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Abstract

Background: The severe form of equine asthma is associated with pathological changes of the peripheral airways and pulmonary parenchyma that are only partly described. Also, the relationship between these structural alterations and the percentage of neutrophils found within the airway lumen, assessed by bronchoalveolar lavage fluid (BALF) cytology, remains ill-defined.

Objective: To examine the histological lesions associated with equine asthma during disease exacerbation and remission, and their relationship with lung function and BALF neutrophilia.

Study design: Observational retrospective study.

Methods: Peripheral lung tissues, BALF cytology, and lung function data from 61 horses (22 controls, 24 asthma exacerbations, and 15 asthma remission) were obtained from an equine pulmonary tissue bank. Two pathologists semi-quantitatively assessed histologic features, including airway wall inflammation, interstitial fibrosis, mucus cell hyperplasia, mucostasis, peribronchiolar metaplasia, presence of granuloma, and the overall severity of these lesions.

Results: Mucostasis, mucus cell hyperplasia, peribronchiolar metaplasia, and interstitial fibrosis were associated with the disease exacerbation ($p<0.05$), and these changes were all attenuated during remission. Airway wall inflammation was greater in horses with asthma in exacerbation compared to horses with asthma remission and control horses ($p<0.05$). Acute (neutrophilic) airway wall inflammation was more frequently detected in asthmatic cases compared to control horses ($p<0.0001$) and was associated with BALF neutrophilia >5% in control horses ($p=0.002$). The degree of bronchiolar inflammation was higher in asthmatic horses in remission stabled and treated pharmacologically compared to those kept on pasture ($p=0.04$).

Main Limitations: Samples obtained from a convenient cohort of horses was studied.

Conclusions: Severely asthmatic horses present parenchymal and peribronchial/peribronchiolar lesions possibly contributing to the obstructive nature of the disease.

5113 words

Introduction

Severe equine asthma (also known as heaves or recurrent airway obstruction) is a chronic obstructive disease characterized by exaggerated contraction, inflammation, and structural alterations of the airways, when susceptible horses are stabled and fed hay. Antigen-induced inflammation of the airways is believed to be responsible for the development of the airway remodeling and associated airway obstruction [1]. The peripheral airways (those < 2mm in diameter) are the most important site of remodeling in severe equine asthma [2-4]. However, the inflammatory cell types present in these small asthmatic airways are not well described. Pulmonary inflammation in general is commonly assessed in the equine species by means of bronchoalveolar lavage fluid (BALF) cytology [5], which samples the lumens of lumens of intermediate and peripheral airways and the alveoli. There is little evidence supporting that BALF cytology correlates with interstitial or peripheral airway wall inflammation and remodeling in horses [2]. Severe equine asthma is characterized by marked BALF neutrophilia (>20-25%) during episodes of exacerbation, in association with increased lung resistance and elastance [6], and increased mucus production or secretion [7]. However, there is no correlation between BALF neutrophilia and lung function [8]. The relationship between BALF neutrophilia and peripheral airway wall pathology is ill-defined, mainly because of the inaccessibility of these airways preventing their assessment in clinical cases. To date, peripheral airway pathology can be evaluated only by means of pulmonary biopsy in living animals (restricted to research purposes), or at necropsy. Identifying a relationship between peripheral airway pathology and BALF cytology or lung function would allow a non-invasive estimation of the processes occurring in the peripheral airways and alveoli or interstitium of asthmatic and healthy horses. Furthermore, it may clarify the prognostic value of the degree of BALF neutrophilia in equine asthma.

Peripheral airway remodeling is a hallmark of severe equine asthma. Early studies on peripheral lung biopsies obtained post-mortem or by thoracoscopy described the alterations occurring in the

submucosa of small peripheral airways [2; 9], and more recent reports have provided histomorphometric evidence showing that structural differences exist at this level. These studies have shown airway smooth muscle, collagen and elastic fiber deposition within the lamina propria in the asthmatic bronchioles when compared to the healthy ones [3; 4; 10]. These lesions are only partially reversible even when prolonged anti-asthma therapy is implemented [11]. Less is known about the histological alterations sustained by peribronchiolar tissues (connective tissue outside the smooth muscle layer), interstitium and alveolar walls of asthmatic horses and their possible reversibility. Moreover, their contribution to airflow obstruction remains ill-defined.

In the present study, we performed a comprehensive histologic evaluation of remodeling and inflammation in peripheral lung tissues of severe asthmatic horses, including samples obtained during exacerbation and remission of the disease, and controls. A subgroup of control horses with >5% BALF neutrophilia but without clinical signs suggestive of lung disease was also studied. We sought to determine the histological lesions associated with equine asthma and whether they differ in horses experiencing exacerbation of the disease when compared to horses in remission of the disease. We studied histological lesions in horses where disease remission had been induced by antigen avoidance strategies or corticosteroids. Finally, the relationship between the histological lesions observed and the BALF cytology and lung function were also studied.

