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**Bronchoalveolar lavage fluid neutrophilia is associated with the severity of pulmonary lesions during equine asthma exacerbations**

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1 **Bronchoalveolar lavage fluid neutrophilia is associated with the severity of pulmonary lesions**  
2 **during equine asthma exacerbations**

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11

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13

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27

28 **Abstract**

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

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

35 **Study design:** Observational retrospective study.

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

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

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

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

52

53

54 5113 words

55

## 56 **Introduction**

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

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

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

89 In the present study, we performed a comprehensive histologic evaluation of remodeling and  
90 inflammation in peripheral lung tissues of severe asthmatic horses, including samples obtained  
91 during exacerbation and remission of the disease, and controls. A subgroup of control horses with  
92 >5% BALF neutrophilia but without clinical signs suggestive of lung disease was also studied. We  
93 sought to determine the histological lesions associated with equine asthma and whether they differ  
94 in horses experiencing exacerbation of the disease when compared to horses in remission of the  
95 disease. We studied histological lesions in horses where disease remission had been induced by  
96 antigen avoidance strategies or corticosteroids. Finally, the relationship between the histological  
97 lesions observed and the BALF cytology and lung function were also studied.

98

## 99 **Materials and Methods**

### 100 *Animals*

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

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

131

132 *Histology*

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

180 **Results**

181 *Animals*

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

190

191 *Agreement*

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

194

195 *Distal lung lesions*

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

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

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

223

#### 224 *Effect of the treatment strategy employed for inducing remission*

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

227 corticosteroids and long-acting  $\beta_2$ -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 $\pm$ S.D.: 10.75% $\pm$ 10.28%  
233 and 23.5% $\pm$ 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  $\leq 1$ ). 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

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

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

266

## 267 **Discussion**

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

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

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

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

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

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

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

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

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

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364 **List of abbreviations**

365 ASM: airway smooth muscle; BAL: bronchoalveolar lavage; BALF bronchoalveolar lavage fluid;

366  $E_L$ : pulmonary elastance; HEPS: hematoxylin-eosin-phloxine-saffron;  $R_L$ : pulmonary resistance;

367 TGF- $\beta$ : tumor growth factor  $\beta$ ; Th: T helper.

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495 **Tables**

496

497 **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 <sub>L</sub> [cm H <sub>2</sub> O/L/s]	0.552±0.223	0.684±0.327	2.522±1.049* <sup>†</sup>
E <sub>L</sub> [cm H <sub>2</sub> O/L]	0.521±0.237	0.649±0.336	4.921±4.547* <sup>†</sup>
BAL neutrophil %	6.3±7.7	16.2±14.6	34.6±26.6* <sup>‡</sup>

498 Data are presented as mean ± S.D. One-way ANOVA with Tukey post-tests was used for statistical

499 analysis. \*: different from controls (p<0.0001). <sup>†</sup>: different from asthma remission (p<0.0001). <sup>‡</sup>:

500 different from asthma remission (p<0.05). R<sub>L</sub>: pulmonary resistance; E<sub>L</sub>: pulmonary elastance;

501 BAL: bronchoalveolar lavage.

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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) <sup>†,‡</sup>
Eosinophilia [range: 0-3]	0.75 (0.5; 2)	0.5 (0; 1)	0 (0; 0.5) <sup>‡</sup>
Mucostasis*	2/22 (9)	3/15 (20) <sup>‡</sup>	15/24 (63) <sup>†,‡</sup>
Mucus cell hyperplasia*	4/22 (18)	7/15 (47) <sup>‡</sup>	16/24 (67) <sup>†,‡</sup>
Peribronchial/peribronchiolar metaplasia*	3/22 (14)	4/15 (27) <sup>‡</sup>	11/24 (46) <sup>†,‡</sup>
Interstitial fibrosis*	4/22 (18)	7/15 (47) <sup>‡</sup>	18/24 (75) <sup>†,‡</sup>
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.

