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Technical and physiological determinants of airway smooth muscle mass in endobronchial biopsy samples of asthmatic horses

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Bullone M, Chevigny M, Allano M, Martin JG, Lavoie J-P. Technical and physiological determinants of airway smooth muscle mass in endobronchial biopsy samples of asthmatic horses. *J Appl Physiol* 117: 806–815, 2014. First published August 7, 2014; doi:10.1152/jappphysiol.00468.2014.—Morphometric analyses of endobronchial biopsies are commonly performed in asthma research but little is known about the technical and physiological parameters contributing to measurement variability. We investigated factors potentially affecting biopsy size, quality, and airway smooth muscle (ASM) content in heaves, an asthma-like disease of horses. Horses with heaves in clinical exacerbation ($n = 6$) or remission ($n = 6$) from the disease and six controls were studied using a crossover design. The effect of disease status, age, bronchodilation, biopsy forceps type, and carina size on total biopsy area (A_{tot}), ASM area (A_{ASM}), ASM% ($A_{\text{ASM}}/A_{\text{tot}}$), and histologic quality were assessed. Concordance among different measuring techniques was also assessed. Compared with other groups, horses with heaves in exacerbation yielded larger biopsies ($P < 0.05$). Better quality biopsies were obtained from carinae of small size compared with large ones ($P = 0.02$), and carina size and forceps type significantly affected the ASM content of the biopsy (interaction, $P < 0.05$). A_{ASM} increased with age only in heaves-affected horses ($r = 0.9$, $P < 0.05$), and ASM% was negatively correlated with pulmonary resistance at 5 Hz in heaves-affected horses ($r = -0.74$, $P = 0.01$), likely because of the increased thickness of the extracellular matrix layer in this group ($P = 0.01$). In conclusion, disease status, carina thickness, and the forceps used may significantly affect biopsy size, quality, and ASM content. Endobronchial biopsies are not appropriate samples for ASM quantification in heaves, and studies measuring ASM mass should not be compared when measuring techniques differ.

endobronchial biopsies; asthma; airway smooth muscle; remodeling; horse

ENDOBONCHIAL BIOPSIES ARE important diagnostic and research tools in respiratory medicine. At present, they are the only means allowing histologic evaluation of central bronchial architecture, inflammation, and remodeling in patients with diseases such as asthma, chronic obstructive pulmonary disease, and cystic fibrosis. Nevertheless, few studies have systematically investigated factors likely affecting biopsy quality, and the measurement of their airway smooth muscle (ASM) content. Also, the effect of lung diseases on biopsy morphological parameters has not been addressed, although endobronchial biopsies are anecdotally known to be easier to obtain in patients with asthma than in healthy individuals (10). Endobronchial biopsy in humans is routinely performed under local anesthesia, following patient premedication with bronchodilat-

ing agents, commonly a β_2 -selective agonist followed or not by atropine (6). However, biopsy collection in the absence of inhaled β -adrenergic agonists has been performed in healthy and asthmatic patients without obvious adverse effect (30). No data are available evaluating the effect of prior bronchodilator treatment on biopsy size and quality. Also, little information is available on the contribution of the biopsy site or the type of forceps used to the quality of the biopsies (15).

Endobronchial biopsies allow the study of ASM quantity and composition, which are considered central to airway obstruction in human asthma (5, 18, 19). Indeed, ASM mass is increased in small and large airways in those with asthma, likely induced by both cellular hyperplasia and hypertrophy (2). However, the lack of correlation between ASM mass in endobronchial biopsies and lung function has been reported in people with mild asthma (22) and in an asthma-like disease of horses (heaves) (16). Whether this is due to the variable makeup of contractile protein in ASM, technical bias due to the biopsy collection, or the measurement method used has not been studied. Interestingly, when ASM mass was evaluated in bronchial biopsies of asthmatics as ASM percentage, a significant decrease was reported after antigen challenge. This finding was completely reversed by pretreating patients with a β_2 -adrenergic agonist for 1 wk (12). The same reduction in ASM mass (%) was observed in heaves after antigen challenge in which bronchodilator pretreatment was not employed (16).

We hypothesized that administration of a bronchodilator prior to biopsy collection ameliorates specimen quality by inhibiting bronchospasm in asthma. The primary objectives were to evaluate 1) the effect of bronchodilation, forceps type, and the size of the carina on histomorphometric analysis of endobronchial biopsies in healthy and diseased airways; 2) the influence of different analysis methods on ASM mass; and 3) the relationship between endobronchial biopsy ASM content and lung function. This study was performed in horses with heaves, because remodeling of the airways in this natural disease has many similarities with that of human asthma (17). This model allows easy and relatively noninvasive bronchial tissue collection from central airways in vivo.

MATERIAL AND METHODS

Animals

Eighteen horses of mixed breed and age (15.1 ± 5.1 yr, mean \pm SD) from the equine research and teaching herds of the Faculty of Veterinary Medicine (Université de Montréal) were studied. Twelve horses had a documented history of heaves, whereas six control horses were considered free of respiratory disease on the basis of history, physical examination, and negative response to hay challenge. This study was approved by the Animal Care Committee of the Université

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de Montréal (Protocol Rech-1324) and conducted in compliance with guidelines of the Canadian Council on Animal Care.

Biopsy Specimen Collection and Processing

A videoendoscope (13 mm Ø, CF-H180AL; Olympus, Richmond Hill, ON, Canada) was passed through a nostril down to the lower airways in horses sedated with detomidine (0.012 mg/kg iv) and butorphanol (0.01 mg/kg iv). Airways were locally anesthetized with lidocaine solution (0.5%). Only one lung was sampled during each experiment. The right lung was biopsied during the first day of the study and the left lung during the second day. Biopsies were collected starting from the most caudal carina available [2.9 or 1.9, as described by Smith et al. (27)] and then moving cranially, until reaching the main carina, following the scheme illustrated in Fig. 1. Importantly, carinae were paired right-left on the basis of their dimensions and position along the bronchial tree to reproduce as much as possible the same conditions during the two experiments. Six biopsy specimens were obtained during each experiment. Biopsy sites were classified as small or large depending on carina size. Three small biopsies from small carinae and three from large carinae were collected during each experiment. The main carina of each horse was biopsied twice, at different sites. Biopsy specimens were formalin-fixed and then enclosed in agar cylinders (Fig. 2). Two 5-µm-thick histologic slides were obtained from each biopsy at 100- to 300-µm distances and stained with hematoxylin phloxine saffron.

