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1 **Short Communication**

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4 **Age-associated change in peripheral airway smooth muscle mass of healthy horses**

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17

18 **Abstract**

19 Peripheral airway smooth muscle (ASM) mass is increased in severe equine asthma, but no
20 information exists on the timing of such a structural alteration during the development of the
21 disease. In order to elucidate the mechanisms driving ASM remodeling during disease, anatomical
22 ASM development has to be evaluated first. This study investigated the morphometric alterations
23 sustained by peripheral ASM during aging in healthy horses. The thickness of the peripheral ASM
24 layer was found to be constant in horses of all ages, but it occupies a greater proportion of the inner
25 wall area in younger than in older horses. This finding suggests that equine airways physiologically
26 experience a decrease in the relative abundance of ASM with age. Failure to do so may play a role
27 in equine asthma development.

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30 *Keywords:* Airway smooth muscle; Development; Foal; Lung; Peripheral airways.

31

32 Severe equine asthma, also known as recurrent airway obstruction (RAO) or heaves, is a
33 chronic obstructive disease affecting adult horses exposed to environmental antigens as found in
34 hay and straw dust. Following exposure, affected horses develop pulmonary inflammation
35 associated with a severe bronchoconstrictive response caused by exaggerated airway smooth muscle
36 (ASM) contraction (Leclere et al., 2011b). Several studies have shown that the ASM mass is
37 increased in severely asthmatic horses, particularly in the peripheral airways, thereby contributing
38 to airflow obstruction (Herszberg et al., 2006; Leclere et al., 2011a; Bullone et al., 2015).

39 Despite the central role of the increased ASM in severe equine asthma, no information is
40 available concerning the timing of these structural alterations during disease development.
41 Moreover, it remains unclear whether this remodeling results from an abnormal growth or from a
42 failure of involution/regression mechanisms normally occurring during airway development in
43 healthy subjects. To clarify these issues, an accurate description of the postnatal ontogenesis of
44 ASM in healthy horses is required. The present study aims to investigate the anatomical alterations
45 sustained by the peripheral ASM with ageing in a cohort of healthy horses.

46
47 Peripheral lung tissues harvested post-mortem from healthy horses were obtained from the
48 Equine Respiratory Tissue Bank¹ or from the histological archives of the authors' institution.
49 Horses were defined as healthy based on history (absence of respiratory signs), blood work results
50 (when available), and histopathological findings. Histological sections of 5 µm thickness were
51 stained with hematoxylin-eosin-phloxyn-saffron (HEPS). Five airways per horse with a major to
52 minor axis ratio <1.5, with an intact epithelium, and with smooth muscle surrounding ≥70% of their
53 circumference were studied. The ASM area, the outer border of ASM, and the internal perimeter
54 length (Pi) were measured in cross-sectionally cut peripheral airways using Image J (NIH). ASM
55 mass was expressed as ASM/Pi (corrected by the internal perimeter to account for variation in
56 airway size) and as ASM% (percentage of the inner airway wall occupied by ASM, where the inner

¹ See: www.ertb.ca (accessed 04/05/2017).

airway wall area was calculated as the difference between the area enclosed by the external border of ASM and the airway lumen area enclosed by Pi; Fig. 1). Measurements were performed by one investigator blinded to the subject identity. For statistical analysis, horses were divided in six age groups (0-6 months, 6-12 months, 1-4 years, 5-10 years, 11-20 years, and >20 years). Further methodological details are provided online (see Appendix A: Supplementary material).

Tissues harvested from 51 healthy horses ranging from 1 day to 32 years of age were studied. Table 1 details their age, sex distribution, and average airway size. ASM/Pi, an indirect measure of ASM thickness, did not change with age in the horses studied ($P=0.3$; Fig. 2A). However, ASM/Pi significantly increased with increasing airway size when airways of all groups were analysed together ($r^2=0.11$, $P<0.0001$; Fig. 2B). ASM%, which represents the percentage of the inner bronchial area occupied by ASM bundles, decreased significantly with ageing ($P=0.02$; Fig. 3A). There was no significant difference in the mean ASM% of foals in the different age groups (see Appendix A: Supplementary material). With the exception of horses aged 11-20 years, in which ASM% decreased with increasing airway size ($P=0.04$), ASM% was not affected by airway size ($P>0.1$; Fig. 3B).