Materials and Methods

Animals

Lung tissues were obtained from an equine pulmonary tissue bank (http://btre.ca). Horses had been euthanized due to the severity of the disease or concurrent medical problems unrelated to the lungs. Horses included into the bank underwent lung function and BAL before euthanasia. However, for samples collected before 2005, BALF cytology data were not collected pre-mortem and only

historical values were available. Due to the difficulty to obtain pulmonary lung tissue from well-characterized horses for research purposes we decided to include these subjects in our study. Inclusion criteria for each animal were the availability of a detailed history, pre-mortem lung function data (pulmonary resistance, $R_{L,}$ and pulmonary elastance, E_L), historical or pre-mortem bronchoalveolar lavage fluid (BALF) cytology results, and at least 5 histological samples corresponding to the 5 regions of the lung identified in **Fig 1**. Controls were included if they have 1) no history of recurrent respiratory distress or systemic or respiratory disorders at the moment of euthanasia or in the past 6 months, 2) pre-mortem or a history of normal eosinophil (<1%) and mast cell (<2%) count at BALF cytology, and 3) a normal lung function (R_L<1 $cmH_2O/L/s$, and E_L<1 cmH_2O/L) at pre-mortem examination. Horses with increased neutrophilia (>5%) at BALF cytology, but otherwise fulfilling the criteria outline above, were included as controls, in as it has previously shown that exposure to hay dust can induce temporary neutrophilia in otherwise healthy animals [10; 12]. Whether control horses had past episodes of respiratory disorders could not be ascertained in all cases (previous owners were unknown in some cases). Horses were classified as severe asthmatics if they had a documented history of 1) repeated and reversible episodes of labored breathing at rest in absence of signs of systemic illness, 2) altered lung function (R_L≥1 $cmH_2O/L/s$, and E_L≥1 cmH_2O/L) and 3) >5% neutrophils at BALF cytology. The status of clinical exacerbation vs. remission at the moment of euthanasia of severely asthmatic horses was defined based on the treatment history and lung function measured pre-mortem (1-7 days before, mean ± S.D.: 2±1 days). Severe asthmatic horses in exacerbation had been stabled and fed hay for 4 weeks or more in absence of treatment and presented increased R_L and E_L. Horses in remission were either kept at pasture for >4 weeks or treated with corticosteroids alone or combined with bronchodilators before euthanasia for at least 2 weeks with normalization of R_L and/or E_L (at least one parameter within normal limits). Exclusion criteria for all horses were the administration of any antimicrobial or antinflammatory drug during the week preceding the euthanasia (except for inhaled/oral corticosteroids for the asthma remission group).

Histology

Lung samples were fixed in 10% neutral-buffered formalin for 48-72 hours before paraffin embedding. Five µm sections were cut and stained with HEPS (hematoxylin-eosin-phloxine-saffron). Experienced veterinary (PH) and human thoracic pathologists (PJ) assessed the following parameters independently: lesion distribution patterns (bronchiolocentric, subpleural, paraseptal, or diffuse), overall severity (0: absent; 1: mild; 2: moderate; 3: severe), eosinophilia (0: no cell; 1: rare cells; 2: few cells; 3: multiple cells), presence of granuloma (present/absent), mucostasis (present/absent), mucus cell hyperplasia (present/absent), peribronchiolar metaplasia (present/absent), interstitial fibrosis (present/absent), and distribution of interstitial fibrosis (bronchiolocentric, diffuse, mixed). Type and severity of bronchial and bronchiolar inflammation were also assessed. The type of inflammation was assessed as: acute, when inflammation was overwhelmingly neutrophilic (and luminal); chronic, when inflammation was overwhelmingly lymphoplasmacytic (and parietal); and mixed, when both types were significantly present. Inflammation was graded using a semi-quantitative scoring system that was based on the subjective assessment of the average degree of leukocytic infiltration and the proportion of affected airways. All slides were read a first time to assess the range of inflammation intensity and establish the number of score categories, and then a second time to grade each case. For individual cases, inflammation was graded as: 0 = absent; 1 = mild, when only a few scattered leukocytes were present multifocally in the wall (lymphocytes and plasma cells) and/or the lumen (neutrophils); 2 = moderate, when a few to several lymphocytes and plasma cells were present circumferentially in the wall and/or neutrophils formed conspicuous aggregates in the lumen; and 3 = severe when numerous lymphocytes and plasma cells were present circumferentially in the wall and/or neutrophils variably filled the lumen. Then, slides from half the cases were randomly selected and re-evaluated to insure repeatability. All 5 sections of the same horse were analyzed together (i.e. the

156 pathologists knew these samples belonged to the same horse). Both pathologists were blinded to the
157 clinical diagnosis of the horses.