Histologic Analysis

Slides were digitized at 20× magnification with the NanoZoomer 2.0-HT system (Hamamatsu Photonics, SZK, Japan). Biopsies were scored 1 (very poor) to 5 (optimal) for histological quality (Fig. 3). Total biopsy area (A_{tot} ; Fig. 4A), ASM area (A_{ASM} ; Fig. 4A), basal membrane length (BM, Fig. 4A), and the distance between epithelium and smooth muscle bundles were measured using ImageJ software (National Institutes of Health, Bethesda, MD). The A_{ASM}/A_{tot} ratio (or ASM%), the A_{ASM}/BM , and the A_{ASM}/BM^2 ratios were calculated for each biopsy. Two further morphological parameters were calculated with newCast software version 4.5.1.324 (Visiopharm, Denmark) using a stereology-based approach (Fig. 4D). ASM volume fraction [$V_{V(ASM)}$] was measured by point counting and expressed as the fraction of the total number of points falling on smooth muscle (P_{ASM}) over the total number of points falling on the biopsy (P_{ref}):

$$V_{V_{ASM}} = \frac{\sum P_{ASM}}{\sum P_{ref}}$$

The volume to surface ratio of smooth muscle per length of basal membrane [$V_{V(ASM)}/S_{V(BM)}$] was calculated by counting points falling on smooth muscle (P_{ASM}) and line intersections with the basal membrane (I_{BM}), as follows:

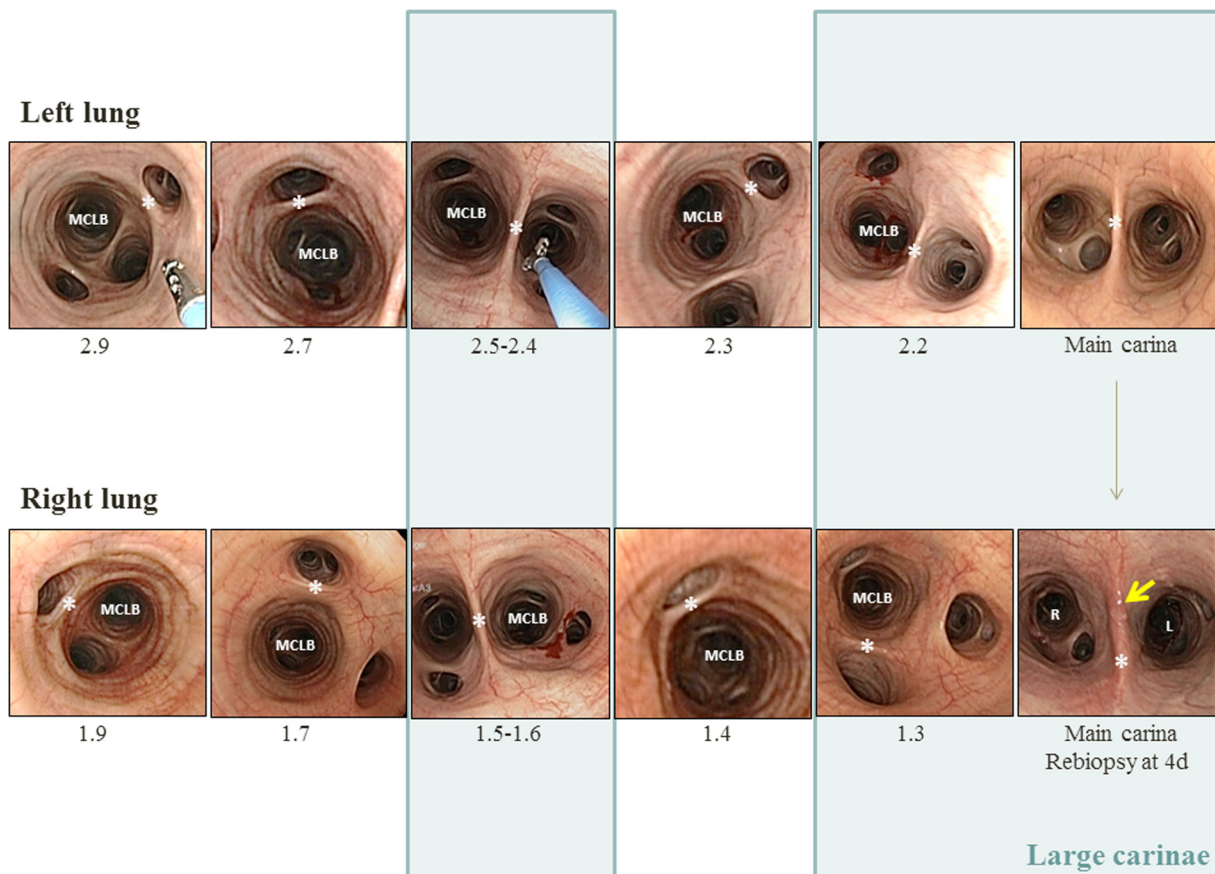


Fig. 1. Biopsy collection scheme. Biopsy sites are indicated with stars. Biopsies were collected from the same sites in all horses. Biopsy sites were classified as small or large depending on carina size. Large carinae were defined as those where the accessory segmental bronchus (only for right lung), first segmental bronchus (or bronchi for the left lung) conducting air to the ventral part of the lung, and intermediate segmental bronchus stem from the main caudal lobar bronchus. Large carinae are shown within the blue boxes. The difference in carina thickness between large and small carinae can be easily appreciated. The yellow arrow indicates the site of the previous biopsy (4 days before) in the main carina; adjacent tissues are visibly inflamed and edematous. MCLB, main caudal lobar bronchus.

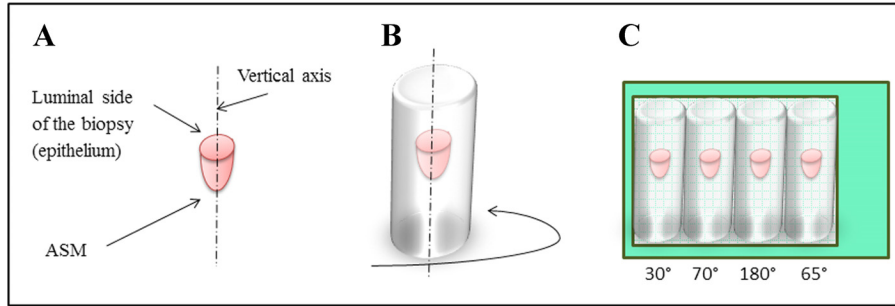


Fig. 2. Biopsy processing protocol. A: agar cylinders of 3 mm diameter and 20 mm length were obtained by slowly pouring a heated (37–40°C) solution of 4% agar into manufactured molds where biopsies were positioned with their vertical axis parallel to the vertical axis of the cylinders under stereoscopic microscope guidance. B: cylinders were allowed to harden at room temperature (about 20°C) for 15 min before being randomly rotated on their vertical axis. A cut was made parallel to a random angle using a radial support. C: agar cylinders were then positioned horizontally into the cassettes for paraffin embedding maintaining their random orientation, with their cut surfaces placed downward.

$$\frac{V_{V_{ASM}}}{S_{V_{BM}}} = \frac{P_{ASM} \cdot l(p)}{2 \sum I_{BM}}$$

where $l(p)$ was a constant determined by the density of line probes and corresponded to 0.02421. A minimum of 200 points was counted for ASM from at least two biopsies per horse. Coefficient of error of the measurements (CE) was <0.1 for all the investigated parameters. All measurements were performed blindly by the same investigator.

