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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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23

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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 ($R_L < 1$ cmH₂O/L/s, and $E_L < 1$
114 cmH₂O/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 ($R_L \geq 1$ cmH₂O/L/s,
121 and $E_L \geq 1$ cmH₂O/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 ± 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 ≤ 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 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 \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 ≤ 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 ± 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 μm [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 ≤ 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- β 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- β expression in equine peripheral lung tissues.
347 However, TGF- β 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.

362

363

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- β : tumor growth factor β ; Th: T helper.

368

369

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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 _L [cm H ₂ O/L/s] | 0.552±0.223 | 0.684±0.327 | 2.522±1.049* [†] |
| E _L [cm H ₂ O/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* [‡] |

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). [†]: different from asthma remission (p<0.0001). [‡]:

500 different from asthma remission (p<0.05). R_L: pulmonary resistance; E_L: 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) ^{†,‡} |
| 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.

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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) |

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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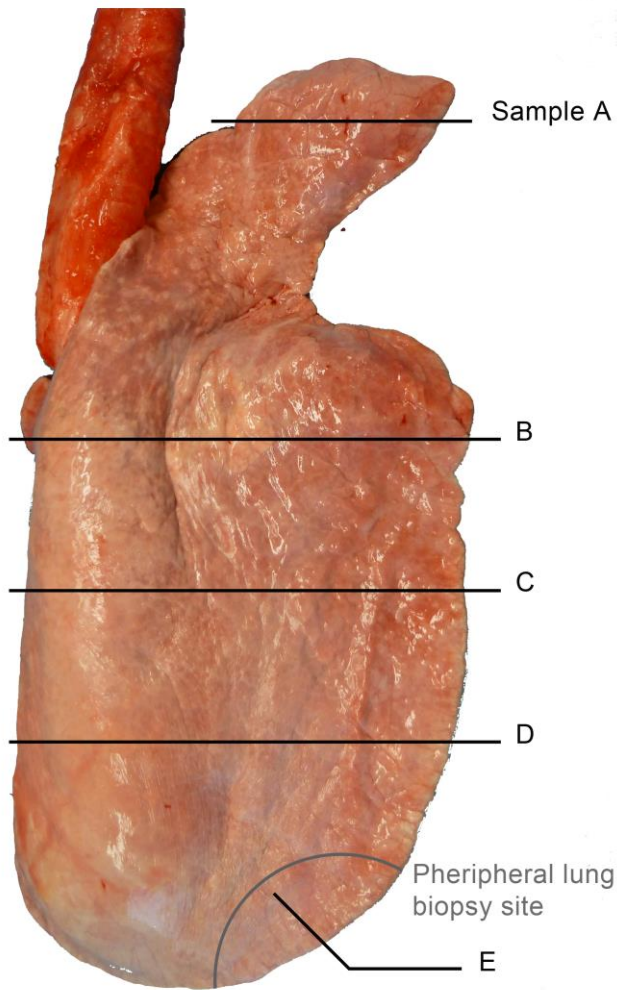
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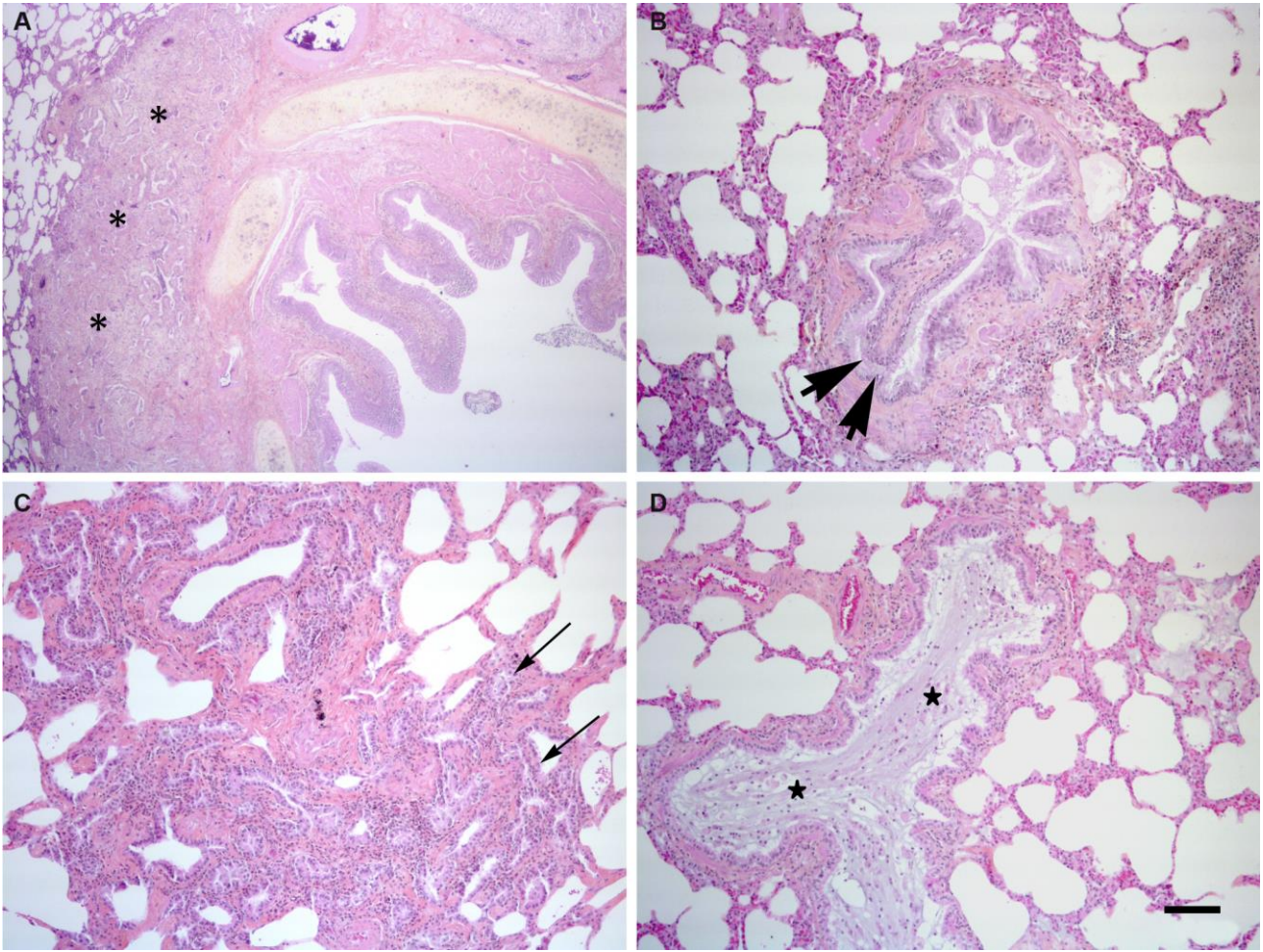
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538 **Figure 1.** Anatomical sites sampled at necropsy for the assessment of distal lung histology. One
539 randomly chosen lung per horse was assessed. A biopsy of 6-8 cm³ in size was harvested at each
540 anatomical site (A, B, C, D, and E) within 2 hours post-mortem and processed for histology.