158

159 *Data analysis*

160 Statistical analysis was performed using Prism 6 software (GraphPad Inc., La Jolla, CA, USA) and
161 GraphPad QuickCalcs (https://graphpad.com/quickcalcs/kappa1/). Inter-observer agreement was
162 evaluated using Kappa Cohen's test. The results of the pathologist with more experience in the
163 assessment of veterinary samples (PH) were used for subsequent analysis. One-way ANOVA and
164 Tukey's post-tests were used for comparing continuous variables (age, lung function parameters,
165 BAL neutrophilia) between the 3 groups. The mean values of ordinal variables (overall severity,
166 eosinophilia, bronchial and bronchiolar inflammation, severity of interstitial fibrosis) in the 3
167 groups were compared with Kruskal-Wallis tests with Dunn's post-tests. Chi squared tests were
168 used for comparing the distribution, expressed as percentages of nominal (type of
169 bronchial/bronchiolar inflammation) or binomial variables (mucostasis,
170 peribronchial/peribronchiolar metaplasia, mucous cell hyperplasia, interstitial fibrosis, granulomas).
171 Mann-Whitney U-test was used for comparing control horses with BALF neutrophilia ≥ or <5%
172 and the treatments to induce disease remission (antigen avoidance vs. pharmacological treatment).
173 Student's t-test was employed to evaluate whether severity of peripheral lung lesions (overall
174 severity ≤1 vs. >1) or the type of peripheral lung inflammatory infiltrate (chronic vs. mixed)
175 significantly affected lung function and BALF cytology. BALF cytology results were correlated
176 using the Spearman or Pearson test with Bonferroni correction for multiple comparisons for each
177 group of horses, depending on data distribution. Horses lacking pre-mortem BALF neutrophil
178 percentage data were excluded from these correlation analyses. Alpha was set at 0.05.

179

Results

Animals

Lung tissues from 61 horses were studied; 22 were classified as controls, 15 as horses with severe asthma in clinical remission, and 24 as horses with severe asthma in exacerbation of the disease. Clinical details of the horses are described in **Table 1**. Pre-mortem BALF neutrophilia data were not available for 3 control horses and for 5 asthmatic horses in exacerbation, for which historical data were used to confirm the diagnosis of asthma. There was no significant difference in age, weight, or sex distribution among groups ($p>0.05$). As expected, horses with asthma in exacerbation had significantly increased R_L, E_L, and BALF neutrophilia compared to the controls ($p<0.001$) and to horses with asthma in remission ($p<0.001$ for R_L and E_L, and $p<0.05$ for BALF neutrophilia).

Agreement

The agreement between the 2 pathologists was fair to optimal for all the histological parameters evaluated (**Supplementary item 1**).

Distal lung lesions

Bronchocentric/bronchiolocentric lesions were observed in 28/29 asthmatic horses (1 horse had diffuse lesions). When lesions were present in control horses, they were also classified as bronchiolocentric (14/22 cases). The overall severity of the pathological processes identified within peripheral lung tissue was greater in asthmatic horses in exacerbation compared to those in remission ($p<0.05$) and control horses ($p<0.001$). Mucostasis, mucus cell hyperplasia, peribronchiolar metaplasia, and interstitial fibrosis were observed more frequently in asthmatic horses whether in exacerbation or in remission, when compared to control horses (**Table 2**). Also,

an increased number of asthmatic horses in exacerbation presented mucostasis, mucus cell hyperplasia, peribronchial/peribronchiolar metaplasia, and interstitial fibrosis compared to asthmatic horses in remission of the disease (**Table 2**). Discrete granulomas were occasionally observed both in asthmatic and in control horses; no micro-organisms were detected with Gram, Gomori's methenamine silver and Ziehl-Neelsen stains.

The severity of bronchial inflammation was greater in asthma exacerbation compared to control animals ($p<0.001$)(**Fig 3A**). The type of bronchial inflammation was, however, differently distributed between asthmatic horses in remission and control animals ($p=0.0003$). Specifically, foci of acute bronchitis were more frequently detected in asthmatic horses compared to controls, where the inflammatory response was either chronic or mixed (**Fig 3B**). The severity of bronchiolar inflammation was greater during asthma exacerbation compared to that observed in control horses ($p<0.001$) and in asthmatic horses during disease remission ($p<0.05$, **Fig 3C**). No differences were observed between the degree of bronchiolar inflammation detected in asthmatic horses in remission and controls. While most horses presented a mild to moderate chronic inflammation of the bronchioles in all groups studied, the proportion of horses with acute bronchiolar inflammation was greater in horses with asthma, both in remission and in exacerbation, compared to controls ($p<0.0001$), and in horses with asthma in exacerbation compared to those in remission of the disease ($p=0.0008$, **Fig 3D**). Eosinophilic infiltration of the peripheral lung was lower in horses with asthma during disease exacerbations compared to control horses ($p<0.001$), while horses with asthma in remission presented variable degrees of pulmonary eosinophilia (**Table 2**).

Effect of the treatment strategy employed for inducing remission

Asthma remission was induced by means of antigen avoidance (alone) in 6/15 horses and by pharmacological treatment (oral corticosteroids, inhaled corticosteroids, or inhaled combinations of

227 corticosteroids and long-acting β_2-agonists) in 7 stabled horses. The management of the 2 remaining
228 horses was undetermined and they were excluded from the statistical analysis investigating the
229 effects of the treatment strategy employed for inducing remission. The degree of bronchiolar
230 inflammation was higher in horses stabled and treated pharmacologically compared to those kept on
231 pasture (p=0.04, **Table 3**). Although BALF neutrophilia was higher in horses treated
232 pharmacologically while stabled compared to horses kept at pasture (mean±S.D.: 10.75%±10.28%
233 and 23.5%±16.72%, respectively), the difference was not statistically significant (p=0.07, unpaired
234 one-way t-test, post-hoc calculation of study power=36.8%) as there were two horses at pasture for
235 1 month with values of BALF neutrophilia still >20%. No difference was observed between the 2
236 groups in terms of overall disease severity, pulmonary eosinophilia, bronchial inflammation,
237 mucostasis, peribronchial/peribronchiolar metaplasia, mucus cell hyperplasia, or interstitial fibrosis.