Respiratory Mechanics

Impulse oscillometry (IOS) was performed as described by van Erck et al. (28) with the Equine MasterScreen IOS system (Jaeger, Würzburg, Germany). In brief, horses breathed through a mask connected to a loudspeaker generating multifrequency impulses. The pressure-flow signal response of the respiratory system superimposed on the animal tidal breathing was measured by a pressure transducer

Biopsy	Score	Description
	1	Unacceptable tissue orientation, there is no continuity between epithelium, ECM and smooth muscle
	2	Good tissue orientation, tissue architecture not completely preserved.
	3	Good tissue orientation, tissue architecture preserved at least in a consistent part of the biopsy (>50%).
	4	Optimal tissue orientation for all the biopsy, minor area where continuity between tissues is lost.
	5	Optimal tissue orientation for all the biopsy, tissue architecture is perfectly conserved. The parenchymal borders of the smooth muscle layer are clearly identifiable

Fig. 3. Histologic quality score of the biopsies. Tissue orientation, architecture, structure preservation, and presence of airway smooth muscle (ASM) were evaluated to classify the biopsies for their quality using a score from 1 (poor quality) to 5 (optimal quality).

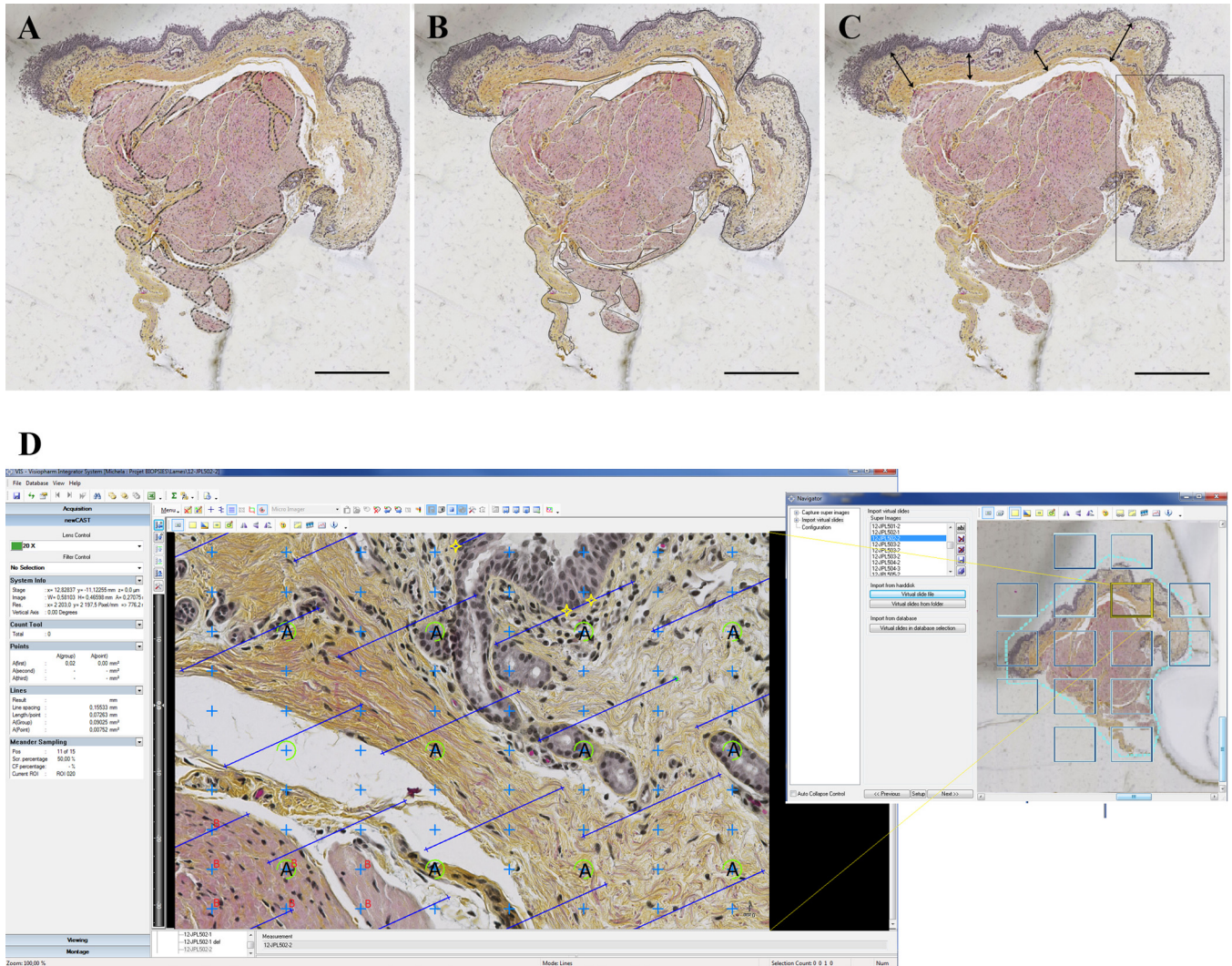


Fig. 4. Histologic analysis of the biopsies. ASM area (A_{ASM}) and total biopsy area (A_{tot}) are clearly outlined for an illustrative biopsy in *A* and *B*, respectively. In *C*, arrows indicate the measure of the distance between the epithelium and the ASM layer. Of note, areas of the biopsies in which collagen fibers were less dense due to significant focal tissue shrinkage or other procedural artifact (as those shown within the black box) were not considered for this type of measure (Scale bar = 0.5mm). *D*: a frame illustrating the software used for stereological analysis of the biopsies. *Left*: a magnification of the yellow area in the small *right* panel. Within the left panel, line grids were used for basal membrane surface density estimation. Note the yellow crosses at the intersection between line probes and basal membrane, indicating intersections with the basement membrane (I_{BM}). Blue crosses were used as probes for ASM mass; blue crosses with green circles were used as probes for reference volume. Crosses marked with letters (*A* for P_{ref} , *B* for P_{ASM}) were used for counts.

connected to a pneumotachograph and placed directly in front of the face mask. Data were acquired for a minimum of 30 s and analyzed (LabManager version 4.53; Jaeger, and FAMOS inc; Meßsysteme, Berlin, Germany) using Fast-Fourier transform to compute the resistance (*R*) and reactance (*X*) of the respiratory system at frequencies down to 1 Hz (25). Only values at frequencies ≤ 10 Hz were analyzed because they have been shown to reliably represent lung function in horses (13, 32). Recordings with a low coherence function were excluded from analyses (13).

