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Original Article

Morphometric analysis of the non-epithelial areas of mouse bronchioles through the normal aging process

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Abstract: Aging is associated with changes in the structure and function of the lung that may increase susceptibility to chronic lung diseases. The aim of this study was the morphometric assessment of the non-epithelial areas of the bronchioles of mouse through the normal aging process. Lungs from CD1 mice at the age of 2, 6, 12, 18, or 24 months were fixed in neutral-buffered formalin and paraffin-embedded. Sections were cut, stained with Masson trichrome, and examined using a light microscope. High-resolution color images were captured using a camera linked to image analysis software to measure areas and lengths. We observed in the bronchioles through the aging process an increase of the total area, an increase of the lumen area, and a decrease of the wall area. In conclusion, our results revealed structural changes in the bronchioles of mouse through the normal aging process. These alterations are likely to contribute to development of chronic lung diseases.

Keywords: Bronchiolar structure, aging, mouse, morphometry

Introduction

Aging is associated with changes in the structure and function of the lung that may increase susceptibility to chronic lung diseases [1, 2]. Alveolar structure has been the factor most frequently analyzed in lung aging-related studies. Senile lungs are characterized by an enlargement of the alveolar airspaces. Alveolar enlargement causes a reduction in maximum achievable flow in the airways during the breathing cycle [3-5].

Chronic respiratory disorders such as chronic obstructive pulmonary disease (COPD), asthma, and cystic fibrosis involve pathological changes in small airways, including epithelial cell injury, inflammation, and obstruction [6, 7]. However, changes in small airways during the aging process have received little attention.

We previously analyzed the cell turnover in the bronchiolar epithelium of mouse through the normal aging process and found a decrease in the total number of epithelial cells and a thinning of the epithelium in the aged mice [8]. The aim of this study was the morphometric assessment of the non-epithelial areas of the bronchioles of mouse through the normal aging process.

Material and methods

Animals and experimental design

Animals and experimental design used were described in a previous paper [8]. Male CD1 mice were examined; they were kept in standard conditions: stainless steel cages, temperature 18-21°C, 55-60% relative humidity, 12:12 h day-night cycle, and provided with food and water ad libitum. Three animals were quickly sacrificed by cervical dislocation at the age of 2, 6, 12, 18, or 24 months. To more consistently control the selection of sample sections, only the right lungs were processed and analyzed. Animal care was provided in accordance with

the principles and procedures outlined in the National Research Council Guide for the Care and Use of Laboratory Animals (8th edition), and in the Mexican Guidelines Z00-062.

Lungs were fixed in 10% neutral-buffered formalin and embedded in paraffin. Serial 5-μm sections were cut, deparaffinized in xylene, and hydrated in graded alcohol solutions. Sections were stained with Masson trichrome by a standard protocol. Analyses were conducted using three tissue sections per animal, taken from the middle of the lung, so that a portion of each lung lobe was included in each section.

Airways are generally classified as a bronchus by the presence of cartilage in the wall, a bronchiole by the absence of cartilage in the wall or a respiratory bronchiole by occasional alveolar outpocketings along the airway wall [9]. The airways analyzed in this study were all noncartilaginous bronchioles. All available bronchioles for each mouse were analyzed. On average, four airways per slide were measured (range 1-10). Only transversely cut bronchioles were analyzed, defined as those showing a short/long diameter ratio greater than 0.6 [10]. Only airways with a visible full perimeter were considered. In cases in which the bronchiole was contiguous to an adjacent vessel, a line was drawn between the two structures to separate them [11].

Bronchioles were excluded from analysis if the entire bronchiole could not be included in the photograph (magnification ×400), if the basement membrane length was > 1000 μm, if the epithelial borders were not well defined, or if the epithelial layer was disrupted [12].

Morphometry

Sections were examined using a Primo Star light microscope (Carl Zeiss Microscopy GmbH, Oberkochen, Germany), and high-resolution color images were captured using a Axio-Cam ICc1 camera (Carl Zeiss Microscopy GmbH) linked to image analysis software Zen lite 2011 (Carl Zeiss Microscopy GmbH) to measure areas and lengths. All analyses were performed on coded slides by a single observer blinded to the age groups.