238

239 *Relationship between BALF inflammation, lung function, and peripheral lung lesions*

240 Asthmatic horses in exacerbation with moderate to severe pulmonary lesions (overall severity >1)
241 had a lower BALF neutrophil percentage (p=0.006, **Fig 4A**) but similar values of R_L (p=0.32) and
242 E_L (p=0.95) compared to those with mild pulmonary lesions (overall severity ≤1). Of these horses,
243 21 out of 24 presented a mixed pulmonary inflammation, which prevented the statistical analysis of
244 the effect of inflammation type on clinical outcomes. BALF neutrophilia was significantly lower in
245 the presence of peripheral mucostasis in this group of horses (p=0.001, **Fig 4B**).

246 Horses with asthma in remission with chronic infiltrates had similar percentages of neutrophil in
247 their BALF (p=0.19), and similar values of R_L (p=0.93) and E_L (p=0.28) than those with a mixed
248 airway inflammatory pattern.

249 Control horses with chronic, mixed, or no evidence of bronchiolar inflammation had similar lung
250 function values (lung resistance, p=0.80; lung elastance, p=0.53). However, they differed for the

percentage of neutrophils in their BALF (p=0.0003, **Fig 4C**). Specifically, control horses with a mixed inflammatory infiltrate in their distal airways (n=6) had a higher percentage of BALF neutrophils (mean±SD: 16.3±5.7, all had BALF neutrophils >5%) compared to those with evidence of chronic or no inflammation at histology. Control horses with >5% neutrophils in their BALF had a significantly greater degree of bronchial and bronchiolar inflammation (p=0.0003 and p=0.002, respectively) and a greater overall severity of pulmonary lesions (p=0.0004) compared to control horses with <5% neutrophils in their BALF. No differences were detected between control horses with less or more than 5% neutrophils in BALF for the parameters eosinophilia (p=0.5), interstitial fibrosis (p=0.4), mucus cell hyperplasia (p=0.2), peribronchial metaplasia (p=0.1), and mucostasis (p=0.05). Raw data are available online in **Supplementary item 2** and **3**. In control horses, BALF neutrophilia correlated significantly with the severity of bronchial and bronchiolar inflammation (r=0.70, p=0.0008, and r=0.50, p=0.03, respectively) and with overall lesion severity (r=0.62, p=0.004).

Results of the relationship between peripheral lung lesions, BALF neutrophilia, and lung function in each group studied are reported in **Supplementary items 4** and **5**.

Discussion

This study provides the first evidence that alterations of the peripheral peribronchial/peribronchiolar tissues and interstitium occur in the distal lung of asthmatic horses with a higher prevalence compared to age-matched controls. These changes may contribute to the development of airflow obstruction, and their presence may explain the lack of a significant correlation between bronchial remodeling and pulmonary resistance or elastance measured during disease exacerbation [4]. Our results also suggest that asthmatic horses with BALF neutrophilia >20% during disease exacerbation are less likely to have severe peripheral pulmonary lesions compared to asthmatic

horses with <20% neutrophils in their BALF (for which we propose the term "paucigranulocytic asthmatic horses"). Mucus plugs preventing saline withdrawal from the most distal airways could explain this finding. Increased percentages of neutrophils in BALF of clinically healthy horses were not associated with bronchial or parenchymal remodeling. However, they were associated with an acute inflammatory process of the terminal airways.

Severe equine asthma is characterized by airway remodeling and inflammation [13]. Previous studies limited to lung tissues from asthmatic horses have described the more severe lesions as being located at the distal level of the bronchial tree [2; 14; 15]. The semi-quantitative assessment of peripheral lung tissue inflammation revealed similar degrees of cellular infiltrate in pulmonary biopsy samples harvested from asthmatic horses and controls [16]. To our knowledge, no study has systematically investigated whether any difference exists in peripheral airway wall inflammation of asthmatic and healthy horses. Using a semi-quantitative and blinded approach, our results confirm that distal airway inflammation is a feature of severe equine asthma and that these changes are more pronounced in the smallest airways. Even among distal airways, the bronchioles (lacking cartilage) sustain more severe inflammatory insults compared to the bronchi. Indeed, 88% of the horses with asthma in exacerbation had acute bronchiolitis graded on average 1.6 out of 2, while acute bronchitis was detected in only 67% of them and graded on average 1 out of 2. Only 27% and 18% of control horses had acute inflammation in their bronchioles and bronchi, respectively, with a mean severity grade of 0.8 and 0.25. The milder degree of inflammation observed in peripheral bronchi compared with adjacent bronchioles appears to be without clinical significance, as it is also observed in healthy animals. The reasons of this finding are not obvious. It is possible that the size of the inhaled antigens responsible for the development of equine asthma could favor their deposition in the most peripheral airways of the lung. For example, the spores of the fungus *Aspergillus fumigatus*, which has been implicated in equine asthma pathogenesis [17-19], have an average size of 2-3.5 μm [20], which allows their deposition in the most distal airways and alveoli.

