligned dot plot showing individual values, medians (bars) and IQRs (fine lines with serifs) of the relative mRNA expression of IL-1β, IL-2, IFN-γ, IL-4, and IL-17A in horses affected by neutrophilic or mixed granulocytic equine asthma. The values on the y-axis represent the relative mRNA expression of specific cytokines, normalized using the ΔΔCt method, based on the reference gene (β-actin) and the reference healthy subject. Statistical significance is shown as ** ($p < 0.01$).

4. Discussion

Multiple immunologic pathways have been implicated in equine asthma, and different immune response types may reflect different EA stages or subtypes. In the present study, the expression of cytokines reflecting innate, Th2 and Th17 immune response types (IL-1β, IL-4, IL-17A) appeared to be associated with different cytological and functional subtypes of EA, supporting the hypothesis that a mixed inflammatory/immune response was involved. Conversely, the role of Th1 response seemed questionable. However, the results of the present study are still preliminary, and further work is needed to confirm our findings and explore the meaning of the identified associations.

In the literature, the immune response type most implicated in the pathogenesis of EA is the Th2 response. Indeed, many authors reported its pivotal role in SEA [1,8,10] or MEA [12], while others did not observe any increase in the Th2 cytokine expression in asthmatic horses [14,15,46]. Therefore, it has been hypothesized that Th2 response may play a role in EA only in specific disease stages, but the studies conducted to test this hypothesis lead to contrasting findings [10,21]. In our study, we evaluated the expression of the Th2 cytokine IL-4. Previous studies reported an increase of IL-4 expression in horses affected by SEA [8–10,20,21,47] and MEA [4]; however, others did not detect any difference between healthy and asthmatic horses [14,15,24]. In the present study, the expression of IL-4 was higher in horses with the mixed granulocytic EA subtype compared to the neutrophilic subtype, and correlated, although only moderately, with increased BALF mast cell percentages, similarly to the findings of previous studies [8,12]. Moreover, IL-4 expression strongly correlated with decreased pulmonary reactance, especially during the expiratory phase of the breath. This finding either demonstrates an association with expiratory flow limitation related to bronchospasm [48], or with the reduction of pulmonary elasticity, typically associated with progressive airway remodeling [7]. Therefore, our results suggest that asthmatic horses with a relevant Th2 response may be more prone to airway hyperresponsiveness associated with BALF mastocytosis and, possibly, to structural remodeling, similar to human asthma [49].

Pro-inflammatory Th1 response has also been widely investigated in the pathogenesis of EA. Some authors reported its implication in MEA [24], in the acute and chronic phases of SEA, and in airway remodeling [21], while others did not find any role of Th1 response in EA pathogenesis [12,14,46]. In the present study, the selected cytokines reflecting Th1 response were IL-2 and IFN-γ. Their expressions did not differ between horses affected by different cytological subtypes of EA, nor were associated with lung function. Only a moderate correlation was observed between IFN-γ and mast cells percentages, analogously to a previous report [4]. In previous studies, IL-2 was either similar in healthy and MEA-affected horses [14] or correlated with BALF neutrophils count [33]. Similarly, contrasting findings were observed concerning IFN-γ expression in EA: some studies reported an increase in SEA [15,20,21,33,47], in MEA [4,24], and in neutrophilic EA [12,21,24,33], while others detected no associations with EA [10,14] or even a decrease in SEA [8]. The results of our study suggest that Th1 response may not play a primary role in lower airway inflammation, nor in lung

Table 3
Tracheal mucus accumulation (TM) scores and results of BALF cytology in the whole study population, and in different equine asthma (EA) subtype groups (neutrophilic EA and mixed EA). Data are presented as median (IQR). Statistically significant differences between groups, based on the Mann-Whitney test, are displayed as ** ($p < 0.01$).

	Whole population (n= 15)	Neutrophilic EA (n=6)	Mixed EA (n=8)
TM score	2 (2 – 4)	2 (2 – 4)	3 (2 – 3)
% Macrophages	44 (40 – 51)	45 (41 – 52)	42 (39 – 50)
% Lymphocytes	33 (25 – 35)	33 (27 – 33)	32 (27 – 34)
% Neutrophils	19 (15 – 23)	20 (18 – 23)	19 (17 – 22)
% Eosinophils	1 (0 – 1)	1 (0 – 1)	1 (1 – 1)
% Mast cells	2 (1 – 3)	2 (0 – 2)**	3 (3 – 4)**

dysfunction. Indeed, the only correlation observed was not strong, and its clinical meaning may be considered cautiously due to the small size of the study population.

Airway hyperreactivity has also been associated with Th17 response [16,27]. In the present study, the expression of IL-17A did not differ between horses affected by different cytological subtypes of EA; only a trend of positive moderate correlation was observed with the BALF eosinophil percentage. This finding is supported by previous literature, reporting its involvement in abnormal immune responses and airway hyperresponsiveness [16], typically associated with BALF eosinophilia [24]. However, previous studies reported an association between IL-17A increased expression and neutrophilic lower airway inflammation [12], both in horses with SEA [16–19,50] and MEA [29]; conversely, other authors did not observe IL-17A increase in MEA-affected horses [27]. Some studies also supported a role of Th17 in EA chronicity [1] and, in humans and mice, it leads to mucous cell metaplasia and airway remodeling [51,52]. According to the results of the present study, this phenomenon may also occur in EA as IL-17A correlated very strongly with decreased reactance, suggesting that it may contribute substantially to lung tissue elasticity loss. However, it remains to be investigated whether the increase of IL-17A contributes to the progression of lung remodeling or they are both consequences of chronic lower airway inflammation.