The parameters analyzed in the bronchioles were (**Figure 1**): lumen area (LA), lumen perimeter (LP), wall area (WA), total area (TA), and total perimeter (TP). LA was the area bounded by the respiratory epithelium. WA represents the area of the bronchiolar wall from the base of the epithelium to the outer adventitia border. TA was the area surrounded by the outer limit of the adventitia. LP and TP were calculated using the approximate formula for the perimeter of an ellipse, $P = 2\pi\sqrt{(a'^2 + b'^2)/2}$ where a' and b' are one-half of the short (a) and long (b) axes of the ellipse [13]. LP and TP were measured only to corroborate results of LA and TA, respectively [14].

Statistical analysis

The results are presented as means ± 1 standard error (SE). Kruskal-Wallis test and One-way ANOVA test were used to determine statistical significance ($P < 0.05$). We used both tests in order to have a robustness check [8]. Least significant difference (LSD) post hoc test was used when significant differences were found between groups. The data were analyzed using the SPSS for Windows software (version 21.0; SPSS, Inc., Chicago, IL, USA).

Results

The results are summarized in **Table 1**. Kruskal-Wallis test and One-way ANOVA test were consistent in their findings in all the analyzed parameters. Kruskal-Wallis test showed a significant difference in the LA among the analyzed ages (Chi square = 13.4, Sig. = 0.009). The results of the ANOVA test were also statistically significant (F = 4.1, Sig. = 0.003). LSD test revealed that the LA of the 6-, 18-, and 24-month-old mice (18482.6 ± 2004.2, 16325.7 ± 1254.2, and 15696.4 ± 1018.6 μm^2, respectively) was significantly greater (*p* values of 0.000, 0.003, and 0.020, respectively) than in mice at 2 months of age (11706.7 ± 880.8 μm^2) (**Figure 2A**, **2B**). The LP was also significantly different among the analyzed ages (Kruskal-Wallis test: Chi square = 12.5, Sig. = 0.014; ANOVA analysis: F = 3.7, Sig. = 0.006). Similarly to the observed with the LA, LSD test revealed that the LP of the 6-, 18-, and 24-month-old mice (458.9 ± 28.1, 443.5 ± 17.4, and 447.1 ± 16.0 μm, respectively) was significantly greater (*p* values of 0.004, 0.004, and 0.006, respectively) than in mice at 2 months of age (376.9 ± 14.2 μm) (**Figure 2C**, **2D**).