Also, the non-ciliated epithelium of the most distal bronchioles could reduce the clearance of external particles that deposit at this level during normal breathing, inducing more severe reactions at this site.

Histological evaluation of the distal airways *in vivo* is limited by their inaccessibility, which prevents the direct assessment of pathological processes occurring at this level [21]. Distal lung sampling is achieved by thoracoscopy or transcutaneously [22; 23]; however, due to the invasiveness of the procedures and related risks, it is done mainly for research purposes. For this reason, BAL is commonly performed as a diagnostic procedure in horses suspected to have severe asthma, with the presence of moderate to severe neutrophilia at BALF cytology (>20-25%) as the only parameter considered for confirming the diagnosis, and thus the presence of peripheral airway pathology [1]. Nevertheless, there is little evidence supporting BALF neutrophilia as a specific marker of the severity of peripheral airway inflammatory disease [15]. Our results suggest that the significance of neutrophilic luminal inflammation varies depending on the clinical condition of the horse. Horses classified as controls in our study and presenting increased percentages of neutrophils at BALF cytology (>5%) had histologic evidence of acute neutrophilic inflammation in their distal airways. Of note, having more than 5% of neutrophils in BALF is considered diagnostic for mild neutrophilic equine asthma (or neutrophilic IAD, *Inflammatory Airway Disease*) when associated with compatible clinical signs [1]. In the present study, as there was no history of lung diseases, these horses were not treated as a separate group. In this perspective, our observations provide the first histologic evidence that BALF neutrophilic inflammation (>5% neutrophils) is associated with acute distal airway inflammation, even in absence of overt clinical signs suggestive of lung diseases. On the other hand, during disease exacerbation, horses with mild pulmonary lesions had higher neutrophil percentages in their BALF cytology compared to horses in exacerbation with severe histologic lesions in their distal lung. Horses with neutrophilia <20%, all had an overall severity score >1, compared to horses with BALF neutrophilia >20% that presented an overall

severity score >1 only in 4/12 cases (33%). As BALF neutrophilia >20% is considered the threshold for the diagnosis of severe asthma based on previous studies [1], we propose the term paucigranulocytic asthma for those severely asthmatic horses presenting with <20% neutrophilia in BALF cytology during disease exacerbations. Of note, all paucigranulocytic cases (7/24, 29% of the group) had pulmonary lesions with an overall severity score ≤1 (mild lesions), suggesting that the number of inflammatory cells is low also within the airway walls and interstitium. The significant association found between BALF neutrophilia <20% and the presence of peripheral mucostasis during episodes of severe equine asthma exacerbations could explain our results as mucus plugs within the peripheral airways may prevent the wash solution reaching the alveoli and terminal non-respiratory bronchioles to be recovered.

Submucosal remodeling occurs in the peripheral airways of asthmatic horses [4; 10]. There is less information concerning peribronchial/peribronchiolar tissues and interstitium, which are commonly overlooked. The presence of chronic bronchoalveolar inflammation suggests that not only the airways but also the alveolar walls may undergo remodeling processes in severe equine asthma. Our findings show that peribronchiolar metaplasia and interstitial bronchiolocentric fibrosis are overrepresented in asthmatic horses compared to healthy animals. The prevalence of these lesions is lower in asthmatic horses during disease remission. While the clinical implication and the mechanisms driving peribronchiolar metaplasia are still ill-defined [24], fibrosis is commonly associated with chronic damage and reparation processes, with increased concentration of TGF-β in lung tissues, and with a Th-2-biased inflammatory response [25]. Th-2 shifted inflammatory response has previously been demonstrated in BALF obtained from horses with asthma [26], while to our knowledge no studies have investigated TGF-β expression in equine peripheral lung tissues. However, TGF-β levels are similar in BALF, BAL cells, and in endobronchial biopsies of healthy and severe asthmatic horses [27; 28], and unaffected by treatment [11]. Th-2 type cytokines are also considered important mediators of mucus cell hyperplasia [29; 30]. In our study, mucostasis and

mucus cell hyperplasia followed the same lesion distribution described for interstitial fibrosis among the groups studied.

In conclusion, severe asthmatic horses present alterations of the peripheral peribronchial/peribronchiolar tissues and interstitium in addition to those already described for the submucosal tissues of peripheral airway walls, which possibly contribute to the obstructive nature of the disease. These changes are mild in asthmatic horses in remission of the disease suggesting they might be, at least partly, reversible. Nevertheless, they remain of a greater magnitude in asthmatic horses in remission of the disease compared to control horses, independently of the treatment strategy adopted to induce disease remission. BALF neutrophilia >5% is associated with acute bronchiolitis in control horses. Contrarily, mild pulmonary lesions and the absence of peripheral mucostasis are associated with a greater (>20%) BALF neutrophilia during equine asthma exacerbations.

List of abbreviations

ASM: airway smooth muscle; BAL: bronchoalveolar lavage; BALF bronchoalveolar lavage fluid; E_L: pulmonary elastance; HEPS: hematoxylin-eosin-phloxine-saffron; R_L: pulmonary resistance; TGF-β: tumor growth factor β; Th: T helper.