The increased ASM mass observed in the peripheral airways of severely asthmatic horses plays a central role in disease development and clinical presentation, as shown by a recent study in which a significant association was found between the degree of peripheral ASM remodeling and disease severity expressed in terms of lung function (Bullone, 2016). As both genetic and environmental factors contribute to severe equine asthma development (Leclerc et al., 2011b), the structural alterations of peripheral airways contributing to airflow obstruction could precede the appearance of clinical signs and occur earlier in a horse's life. Interestingly, the mild form of equine asthma known as inflammatory airway disease (IAD), which is common among young athletic horses and is considered a risk factor for the development of the severe form of the disease

83 (Bosshard and Gerber, 2014), is characterised by airway hyperreactivity, i.e. an exaggerated
84 bronchoconstrictive response. To elucidate the mechanisms driving ASM remodeling in equine
85 asthma, normal smooth muscle development needs to be evaluated first. Our study provides the first
86 data on peripheral ASM postnatal ontogenesis in the equine species. These results show that
87 peripheral airways of similar size have an ASM layer characterised by a constant thickness.
88 However, a higher proportion of the inner bronchial area is occupied by ASM in the fast-growing
89 foals compared to what is observed in adult horses. Such age-related decrease of ASM% can be
90 caused by an increased epithelial or lamina propria area, or both. Previous studies have reported an
91 unchanged peripheral ASM thickness in children vs. men (Hislop and Haworth, 1989), which is in
92 agreement with our results. An age-related decrease in peripheral ASM bundle size (corrected by
93 airway dimensions) was reported in rhesus monkeys which, if occurring in man as well, might
94 explain the increased airway hyperreactivity observed during childhood (Tran et al., 2004). In fact,
95 in airways of similar size, the ability of the ASM to contract and induce bronchospasm is
96 proportional to its mass. Increased ASM% has also been reported in young vs. old rabbits
97 (Ramchandani et al., 2000), while the same was not observed in swine (Murphy et al., 1991).
98 However, the latter study was limited to the large conducting airways. As a limitation of the current
99 study, our data are limited to small peripheral airways, and therefore, the observed effects of airway
100 size may not reflect what occurs when the entire bronchial tree is considered.

101

102 In conclusion, our study provides fundamental information on the anatomical development
103 of ASM mass in healthy horses and paves the way for detecting asthma-related alterations in this
104 process. The increased ASM% observed in young horses deserves attention as it could be associated
105 with increased hyperresponsiveness and may be implicated in the pathogenesis of mild forms of
106 equine asthma.

107

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115 **Appendix A: Supplementary material**

116 Supplementary data associated with this article can be found, in the online version, at doi: ...

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118 **Conflict of interest statement:**

119 None of the authors of this paper has a financial or personal relationship with other people or
120 organisations that could inappropriately influence or bias the content of the paper.

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164

165 **Table 1.**

166 Details of the horses in the different age groups.

	0-6 months	6-12 months	1-4 years	5-10 years	11-20 years	>20 years
<i>n</i>	11	7	9	8	9	7
Age ^a	0.5 ± 1 months	8 ± 2 months	2 ± 1 years	8 ± 2 years	16 ± 3 years	25 ± 3 years
Sex ^b	4:7	3:4	5:3	3:4	6:3	3:4
Pi [μm] ^a	876 ± 232	817 ± 318	851 ± 343	1022 ± 578	934 ± 685	1026 ± 407

167 ^a Expressed as mean ± standard deviation.

168 ^b Expressed as female:male ratio.

169 Pi: internal perimeter.

170 **Figures**

171

172 Fig. 1. Histomorphometric parameters assessed on peripheral airways. ASM% was calculated as
173 $(\text{ASM area}/[\text{ASM}_{\text{out}} - \text{airway lumen area}]) \times 100$. Airway lumen area is the area enclosed by Pi.
174 Scale bar: 50 μm. ASM: airway smooth muscle; ASM_{out}: area enclosed by the outer border of the
175 airway smooth muscle layer; Pi: internal perimeter.

176

Fig.

177

178 2. Effect of age (A) and airway size (B) on peripheral ASM thickness. Up to 10-fold variations were
 179 observed for ASM/Pi values measured in airways of similar size in the same subject. ASM/Pi:
 180 thickness of the peripheral airway smooth muscle layer. ASM: airway smooth muscle. Pi: internal
 181 perimeter.

182

Fig.

183

184 3. Effect of age (A) and airway size (B) on the percentage of peripheral inner airways occupied by
 185 ASM bundles. (B) Red line: horses 0-6 month old. Orange line: horses 6-12 month old. Light blue
 186 line: horses 1-4 year old. Blue line: horses 5-10 year old. Green line: horses 11-20 year old. Black
 187 line: horses >20 year old. ASM: airway smooth muscle. ASM%: percentage of peripheral inner
 188 airways occupied by ASM bundles. Pi: internal perimeter.

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