Pulmonary innate immunity also appears to be involved in the pathogenesis of EA [15,53,54]. The increased expression of pro-inflammatory cytokines was reported in the BALF of SEA-affected horses in several studies [4,13,15,20,23,53–55], while contrasting results were found in MEA-affected horses [4,12,14,24,26,27]. In the present study, the expression of IL-1β positively correlated with the percentage of neutrophils in the BALF, confirming the findings of previous reports [4,12,20,23,26,27,42,55]. Interestingly, IL-1β was the only cytokine correlated with increased expiratory resistance: as resistance depends on airway caliber and architecture, its increase may be associated with bronchoconstriction and/or structural changes. Moreover, EA mainly affects the expiratory phase of the breath [56], which can further explain the correlations detected by this study.

Overall, the results of the present study agree with some previous reports and are in contrast with others. The inconsistency of the literature on EA immunopathology is probably due to the subcategorization of EA based on its severity rather than on different disease types [33]. Indeed, horses sharing the same diagnosis may differ in their underlying pathogenetic mechanisms: for example, clinical signs may arise at a young age (early-onset) or as an adult/elderly (late-onset), may differ in the allergic or non-allergic phenotype, and may be associated or not with other atopic disorders [26,57]. It has been demonstrated that BALF cytokine expression varies with time and is influenced by avoidance or exposure to allergens [10,20,21,42], making it difficult to standardize and compare results obtained by different studies. For this reason, to allow EA immunophenotyping, studies should be performed at several time points and be based on more sensitive and complete approaches, such as genome-wide transcriptomics. In the present study, only a limited spectrum of genes was considered, due to budget limitations. Therefore, we decided to select one or two cytokines as representatives of each immune response type previously implicated in EA. Therefore, generalizations of our findings should be avoided, and interpretations should be considered cautiously. Cytokine expression can be influenced by another important factor, which is the wide range of age [58,59] of EA-affected horses; in the present study, horses between 2 and 20 years old were included, which may have biased the conducted analyses. However, to overcome this limitation, we have considered age as a confounding variable in the correlation analyses between cytokine expressions, BALF cytology and lung function. More numerous and better age-grouped populations are needed to confirm our findings.

Some other limitations deserve to be discussed in the present study. Normalization of mRNA cytokine expression was based on one single reference gene, and a single control horse. The reference gene used in this study was β-actin, traditionally utilized in similar equine studies [13,14,20,33,41,42]; however, in one study, low stability of β-actin in MEA-affected horses treated by corticosteroids was reported, suggesting the use of more suitable reference genes [12]. Nevertheless, in the present study, recent pharmacological treatment represented an exclusion criterion, eluding possible biases. It should be mentioned that, to inhibit the cough reflex during endoscopy and BALF collection, a 0.5 % lidocaine solution was sprayed at the level of the bronchial bifurcation. A previous study confirmed the efficacy and importance of this procedure, but also showed that it may lead to an increase of BALF neutrophil percentages [60]. Its influence on mRNA cytokine expression cannot be excluded, but it was presumably equal among all horses.

The lack of a control group of healthy horses remains the main limitation of the study: unfortunately, due to ethical issues, it was not possible to collect BALF from horses without the clinical indication for performing it, as it represents a relatively invasive procedure. The only healthy horse, used as reference patient in our study, underwent BALF collection as part of a poor athletic performance protocol to exclude pulmonary disorders; due to the absence of history of respiratory signs throughout the horse's life, the normal findings at clinical examination, and the within-ranges BALF cytology, we were confident that the selected horse was healthy. Due to the lack of a control group, the present study could not compare EA-affected and healthy horses, but rather focused on the evaluation of cytokine expression differences between various EA subtypes, and their associations with different inflammatory cells and lung function impairment. Since lung function was evaluated only in 8 horses, due to the lack of availability of the oscillometry device during some periods of the study, the reported correlations should be interpreted cautiously. However, despite the limited population, the novelty of these preliminary results deserves to be highlighted. Indeed, this is the first study reporting the associations between cytokine expressions and lung function in horses; in order to understand whether the detected correlations are due to bronchoconstriction or chronic pulmonary remodeling, further studies should be conducted including endobronchial ultrasound and histomorphometric analysis of endobronchial biopsies.

5. Conclusions

The results of the present study support the hypothesis that multiple immune response types are involved in EA. While innate immune response seems to be associated with airway neutrophilic inflammation, Th2 type response seems to be involved in the increase of airway mast cells. Moreover, lung function impairment may correlate with different immune response types: the involvement of innate response seems to be associated with increased airway resistance, reflecting a reduction of airway caliber, while Th2 and Th17 responses may play a role in reactance decrease, either due to acute bronchospasm or progressive loss of lung tissues elasticity. Due to the several limitations of the present study, further research is needed to confirm or confute our findings. Moreover, the possible causative relationships between the increase of cytokine expressions, airway inflammation and lung dysfunction still need to be verified.

Data statement

The data presented in this study are available upon request from the corresponding author.

CRediT authorship contribution statement

Chiara Maria Lo Feudo: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Luca Stucchi:** Writing – review & editing, Supervision, Investigation. **Chiara Bazzocchi:** Writing – review & editing, Validation, Methodology, Investigation, Data curation. **Anna Lange**

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Declaration of competing interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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