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Tables

Table 1. Details of the horses studied.

	Controls	Asthma remission	Asthma exacerbation
N	22	15	24
Age [years]	20.4±5.6	22.4±5.9	23.2±6.2
Sex (F/M)	18/4	9/5	18/6
R_L [cm H_2O/L/s]	0.552±0.223	0.684±0.327	2.522±1.049*,†
E_L [cm H_2O/L]	0.521±0.237	0.649±0.336	4.921±4.547*,†
BAL neutrophil %	6.3±7.7	16.2±14.6	34.6±26.6*,‡

Data are presented as mean ± S.D. One-way ANOVA with Tukey post-tests was used for statistical analysis. *: different from controls ($p<0.0001$). †: different from asthma remission ($p<0.0001$). ‡: different from asthma remission ($p<0.05$). R_L: pulmonary resistance; E_L: pulmonary elastance; BAL: bronchoalveolar lavage.

514 **Table 2.** Prevalence and severity of peripheral lung lesions.

Parameter assessed	Group		
	Control (n=22)	Asthma remission (n=15)	Asthma exacerbation (n=24)
Overall severity [range: 0-3]	0.5 (0; 1)	0.5 (0.5; 1)	1.5 (1; 1.5)†,‡
Eosinophilia [range: 0-3]	0.75 (0.5; 2)	0.5 (0; 1)	0 (0; 0.5)‡
Mucostasis*	2/22 (9)	3/15 (20)‡	15/24 (63)†,‡
Mucus cell hyperplasia*	4/22 (18)	7/15 (47)‡	16/24 (67)†,‡
Peribronchial/peribronchiolar metaplasia*	3/22 (14)	4/15 (27)‡	11/24 (46)†,‡
Interstitial fibrosis*	4/22 (18)	7/15 (47)‡	18/24 (75)†,‡
Granulomatous lesions*	1/22 (4)	2/15 (13)	1/24 (4)

515 Overall severity and eosinophilia are expressed as median (interquartile range) * Results are
516 presented as the number of cases in which the lesion was present/total number of cases
517 (percentage). †: different from asthma remission. ‡: different from control.

518

519

520 **Table 3.** Effect of the strategy employed to induce disease remission on peripheral lung lesions.

	Horses with asthma in remission	
	Pasture (antigen avoidance) (n=6)	Stabling and pharmacological treatment (n=7)

Overall severity [range: 0-3]	0.5 (0.375; 1)	0.5 (0.5; 1)
Eosinophilia [range: 0-3]	0.5 (0; 1.875)	0.5 (0.5; 1)
Bronchial inflammation [range: 0-3]	0.25 (0; 0.625)	0.5 (0.5; 0.5)
Bronchiolar inflammation [range: 0-3]	0.75 (0.5; 1)	1 (1; 1)†
Mucostasis*	1/6 (17)	1/7 (14)
Mucus cell hyperplasia*	3/6 (50)	2/7 (28)
Peribronchial/peribronchiolar metaplasia*	2/6 (33)	2/7 (28)
Interstitial fibrosis*	2/6 (33)	3/7 (43)
Granuloma*	0/6 (0)	2/7 (28)

Overall severity, eosinophilia, and airway inflammation are expressed as median (interquartile range). * Results presented as the number of cases in which the lesion was present/total number of cases (percentage). †: different from pasture (p=0.04).