Study Design

A crossover case-control study was performed. Six horses with heaves in clinical exacerbation of the disease (HE) and six control horses (C) were stabled and exposed to hay and dust starting 2 wk before the study period. Six additional horses with heaves were kept at pasture and administered dexamethasone (0.06 mg/kg PO q 24 h) starting 1 wk before the first biopsy collection and for the duration of the study to induce clinical remission of the disease (HR).

Horses were studied twice, at a 4-day interval, with and without administration of a bronchodilator agent (N-butylscopolammonium bromide, 0.3 mg/kg, Buscopan; Boehringer Ingelheim, Germany) (4). The order of treatment (bronchodilator vs. placebo) was randomly determined for each subject. Lung function was evaluated using IOS before (baseline) and 15 min after sedation, independently of whether or not they received the bronchodilator 5 min after sedation. During the first series of experiments, three of the six biopsies randomly chosen for each horse were obtained with a smooth oval disposable biopsy forceps (2.85 mm in diameter, FB-234U; Olympus), whereas the remaining three were taken with an alligator jaw disposable forceps (2.85 mm in diameter, FB-214U; Olympus). During the second series, forceps selection was simply inverted.

Statistics

Statistical analysis was performed with SAS v.9.3 (SAS, Cary, NC) and Prism 5 (GraphPad Software, La Jolla, CA). The effect of technical parameters (bronchodilator administration, forceps type,

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carina size, and disease status) on morphological variables (A_{tot} , A_{ASM} , and $\text{ASM}\%$) was studied with a repeated-measures linear model. The effect of technical parameters on biopsy quality was assessed with a generalized estimating equation model for ordinal variables. Technical parameters were always considered as intrasubject factors. The effect of multiple uses of the forceps on biopsy quality was assessed with the Cochran-Mantel-Haenszel test, considering repeated measures observed for every subject. Rebiopsy parameters were analyzed with two-way ANOVA and a Bonferroni post test. One-way ANOVA or χ^2 test was used for comparison of continuous variables or proportions between the three groups. Paired t -tests were used for comparison of lung function parameters before and after bronchodilator administration within the same group. Correlations were assessed with a Pearson test when $n > 8$ and data were distributed normally (Kolmogorov-Smirnov test) or Spearman test when $n < 8$ or data were not normally distributed. For all analysis, a value of $P < 0.05$ was considered significant.

RESULTS

Biopsy specimens. The procedure was well tolerated by all horses, with only mild bleeding occasionally noticed at the biopsy site. A total of 216 biopsies were collected during the study period, all of which were considered of appropriate size. Two biopsies were lost during the embedding process; 214 biopsies were finally analyzed.

Overall, the quality of 39.2% of biopsies was considered to be good to optimal (scores 4 and 5), 20.6% as partially suitable for assessment (score 3), and 40.2% were poor or very poor quality (scores 2 and 1) and not suitable for analysis. Smooth muscle bundles were identified in 84.6% of biopsies, with no significant differences between groups ($P = 0.98$).

Technical variables. Disease status had a strong effect on A_{tot} ($P = 0.003$), with the size of the biopsies collected from heaves-affected horses during exacerbations being significantly larger than biopsies obtained from both control horses and those with heaves in remission ($P < 0.05$). No significant difference was found between A_{tot} of heaves asymptomatic and control horses (Fig. 5A). Biopsy A_{ASM} ($P = 0.38$; Fig. 5B), $\text{ASM}\%$ ($P = 0.87$; Fig. 5C), and the quality score of biopsies ($P = 0.49$; Fig. 5D) were not affected by disease status.

Bronchodilation did not affect A_{tot} ($P = 0.16$), A_{ASM} ($P = 0.2$), $\text{ASM}\%$ ($P = 0.64$), or biopsy quality score ($P = 0.11$).

The size of the carina and the forceps type did not significantly affect A_{tot} ($P = 0.73$ and $P = 0.3$, respectively), A_{ASM} ($P = 0.46$ and $P = 0.85$, respectively), or $\text{ASM}\%$ ($P = 0.49$ and $P = 0.5$, respectively). However, significant interactions were found between the size of the carina and the forceps used, both for A_{ASM} and $\text{ASM}\%$ ($P = 0.046$ and $P = 0.04$, respectively). This was due to a trend for A_{ASM} ($P = 0.06$) and $\text{ASM}\%$ ($P = 0.052$) to be lower in large carinae compared with small ones with smooth oval forceps, but not with alligator jaw forceps ($P = 0.47$ and $P = 0.36$, respectively). There was also a trend ($P = 0.058$) for $\text{ASM}\%$ to be higher in biopsies obtained from small carinae with smooth oval forceps compared with those obtained with jaw alligator forceps. No difference was found between mean $\text{ASM}\%$ of biopsies obtained from large carinae with smooth oval forceps compared with those obtained with jaw alligator forceps ($P = 0.36$).

The quality of biopsies was not influenced by the forceps used ($P = 0.08$). However, the size of the carina had a significant effect on histologic quality of the biopsies ($P = 0.02$). There was a 2.2 times greater probability of obtaining an optimal quality biopsy when the sampling site was a small carina compared with a large one. Reutilizing the same forceps for collecting biopsies from 6 to 8 horses did not result in significant changes detectable on biopsy quality ($P = 0.61$) or biopsy size ($P = 0.41$).

Only the main carina was biopsied twice at a 4-day interval in each horse, and at a distance allowing the avoidance of a biopsy being obtained that overlapped the site of the first biopsy taken. Rebiopsy did not significantly affect A_{tot} ($P = 0.19$), however, a significant effect of the group was observed for A_{tot} ($P = 0.009$). Interestingly, A_{tot} was increased in rebiopsies of 67% of horses with heaves in exacerbation and controls, but not in horses with heaves in clinical remission. $\text{ASM}\%$ was significantly decreased in rebiopsies ($P = 0.005$) and a similar trend was observed for A_{ASM} ($P = 0.05$). Biopsy quality was similar in the first and second biopsies ($P = 0.65$).