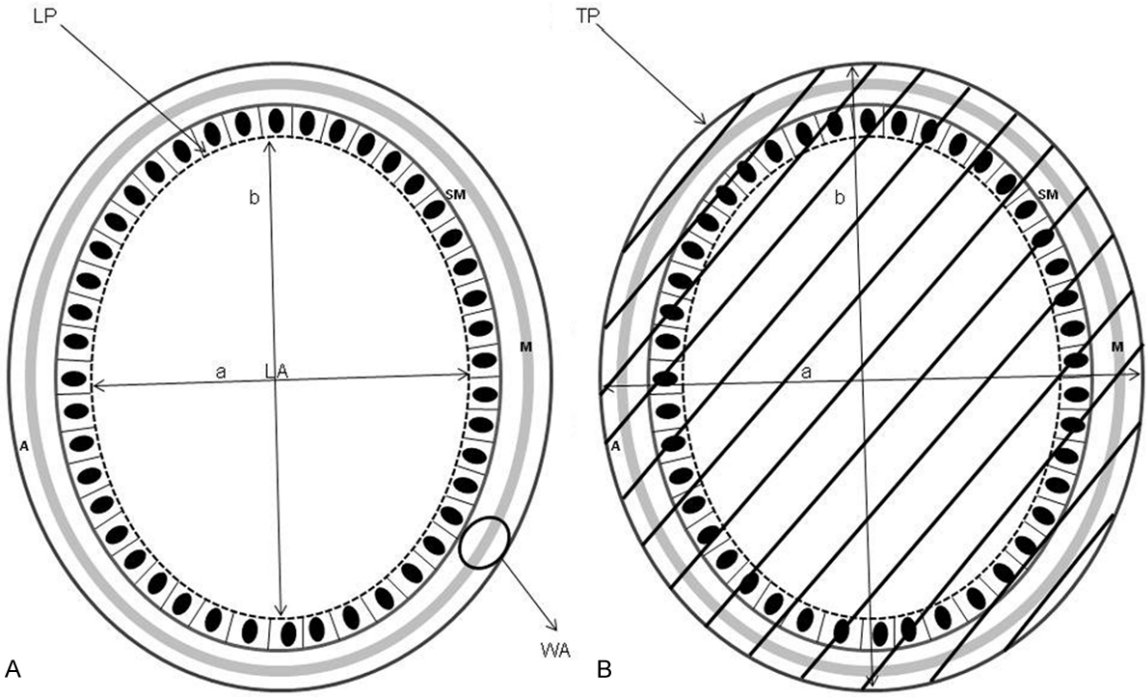

Figure 1. Schematic representation of airways showing morphometric measurements. (A) LA, lumen area: area bounded by the respiratory epithelium; WA, wall area: area of the bronchiolar wall from the base of the epithelium to the outer adventitia border. (B) Diagonal lines, total area (TA): area surrounded by the outer limit of the adventitia. Lumen perimeter (LP in A) and total perimeter (TP in B) were calculated using the formula $P = 2\pi\sqrt{(a'^2 + b'^2)/2}$ where a' and b' are one-half of a (short axis) and b (long axis). SM, submucosa; M, muscular layer; A, adventitia.

Table 1. Morphometric parameters of bronchioles of mouse through the normal aging process

Parameter	Age (months)				
	2	6	12	18	24
Lumen area (μm^2)	11706.7 ± 880.8	18482.6 ± 2004.2[a]	13975.0 ± 1765.4	16325.7 ± 1254.2[a]	15696.4 ± 1018.6[a]
Lumen perimeter (μm)	376.9 ± 14.2	458.9 ± 28.1[a]	395.8 ± 28.9	443.5 ± 17.4[a]	447.1 ± 16.0[a]
Wall area (μm^2)	5240.7 ± 474.3	4703.7 ± 348.3	4778.1 ± 482.9	3686.6 ± 190.7[a,b]	5041.7 ± 434.2[d]
Total area (μm^2)	25808.0 ± 1413.2	26835.5 ± 1552.3	28072.4 ± 1955.2	24939.3 ± 1238.9	31292.9 ± 1457.2[a,b,d]
Total perimeter (μm)	572.0 ± 17.8	571.1 ± 17.2	597.8 ± 24.2	559.2 ± 14.8	632.8 ± 16.6[a,b,d]

Data reported as mean ± standard error (SE). Data were analyzed by the least significant difference (LSD) test. Results were considered statistically significant at $P < 0.05$. [a]Statistically significant compared with 2-month-old mice; [b]Statistically significant compared with 6-month-old mice; [c]Statistically significant compared with 12-month-old mice; [d]Statistically significant compared with 18-month-old mice.

The WA was significantly different among the analyzed ages (Kruskal-Wallis test: Chi square = 11.0, Sig. = 0.026; ANOVA test: F = 3.0, Sig. = 0.018). According to the LSD test, the WA in mice at 18 months of age (3686.6 ± 190.7 μm^2) was significantly smaller than in mice at 2 and 6 months of age (5240.7 ± 474.3 and 4703.7 ± 348.3 μm^2, respectively; *p* values of 0.002 and 0.039, respectively). The WA in mice at 24 months of age (5041.7 ± 434.2 μm^2) was significantly greater than in the 18-month-old mice (P = 0.009) (**Figure 3**).