Figures

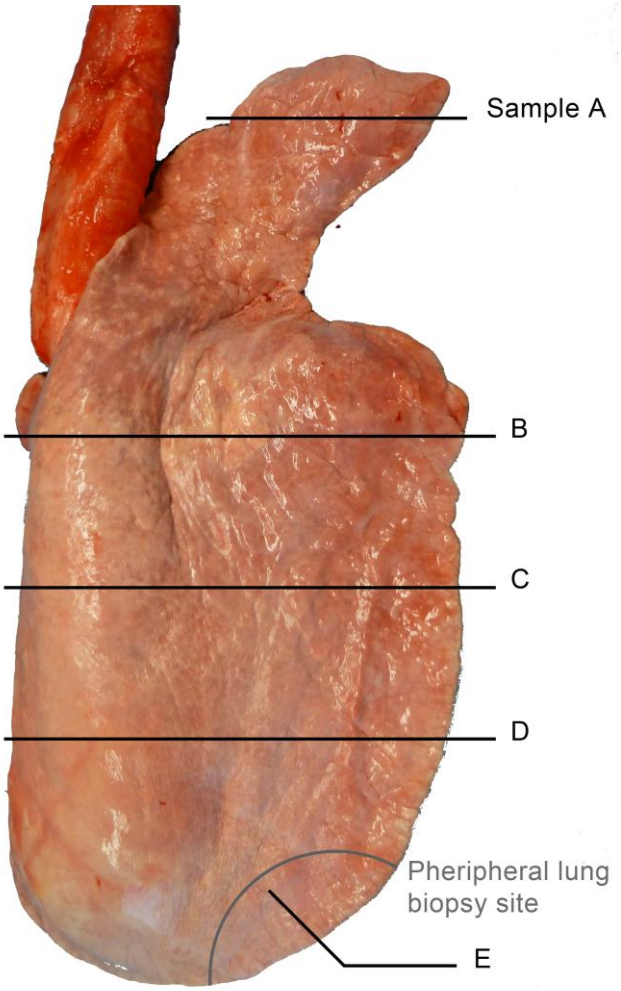
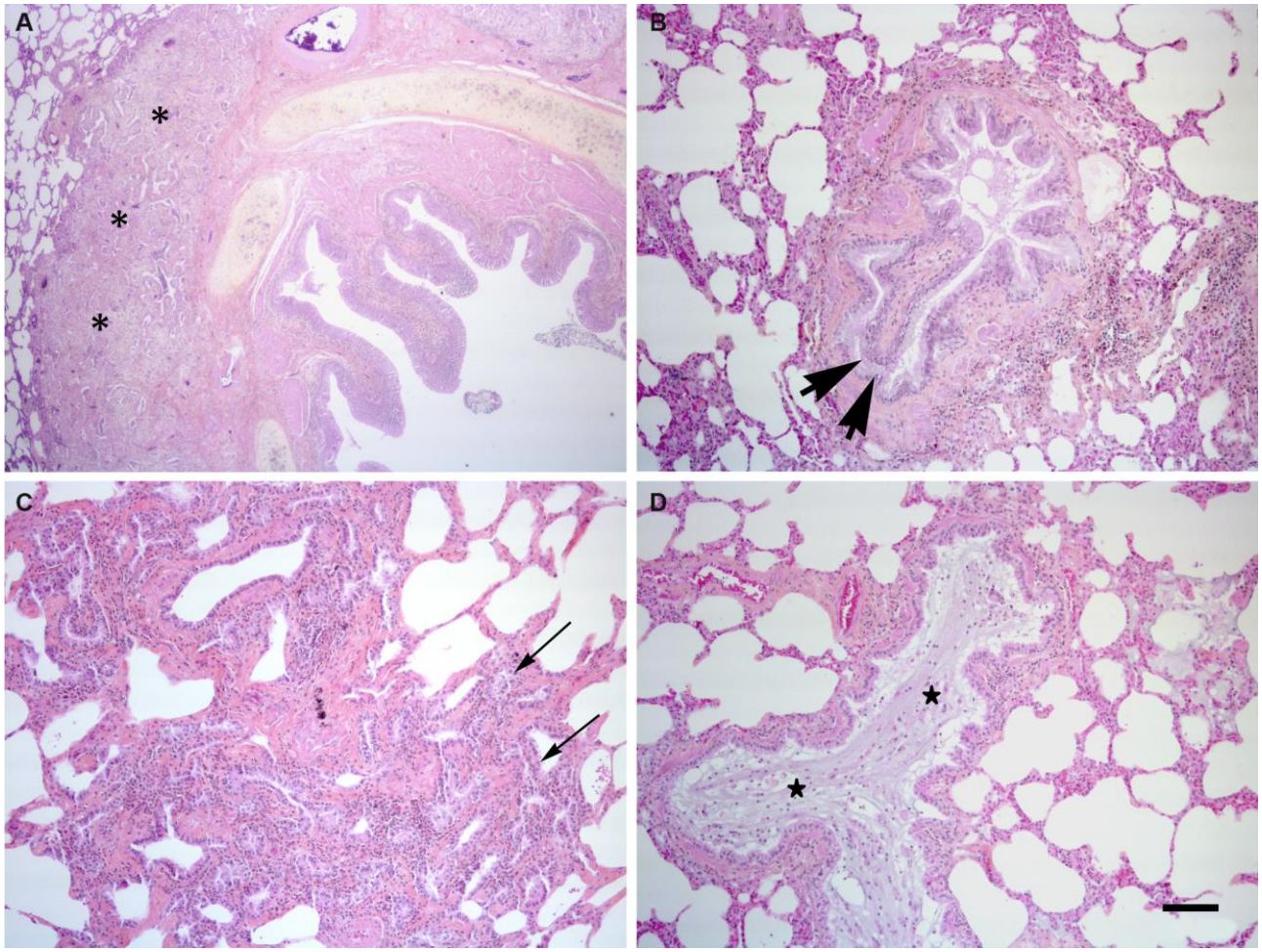


Figure 1. Anatomical sites sampled at necropsy for the assessment of distal lung histology. One randomly chosen lung per horse was assessed. A biopsy of 6-8 cm^3 in size was harvested at each anatomical site (A, B, C, D, and E) within 2 hours post-mortem and processed for histology.



542

543 **Figure 2.** Histological lesions observed in asthmatic horses. A) Interstitial fibrosis (asterisks), 2.5x.
544 B) Mucus cell hyperplasia (arrowheads), 10x. C) Peribronchial metaplasia (arrows), 10x. D)
545 Mucostasis (stars), 10x. HEPS staining. Scale bar: 400 µm in panel A; 100 µm in panels B, C, and
546 D.