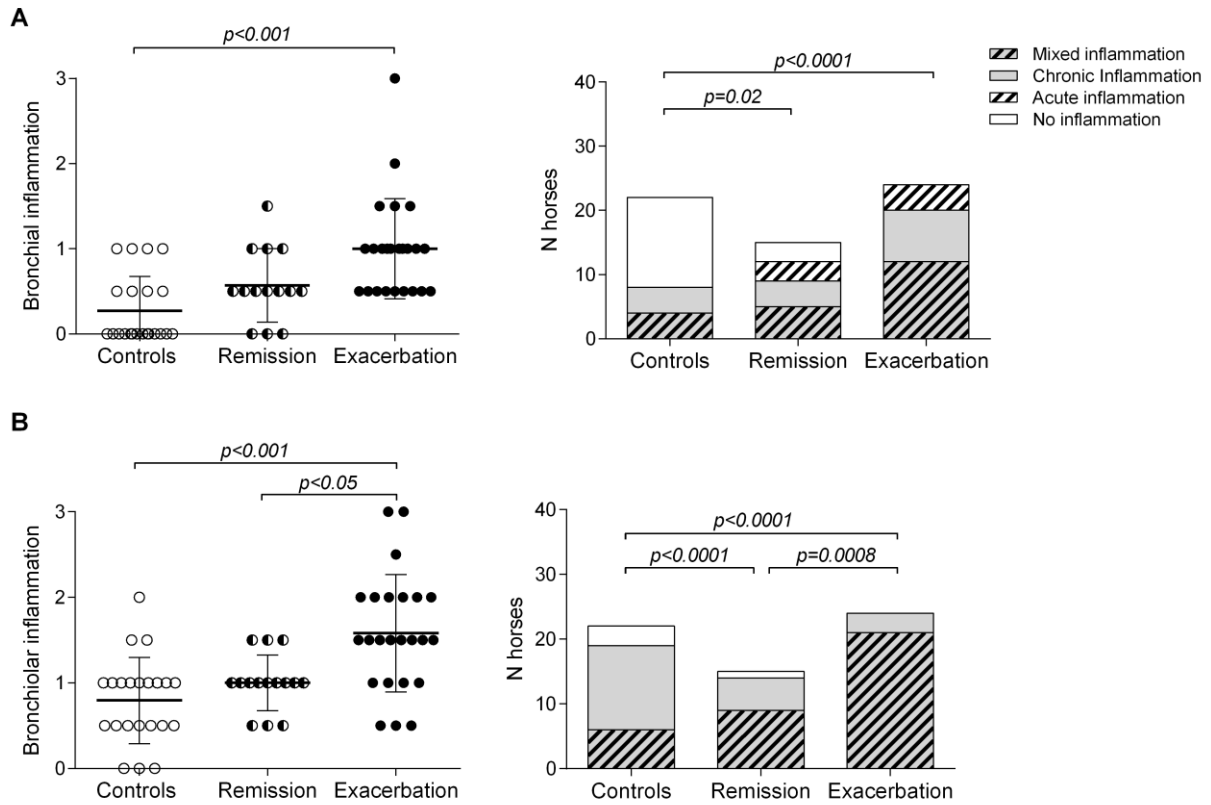
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543 **Figure 2.** Histological lesions observed in asthmatic horses. A) Interstitial fibrosis (asterisks), 2.5x.
544 B) Mucus cell hyperplasia (arrowheads), 10x. C) Peribronchial metaplasia (arrows), 10x. D)
545 Mucostasis (stars), 10x. HEPS staining.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