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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) <sup>†</sup>
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)

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

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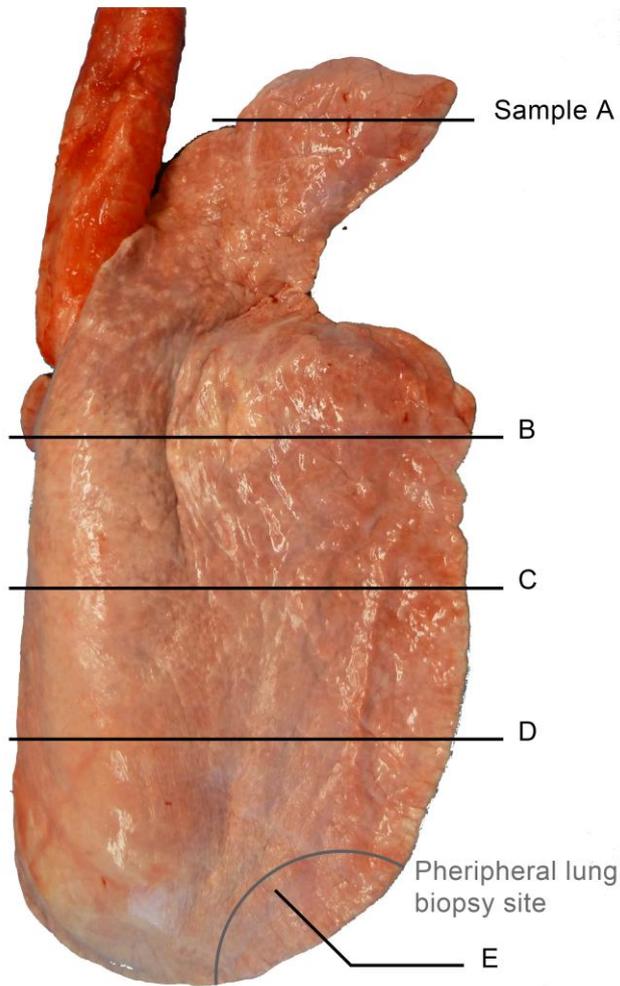
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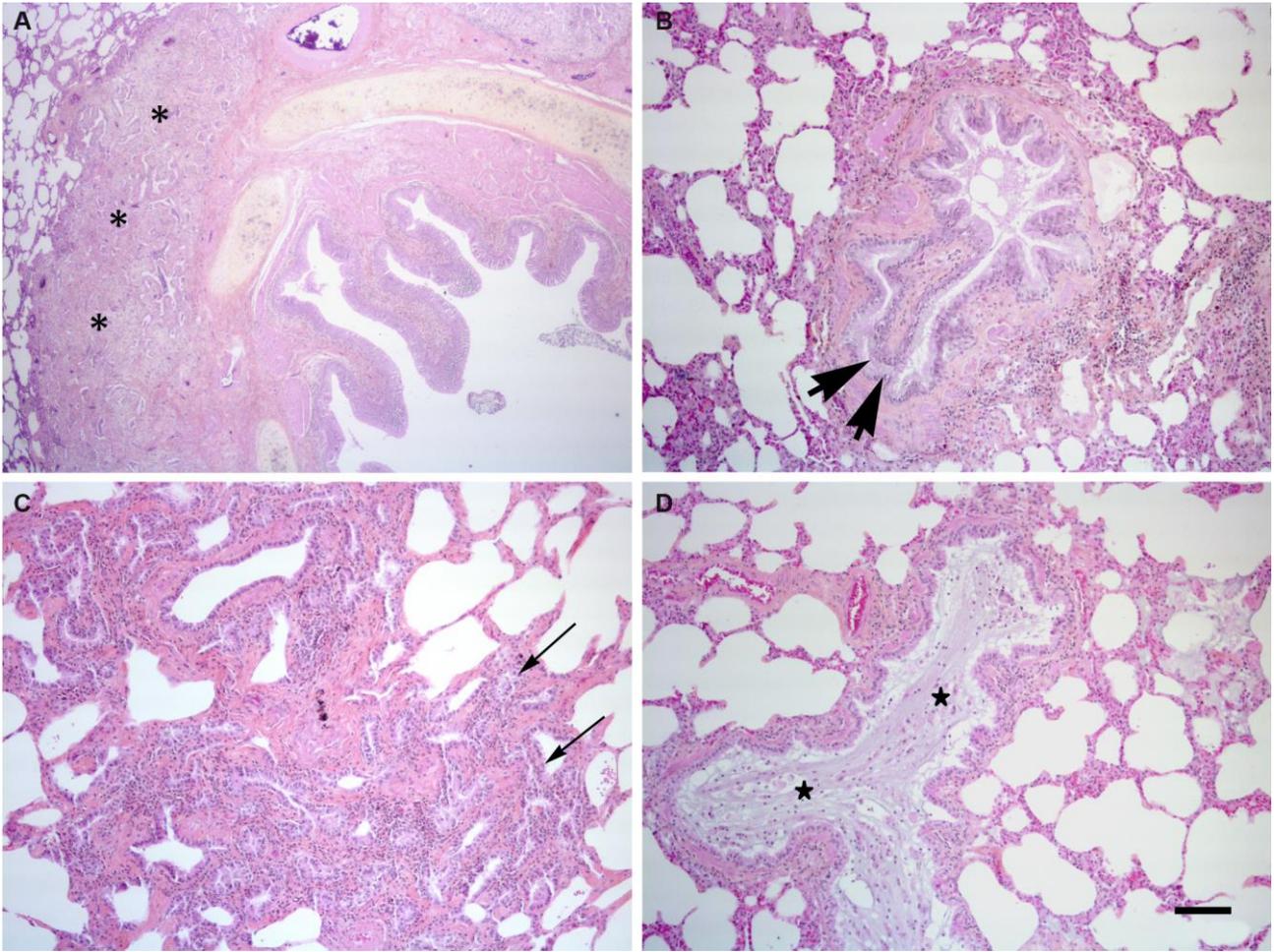
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538 **Figure 1.** Anatomical sites sampled at necropsy for the assessment of distal lung histology. One  
539 randomly chosen lung per horse was assessed. A biopsy of 6-8 cm<sup>3</sup> in size was harvested at each  
540 anatomical site (A, B, C,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