Analysis methods. When only good-quality biopsies (grades 3 to 5) were analyzed, smooth muscle content increased significantly compared with all biopsies analyzed together, independently of the parameter used for its quantification (from 0.54 ± 0.31 to $0.68 \pm 0.33 \text{ mm}^2$ for A_{ASM} , $P = 0.0003$; and from $25 \pm 7\%$ to $32 \pm 6\%$ for $\text{ASM}\%$, mean \pm SD; $P = 0.005$). A_{ASM} and $\text{ASM}\%$ increased on average 26% and 23%, respectively, after exclusion of poor-quality biopsies. Also, intrasubject variability expressed as the coefficient of variation decreased significantly when only good-quality biopsies were taken into account ($P = 0.002$ with an average 25% reduction in variability for A_{ASM} , and $P = 0.0001$ with an average 35% reduction in variability for $\text{ASM}\%$). Comparison between all and only good-quality biopsies was not performed for A_{ASM}/BM , $A_{\text{ASM}}/\text{BM}^2$, $V_{V(\text{ASM})}$, and $V_{V(\text{ASM})}/\text{SV}(\text{BM})$ because these parameters were calculated only for good-quality biopsies. Considering only good-quality biopsies, intrasubject variability of smooth muscle quantification varied significantly depending on the analysis method used ($P = 0.02$), and it was higher when expressed as A_{ASM} compared with $\text{ASM}\%$ or $V_{V(\text{ASM})}$ for the same biopsy ($P < 0.05$).

In good-quality biopsies, correlations between $\text{ASM}\%$ and its stereological equivalent, $V_{V(\text{ASM})}$, and between ASM/BM

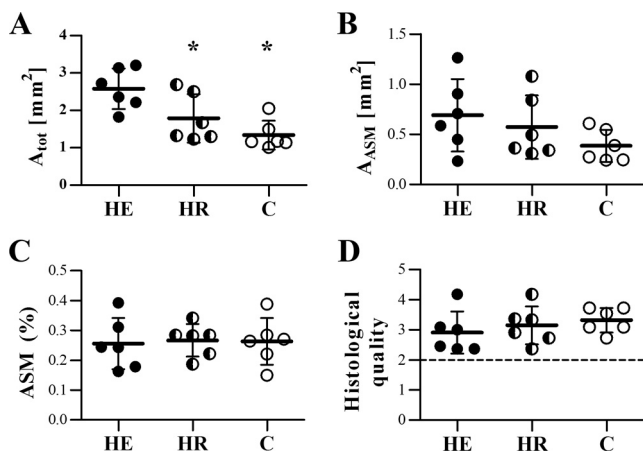


Fig. 5. Effect of disease status on A_{tot} (A), A_{ASM} (B), $\text{ASM}\%$ ($A_{\text{ASM}}/A_{\text{tot}}$) (C), and histological quality (D) of biopsies. Each point represents the mean value of all biopsies obtained from each animal (quality 1 to 5), excluded the rebiopsy site of the main carina. In B and C, biopsies in which ASM was not present were included and the value of 0.00 was used for calculating the means. HE, heaves exacerbation; HR, heaves remission; C, controls.

Table 1. Correlation among different measuring methods

	Control	Heaves Remission	Heaves Exacerbation	All Groups	Difference Between Groups
Correlation					
A_{ASM} , mm ² - ASM ratio, %	0.186 (0.62)	0.383 (0.44)	0.424 (0.41)	0.084 (0.42)	
A_{ASM} , mm ² - A_{ASM}/BM , mm	0.082 (0.76)	0.778 (0.15)	0.355 (0.46)	0.118 (0.38)	
A_{ASM}/BM , mm - ASM ratio, %	0.123 (0.70)	0.007 (0.93)	0.606 (-0.27)	0.045 (0.47)	
A_{ASM}/BM^2 - ASM ratio, %	0.075 (0.77)	0.041 (0.83)	0.598 (-0.32)	0.073 (0.44)	
$V_{V(ASM)}$ - ASM, mm ²	0.129 (0.69)	0.581 (0.29)	0.26 (0.62)	0.236 (0.29)	
$V_{V(ASM)}$ - ASM ratio, %	0.016 (0.89)	0.015 (0.89)	0.004 (0.98)	<.0001 (0.93)	
$V_{V(ASM)}$ - A_{ASM}/BM , mm	0.008 (0.92)	0.003 (0.96)	0.20 (0.61)	0.0007 (0.72)	
$V_{V(ASM)}$ - A_{ASM}/BM^2 , mm	0.031 (0.85)	0.011 (0.91)	0.565 (0.30)	0.004 (0.64)	
$V_{V(ASM)}$ - $V_{V(ASM)}/S_{V(BM)}$, mm	0.488 (-0.36)	0.093 (0.74)	0.014 (0.90)	0.011 (0.57)	
$V_{V(ASM)}/S_{V(BM)}$, mm - A_{ASM} , mm ²	0.699 (0.20)	0.485 (0.36)	0.43 (0.66)	0.08 (0.42)	
$V_{V(ASM)}/S_{V(BM)}$, mm - ASM ratio, %	0.80 (-0.13)	0.005 (0.94)	0.33 (0.55)	0.035 (0.51)	
$V_{V(ASM)}/S_{V(BM)}$, mm - A_{ASM}/BM , mm	0.051 (0.64)	0.039 (0.83)	0.028 (0.86)	0.0001 (0.78)	
$V_{V(ASM)}/S_{V(BM)}$, mm - A_{ASM}/BM^2 , mm	0.927 (-0.05)	0.098 (0.73)	0.339 (0.58)	0.002 (0.66)	
A_{ASM} , mm ²					0.04*
ASM ratio, %					0.96*
A_{ASM}/BM , mm					0.32*
A_{ASM}/BM^2					0.70*
$V_{V(ASM)}$					0.72*
$V_{V(ASM)}/S_{V(BM)}$, mm					0.41*

ASM, airway smooth muscle; A_{ASM} , ASM area; BM, basal membrane; $S_{V(BM)}$, basal membrane surface density; $V_{V(ASM)}$, ASM volume fraction; $V_{V(ASM)}/S_{V(BM)}$, smooth muscle volume per surface of basal membrane. Data are expressed as the P (r) values resulting from Spearman (controls, heaves remission and heaves exacerbation) or Pearson (all groups) correlation tests or as P values from one-way ANOVA test. Overall, there was poor correlation among different measuring methods, except for $V_{V(ASM)}$ and ASM%, and $V_{V(ASM)}/S_{V(BM)}$ and A_{ASM}/BM . Also, using different methods may lead to different results: a significant difference was indeed observed among the 3 groups for A_{ASM} values ($P = 0.04$) but not for the other measuring techniques ($P > 0.05$). *Via one-way ANOVA. Bold characters identify statistically significant differences.

and its stereology equivalent, were significant in all groups, confirming the appropriateness of our counts (Table 1). However, correlations among different measuring techniques are poor. Also, the measuring technique can significantly affect the outcome of the study (Fig. 6).