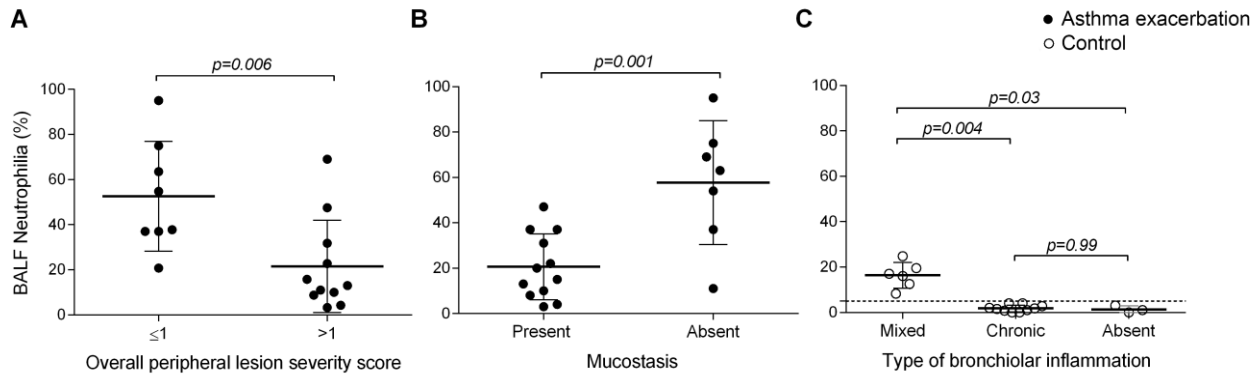
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549 **Figure 3.** Peripheral airway inflammatory infiltrate. Severity of bronchial (A) and bronchiolar (B)
 550 inflammation in the three groups of horses studied is reported in the left panels, while inflammation
 551 type is shown in the right panels.

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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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