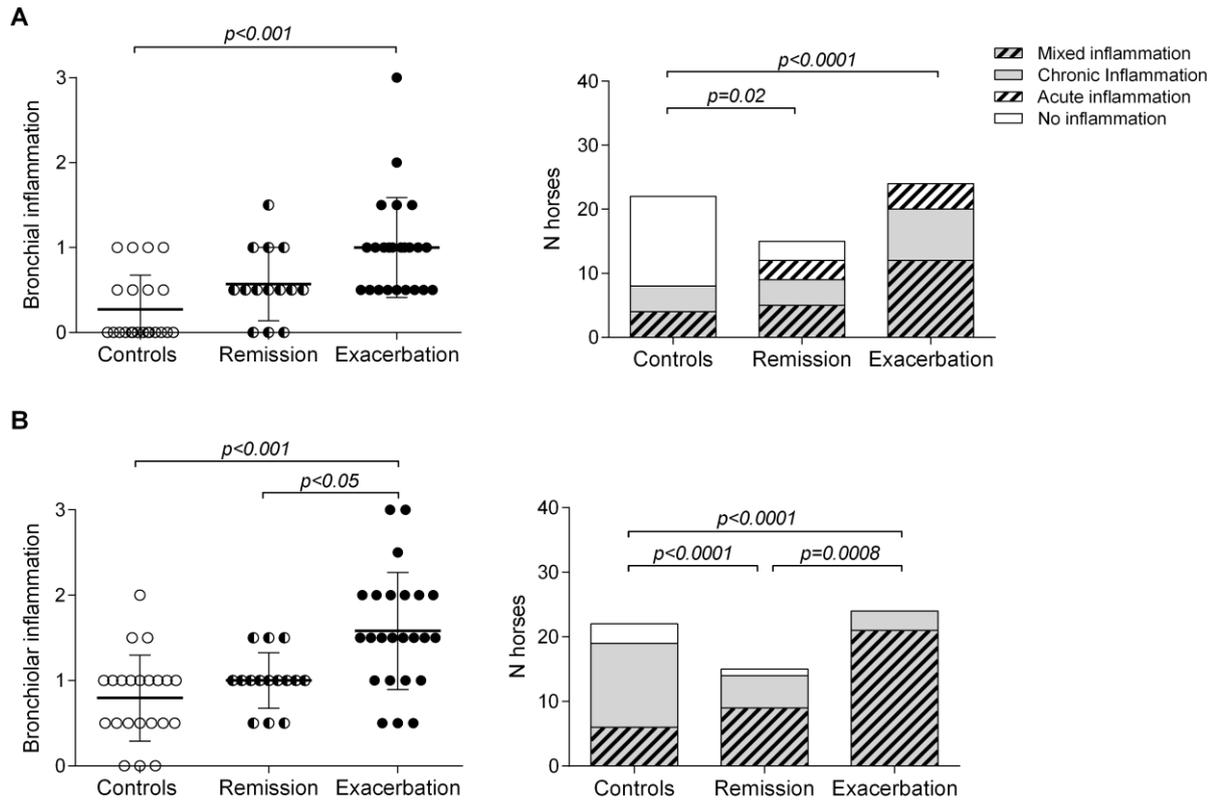
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543 **Figure 2.** Histological lesions observed in asthmatic horses. A) Interstitial fibrosis (asterisks), 2.5x.  
544 B) Mucus cell hyperplasia (arrowheads), 10x. C) Peribronchial metaplasia (arrows), 10x. D)  
545 Mucostasis (stars), 10x. HEPS staining.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