Structure-function relationships and other physiological variables. There was a significant difference in total lung resistance (R_3 , $P = 0.02$), reactance (X_3 , $P = 0.009$), and $R_5:R_{10}$ ratio ($P = 0.01$) among the three groups of horses at baseline (Table 2). Higher values of R_3 , X_3 , and $R_5:R_{10}$ were observed in horses with heaves in exacerbation compared with controls ($P < 0.05$). Values of airway function (Z_5 , R_3 , X_3) were not significantly different during the 2 days of experimentation for each group ($P > 0.05$). Sedation with detomidine and butorphanol caused a significant increase in inspiratory resistance values, suggesting upper airway obstruction. Buscopan administration, but not placebo treatment, caused a bronchodilation in horses with heaves whether in exacerbation or in

remission of the disease, as shown by the significant decrease in the $R_5:R_{10}$ ratio (29) (Table 2 and Fig. 7).

When only good-quality biopsies were included in the analysis, baseline R_5 values were positively correlated with ASM% ($r = 0.84$, $P = 0.03$) in control horses, but only when bronchodilation was induced (Fig. 8A). Interestingly, a negative correlation was observed between baseline R_5 and ASM% in heaves-affected horses after bronchodilation (horses with heaves in exacerbation and remission of the disease pooled together, $P = 0.01$, $r = -0.74$; Fig. 8B). The negative correlation could be explained by an increased volume of the extracellular matrix in heaves-affected horses, because the distance between epithelium and smooth muscle bundles was increased compared with controls (0.143 ± 0.049 mm in horses with heaves, 0.082 ± 0.031 mm in controls, mean \pm SD; $P = 0.01$; Fig. 9) and the external border of the smooth muscle bundle was less frequently identifiable in biopsies from these animals (32% and 13% of biopsies from controls and horses in exacerbation, respectively).

When only good-quality biopsies were considered, A_{ASM} was strongly correlated with age in both groups of horses with heaves (exacerbation $P = 0.04$, $r = 0.90$; remission $P = 0.03$, $r = 0.91$) but not in control horses ($P = 0.85$, $r = 0.10$). Moreover, the regression lines derived from these data were significantly different between horses with heaves in exacerbation and remission of the disease (similar slopes, $P = 0.09$; but different intercepts, $P = 0.02$), as well as between horses with heaves in exacerbation and controls (different slopes, $P = 0.049$). No significant differences were observed between horses with heaves in remission and controls, possibly because of the absence of very old horses in the control group (Fig. 10A). The same trend was observed for A_{tot} , but correlation coefficients did not reach significance ($r = 0.82$, $P = 0.09$ for horses with

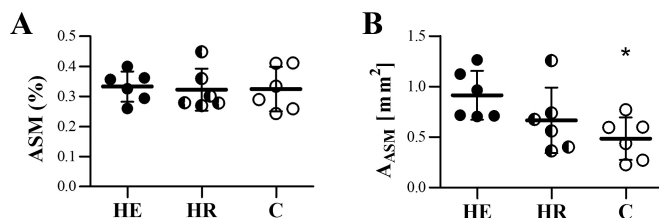


Fig. 6. ASM remodeling expressed with two different measuring techniques in the same samples. Only good-quality biopsies were used (score >2). In A, data are expressed as ASM%, and no difference is observed among the three groups ($P = 0.96$). In B, data are expressed as A_{ASM} (mm²), and a significant difference is detected among the three groups ($P = 0.04$), with A_{ASM} of controls (C) being significantly lower than those of horses with heaves in exacerbation (HE, $P < 0.05$).

Table 2. Lung function measured by impulse oscillometry system (IOS)

	Control	Heaves Remission	Heaves Exacerbation	P
Baseline				Group effect
R ₃	0.0648 (±0.0177)	0.0805 (±0.0308)	0.1340 (±0.0381)*	0.02
X ₃	0.0074 (±0.0089)	-0.008167 (±0.0301)	-0.05683 (±0.0562)*	0.009
R ₅ :R ₁₀ placebo	0.7052 (±0.19)	0.9309 (±0.2325)	1.214 (±0.1477)*	0.01
R ₅ :R ₁₀ buscopan	0.8526 (±0.167)	1.219 (±0.696)	1.215 (±0.217)	0.06
Z ₅ placebo	0.0720 (±0.0228)	0.09167 (±0.0204)	0.1050 (±0.0302)	0.08
Z ₅ buscopan	0.0700 (±0.0187)	0.0767 (±0.025)	0.09833 (±0.0319)	0.25
15 min postsedation				
R ₅ :R ₁₀ placebo	0.7761 (±0.059)	0.952 (±0.196)	1.16 (±0.289)	0.13
R ₅ :R ₁₀ buscopan	0.8854 (±0.198)	0.7447 (±0.132)†	0.904 (±0.172)†	0.21
Z ₅ placebo	0.1167 (±0.055)	0.08167 (±0.0256)	0.1000 (±0.0473)	0.39
Z ₅ buscopan	0.085 (±0.051)‡	0.0740 (±0.0114)	0.095 (±0.0191)	0.35

IOS was performed before and 15 min after sedation. Bronchodilator (buscopan) or placebo was administered 5 min after sedation. Data are expressed as mean (± SD). Resistance (R), reactance (X), and impedance (Z) values are expressed in kPa-liter⁻¹·s⁻¹. P values reported express whether differences were observed among the three groups. *Different from control group (one-way ANOVA and Dunn's post test). †Different from R₅:R₁₀ buscopan at baseline (P = 0.03 for HR and P = 0.02 for HE, paired t-test). ‡Tends to be different from Z₅ placebo at 15 min postsedation (P = 0.07, paired t-test). Bold characters identify statistically significant differences.

heaves in exacerbation; r = 0.63, P = 0.17 for horses with heaves in remission; and r = 0.08, P = 0.9 for controls). However, a significant difference was found between the regression lines constructed for the three groups from A_{tot} values plotted vs. age (similar slopes, P = 0.6; different intercepts, P = 0.0009; Fig. 10B).