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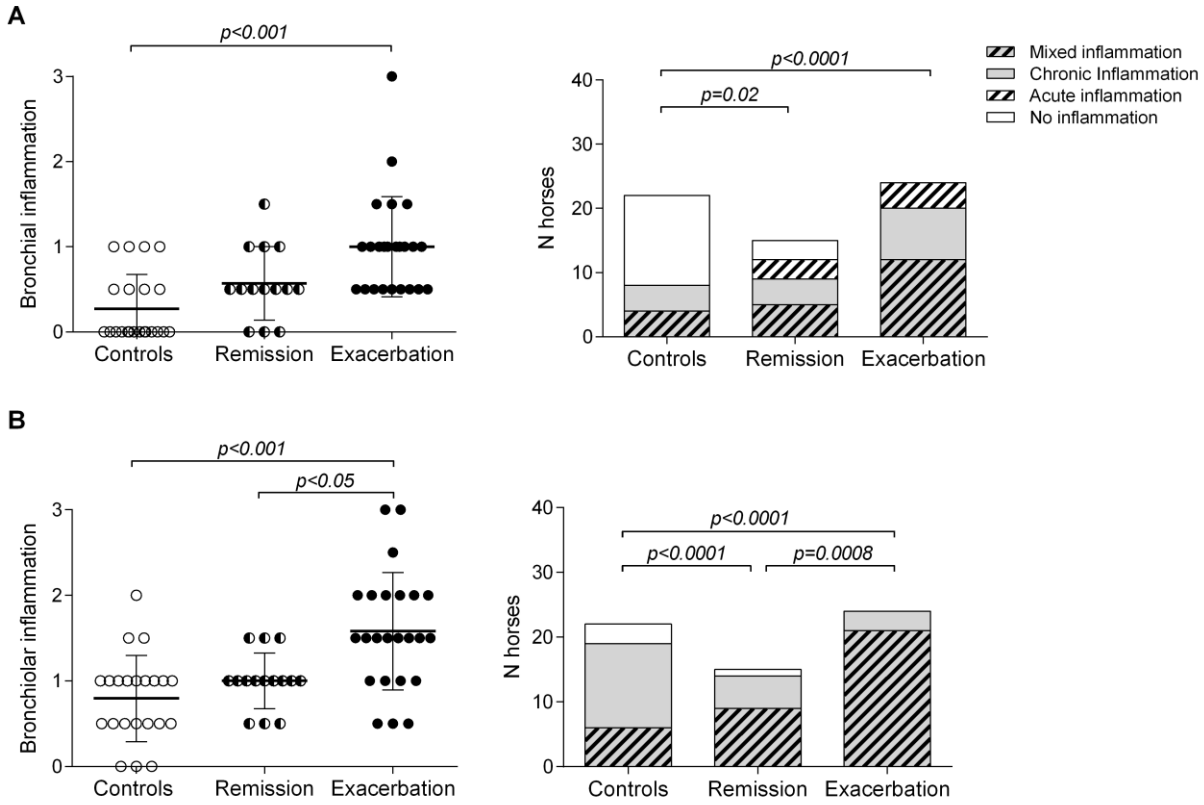
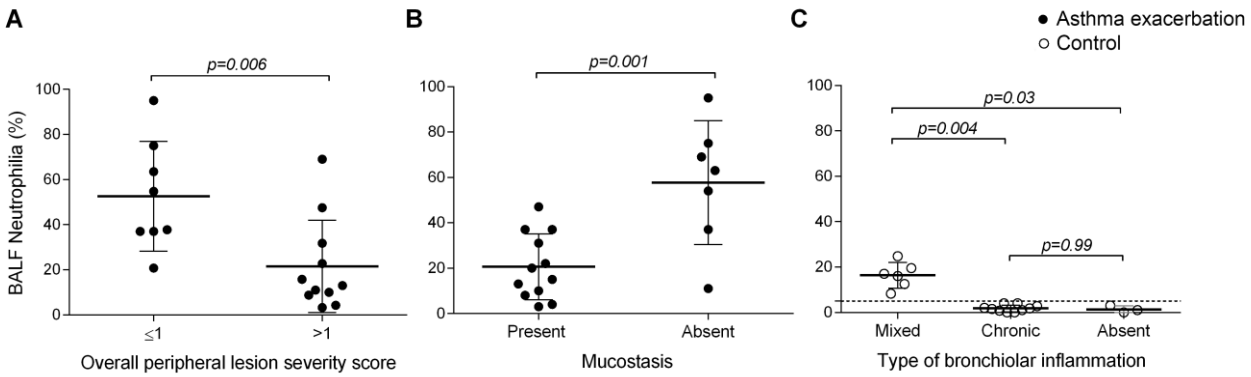


548

549 **Figure 3.** Peripheral airway inflammatory infiltrate. Severity of bronchial (A) and bronchiolar (B)
550 inflammation in the three groups of horses studied is reported in the left panels, while inflammation
551 type is shown in the right panels.

552



553

554 **Figure 4.** Determinants of BALF neutrophilia in asthmatic and control horses. Effect of the
555 histological severity of pulmonary lesions (A) and of the presence of peripheral mucostasis (B) on
556 BALF neutrophil percentage in horses with asthma in exacerbation of the disease. Effect of the type
557 of bronchiolar inflammatory infiltrate on BALF neutrophil percentage in control horses (C). The
558 dashed line identifies 5% of neutrophils in BALF, currently considered as the cutoff for the
559 diagnosis of equine asthma.

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