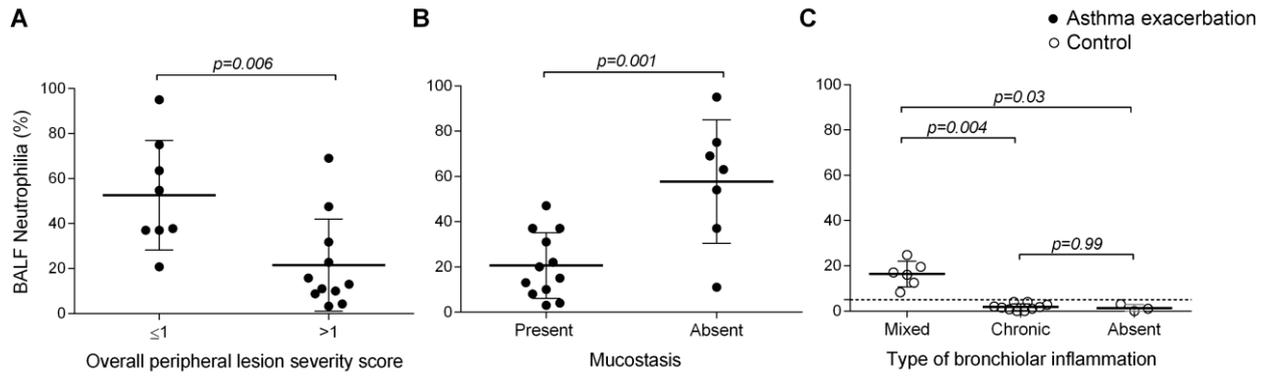
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549 **Figure 3.** Peripheral airway inflammatory infiltrate. Severity of bronchial (A) and bronchiolar (B)  
 550 inflammation in the three groups of horses studied is reported in the left panels, while inflammation  
 551 type is shown in the right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

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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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