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### Age associated changes in peripheral airway smooth muscle mass of healthy horses

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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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7	Michela Bullone, Morgane Pouyet, Jean-Pierre Lavoie <sup>*</sup>
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10	Department of Clinical Sciences, Université de Montréal, 3200 rue Sicotte, St-Hyacinthe, J2S 2M2,
11	Quebec, Canada
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15	*Corresponding author: Tel: +1 450 7738521.
16	E-mail address: jean-pierre.lavoie@umontreal.ca (JP. Lavoie).

### 18 Abstract

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

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

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

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

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Peripheral lung tissues harvested post-mortem from healthy horses were obtained from the 47 Equine Respiratory Tissue Bank<sup>1</sup> or from the histological archives of the authors' institution. 48 Horses were defined as healthy based on history (absence of respiratory signs), blood work results 49 (when available), and histopathological findings. Histological sections of 5 µm thickness were 50 stained with hematoxylin-eosin-phloxyn-saffron (HEPS). Five airways per horse with a major to 51 minor axis ratio <1.5, with an intact epithelium, and with smooth muscle surrounding  $\geq$ 70% of their 52 53 circumference were studied. The ASM area, the outer border of ASM, and the internal perimeter length (Pi) were measured in cross-sectionally cut peripheral airways using Image J (NIH). ASM 54 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

<sup>1</sup> See: <u>www.ertb.ca</u> (accessed 04/05/2017).

57 airway wall area was calculated as the difference between the area enclosed by the external border 58 of ASM and the airway lumen area enclosed by Pi; Fig. 1). Measurements were performed by one 59 investigator blinded to the subject identity. For statistical analysis, horses were divided in six age 60 groups (0-6 months, 6-12 months, 1-4 years, 5-10 years, 11-20 years, and >20 years). Further 61 methodological details are provided online (see Appendix A: Supplementary material).

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Tissues harvested from 51 healthy horses ranging from 1 day to 32 years of age were 63 studied. Table 1 details their age, sex distribution, and average airway size. ASM/Pi, an indirect 64 measure of ASM thickness, did not change with age in the horses studied (P=0.3; Fig. 2A). 65 However, ASM/Pi significantly increased with increasing airway size when airways of all groups 66 were analysed together ( $r^2=0.11$ , P<0.0001; Fig. 2B). ASM%, which represents the percentage of 67 the inner bronchial area occupied by ASM bundles, decreased significantly with ageing (P=0.02; 68 69 Fig. 3A). There was no significant difference in the mean ASM% of foals in the different age 70 groups (see Appendix A: Supplementary material). With the exception of horses aged 11-20 years, 71 in which ASM% decreased with increasing airway size (P=0.04), ASM% was not affected by 72 airway size (P>0.1; Fig. 3B).

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74 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 75 which a significant association was found between the degree of peripheral ASM remodeling and 76 disease severity expressed in terms of lung function (Bullone, 2016). As both genetic and 77 environmental factors contribute to severe equine asthma development (Leclere et al., 2011b), the 78 structural alterations of peripheral airways contributing to airflow obstruction could precede the 79 80 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 81 horses and is considered a risk factor for the development of the severe form of the disease 82

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

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

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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:						
117							
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119	None of the authors of this paper has a financial or personal relationship with other people or						
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  of Applied Physiology 97, 2364-2371.
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# 165 **Table 1.**

	0-6 months	6-12 months	1-4 years	5-10 years	11-20 years	>20 years
n	11	7	9	8	9	7
Age <sup>a</sup>	$0.5 \pm 1 \text{ months}$	$8 \pm 2$ months	$2 \pm 1$ years	$8 \pm 2$ years	$16 \pm 3$ years	$25 \pm 3$ years
Sex <sup>b</sup>	4:7	3:4	5:3	3:4	6:3	3:4
Pi [µm] <sup>a</sup>	$876\pm232$	$817\pm318$	851 ± 343	$1022\pm578$	$934\pm685$	$1026\pm407$

166 Details of the horses in the different age groups.

167 <sup>a</sup> Expressed as mean  $\pm$  standard deviation.

<sup>b</sup> Expressed as female:male ratio.

169 Pi: internal perimeter.

# 170 **Figures**



Fig. 1. Histomorphometric parameters assessed on peripheral airways. ASM% was calculated as
(ASM area/[ASM<sub>out</sub> - airway lumen area])\*100. Airway lumen area is the area enclosed by Pi.

- 174 Scale bar: 50 μm. ASM: airway smooth muscle; ASM<sub>out</sub>: area enclosed by the outer border of the
- airway smooth muscle layer; Pi: internal perimeter.



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



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