DISCUSSION

Previous studies reported a decrease in ASM mass in endobronchial biopsies of patients with asthma and horses with heaves after antigen exposure (12, 16). We postulated that methodological factors contributed to these findings, and therefore we investigated the effect of technical and physiological variables possibly affecting endobronchial biopsy quality and morphology. Contrary to what we had hypothesized, bronchodilation did not improve biopsy morphology or quality; however, it allowed normalization of the structure-function relationships in our study. Indeed, when analyzing only biopsies

obtained after bronchodilation, we detected significant negative correlations between ASM content of the biopsies (ASM%) and lung function in horses with heaves and controls, clearly linked to the remodeling features of the bronchial extracellular matrix. Also, biopsies obtained during heaves exacerbation (comparable to asthmatic attacks) were significantly larger (increased A_{tot}) than those obtained during the remission phase of the disease (comparable to the controlled asthmatic state) or from control horses, indicating that disease status is a critical determinant of commonly employed histomorphometric parameters. Biopsies from medium and small carinae increase the probability of obtaining good quality biopsies with greater quantity of smooth muscle, especially when forceps with smooth cutting surfaces are employed. Nevertheless, quantification of airway smooth muscle mass is not reliable when performed on equine endobronchial biopsies, independently on the sampling techniques and analysis methods. Indeed, because even larger forceps rarely sample the full depth of the smooth muscle layer, ASM mass assessment can be biased if growth of smooth muscle cells is directed toward the adventitial layer.

Effect of Technical Variables on Biopsy Size and Quality

The size and quality of biopsy specimens can greatly affect the assessment of morphological and histopathological features (10). We used the largest biopsy forceps instrument passing

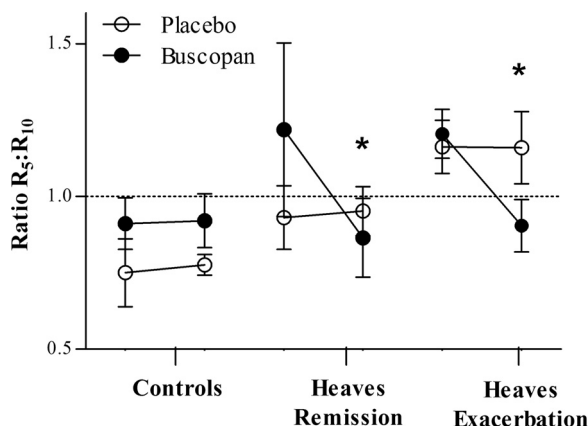


Fig. 7. Bronchodilator effect on R₅:R₁₀ ratio. When horses received placebo (saline, white points), no change was observed in the R₅:R₁₀ ratio at baseline and 15 min after sedation/placebo, meaning that airflow obstruction was not significantly affected by sedation/placebo administration in the three groups. When horses received bronchodilator (buscopan, black points), the R₅:R₁₀ ratio did not change in control horses but it significantly decreased in horses with heaves (both in exacerbation and in remission, P < 0.05), indicating that airway obstruction was reduced after sedation/bronchodilator treatment in this group of animals.

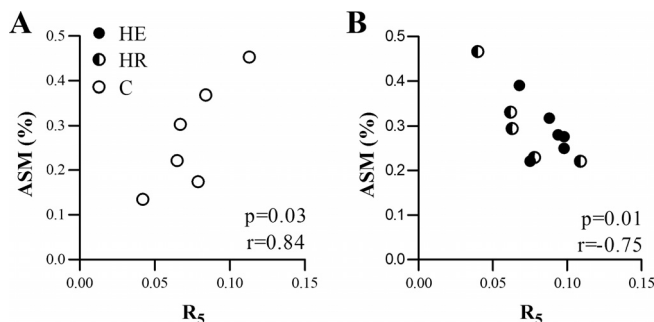


Fig. 8. Correlation between ASM% and baseline peripheral lung resistance during days in which buscopan was administered prior to endobronchial biopsy collection in control (A) and heaves-affected horses (B).

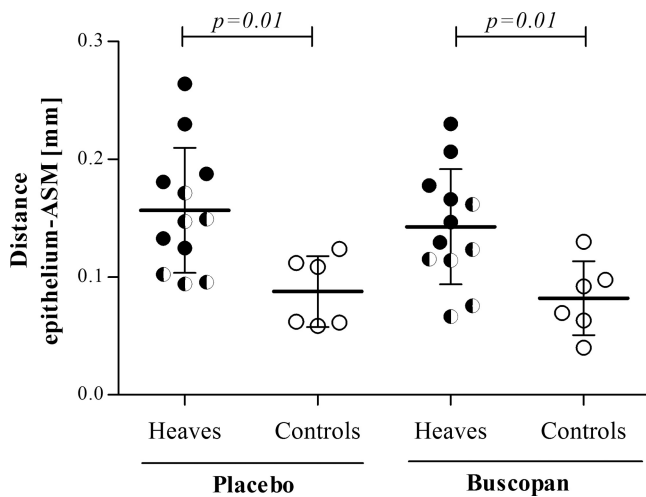


Fig. 9. Extracellular matrix thickness, evaluated as the distance between the bronchial epithelium and the ASM layer, is greater in horses with heaves compared with controls. Bronchodilation treatment had no significant effect on this parameter.

through the working channel of the endoscope to maximize biopsy size (10, 23), which explains why we obtained larger biopsies than those generally obtained in humans [on average, A_{tot} was 2 mm² in horses and 1 mm² in humans (15)]. However, we likely sampled the bronchial tissues to a similar depth, because the central equine airways are also larger than human ones. Indeed, ~60% of our attempts provided samples suitable for histologic assessment, which is a similar result to that observed for adults with asthma (15). A_{tot} was significantly affected by disease status, with horses during disease exacerbations (similar to asthma attacks) yielding larger biopsies than control horses or horses in clinical remission of the disease. At first sight, these results contrast with findings in human in which no significant differences were found in A_{tot} of biopsies from asthmatic and control patients (15, 23), or from patients with severe and moderate asthma (11, 22). However, in these studies, patients with asthma were under treatment or their disease was stable for months prior to sampling. Our results are thus in agreement with these findings because no significant differences were shown in the size of biopsies between horses with heaves in remission of the disease and controls. We attributed the increased A_{tot} of biopsies of subjects with heaves in exacerbation to the increased tissue inflammation during the active phase of the disease, leading to edema and tissue fragility. Very little is known about tissue morphology during spontaneous asthmatic attacks in humans due to ethical, management, and safety concerns, and equine heaves is perhaps the only animal disease that allows studying this aspect of the disease prospectively.

Biopsy quality was significantly affected only by carina size. Smaller carinae were 2.2 times more likely to provide good-quality biopsies than larger ones. Despite large forceps being used, main carina and carinae of the first generation of equine bronchi are often too thick to allow optimal forceps gripping, especially in symptomatic horses (14). On the other hand, contrary to what is reported in humans (15), almost all of the accessible carina sites in the horse are large enough to provide good-quality endobronchial biopsies of the airway wall. This finding may explain why in targeting smaller carinae improves

the histological quality of the biopsy and the possibility of sampling the entire ASM layer in horses.

Effect of Technical Variables on ASM Content

In our study, endobronchial biopsy ASM mass was not significantly affected by disease status when all biopsies were analyzed together. Despite an obvious trend being observable for A_{ASM} , differences did not reach statistical significance, likely due to the relatively small number of animals studied and the high intragroup variability of the data. However, excluding poor-quality biopsies, we found a significant effect of disease status only when ASM was quantified as A_{ASM} . Similarly, differences between A_{ASM} values of horses with heaves in exacerbation and in remission were not statistically significant, possibly because of lack of statistical power. We are aware of a single previous study in which a significant difference was found between A_{ASM} of patients with moderate and severe asthma (22). More often ASM remodeling is reported as ASM%, and differences between asthmatics and controls are observed in most (1, 23, 26) but not all (15, 21, 26) studies investigating this aspect. ASM% did not change significantly among groups in our study. Importantly, biopsies were collected from the same sites in all subjects to avoid differences arising as a consequence of the different proportion of small vs. large carinae sampled. Indeed, we have shown that carina size significantly affects ASM content of the biopsy, especially when forceps with smooth cutting surfaces are employed.

Rebiopsy Findings

The same carina may be biopsied twice or more in prospective human studies, due to the limited number of sites available to perform endobronchial biopsies (15). Because the effects of rebiopsying the same site on biopsy size or morphology are not known (10), we sampled the main carina on two occasions 4 days apart. Rebiopsy was associated with a generalized increase in A_{tot} , which we hypothesized was the consequence of the inflammation induced by the first biopsy, because this effect was prevented by the administration of dexamethasone (group in remission of the disease). Also, ASM mass decreased in rebiopsies, indicating that inflammation-induced submucosal edema possibly increased the distance between epithelium and ASM, as previously shown in asthmatics (22). As a consequence, short-term rebiopsy of the same site should be

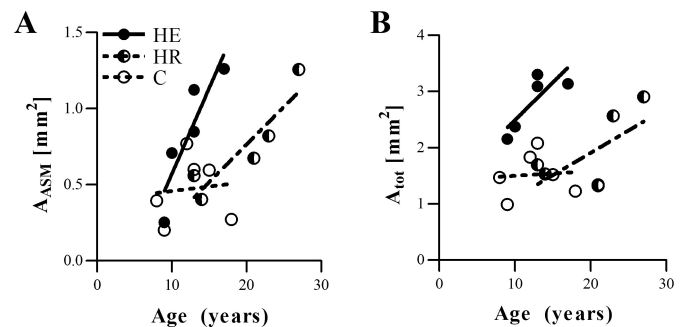


Fig. 10. Correlation between age of horses and A_{ASM} (A) or total biopsy area (B). In horses with heaves, but not in controls, age is significantly correlated with A_{ASM} . Interestingly, disease exacerbation significantly shifts the regression line toward the left. A similar trend can be observed for total biopsy area (see the text for further details).

avoided when investigating ASM mass or extracellular matrix content, even when performed at sites that visually appear to lack inflammation. Further studies may clarify how much time should elapse before a rebiopsy could be obtained without significant effect on tissue morphology in the absence of anti-inflammatory therapy.

Comparison Among Different Measuring Techniques

Endobronchial biopsy is the gold standard for the study of central airway remodeling (3). However, the high intersubject (up to 70%) and intrasubject (20%) biological variability (10), and the lack of standardization of methods employed for tissue structure analysis (9) limit the usefulness of these measurements. Remarkably, identification of external ASM boundaries, which is required to ensure that the full thickness ASM was sampled, does not represent a parameter constantly considered for biopsy quality assessment. Stereology has been proposed as the method of choice for lung morphometric studies because it provides accurate quantitative data (8). However, it requires that the reference space is known and sampling is unbiased (20). In endobronchial biopsies, the reference space is unknown, and sampling is biased because it is limited to carinae and by the penetrating capacity of the forceps used (31). Basal membrane length should be employed as a correction factor because it avoids the reference trap when the reference space is unknown (8). However, there is no method available to correct for the incomplete and variable (nonquantifiable) sampling of the ASM layer. For these reasons and to assure unbiased analysis, we suggest that future studies include only biopsies in which the external border of the ASM is clearly identifiable. A further confounding factor when comparing different studies is the measurement unit used. We have shown that data obtained using different measurement units on the same samples do not correlate as had been previously reported for human patients (15). For these reasons, direct and deliberate comparison of studies employing different units should be avoided.

Structure-Function Relationships

As a secondary outcome, we analyzed the relationship between structural ASM parameters and physiologic functional data. We showed that values of resistance at 5 Hz correlated with ASM% in both controls and heaves-affected horses only after bronchodilation. These findings suggest that bronchospasm is induced by the biopsy procedure at least in control animals, which can possibly alter tissue remodeling measurements. In agreement with our findings, excluding data of asthmatic children unresponsive to bronchodilator therapy ameliorated the correlation between ASM mass in endobronchial biopsies and lung function (24), thereby supporting the fact that inhibiting bronchoconstriction normalizes ASM remodeling data. In horses with heaves, less smooth muscle (%) was present in the biopsy as lung resistance increased. This was likely caused by the thickening of the extracellular matrix layer, which widens the distance between epithelium and smooth muscle, as is also reported to occur in people with asthma (1). In healthy people, ASM has been shown to increase linearly with airway diameter during infancy, but when adult age is reached, the proportion of ASM within the airway wall stabilizes (7). The same seems to be true for healthy horses. On the other hand, aging (or disease duration) progressively in-

creases the absolute quantity of ASM (A_{ASM}) in endobronchial biopsy samples of horses with heaves, but not its ASM% value. The reasons for these findings are still unclear, but likely reflect changes in tissue viscoelasticity and the shape of the carinae with aging. Furthermore, the significant difference observed between the A_{ASM} regression lines of horses with heaves in exacerbation and in remission indicates that such increase in A_{ASM} occurs even more rapidly during active phases of the disease, possibly driven by inflammation, which increases tissue fragility.

Conclusions and Perspectives

In conclusion, our study provides evidence that although bronchodilation does not affect endobronchial biopsy morphological parameters, it does improve the relationship between ASM in the central airways and lung function. Horses with heaves produce larger biopsies during disease exacerbations, with ASM mass being positively correlated with age and negatively correlated with disease severity as measured by lung resistance. However, endobronchial biopsy provides inappropriate samples for quantitative studies of ASM remodeling, at least in horses.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.B., J.G.M., and J.-P.L. conception and design of research; M.B., M.C., and M.A. performed experiments; M.B. analyzed data; M.B. and J.-P.L. interpreted results of experiments; M.B. prepared figures; M.B. drafted manuscript; M.B., J.G.M., and J.-P.L. edited and revised manuscript; M.B., M.C., M.A., J.G.M., and J.-P.L. approved final version of manuscript.

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