There was a significant difference in the TA among the analyzed ages (Kruskal-Wallis test: Chi square = 12.0, Sig. = 0.017; ANOVA analysis: F = 2.8, Sig. = 0.026). TA in mice at 24 months of age (31292.9 ± 1457.2 μm^2) was significantly greater than in mice at 2, 6, and 18 months of age (25808.0 ± 1413.2, 26835.5 ± 1552.3, and 24939.3 ± 1238.9 μm^2, respectively; *p* values of 0.009, 0.034, and 0.002, respectively) (**Figure 4A**, **4B**). Finally, the TP was significantly different among the analyzed ages (Kruskal-Wallis test: Chi square = 11.8, Sig. = 0.019; ANOVA test: F = 2.8, Sig. = 0.027). Similarly to the observed with the TA, the TP in mice at 24 months of age (632.8 ± 16.6 μm) was significantly greater than in mice at 2, 6, and 18 months of age (572.0 ± 17.8, 571.1 ±

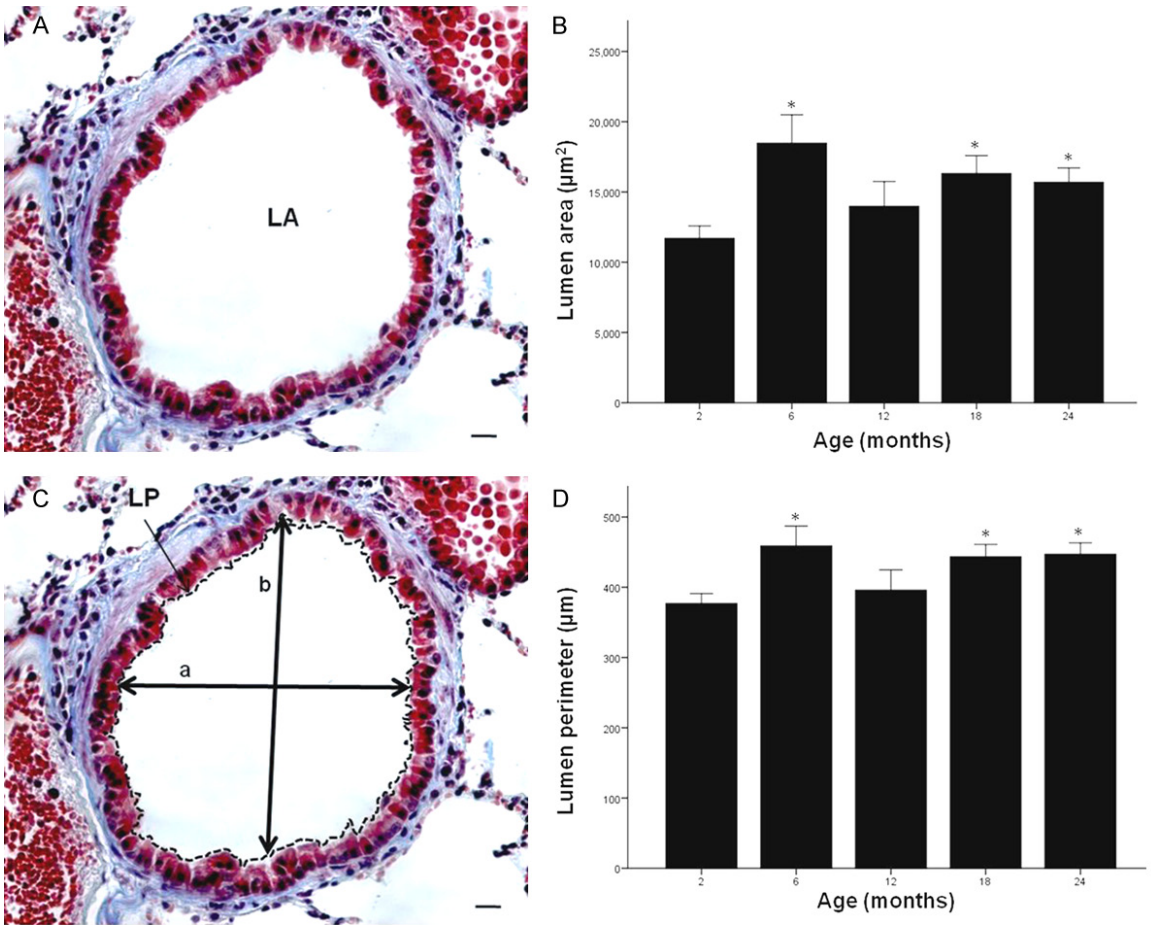

Figure 2. Morphometric analysis of the lumen area and the lumen perimeter of bronchioles from healthy 2-, 6-, 12-, 18-, and 24-month-old mice. (A) Lumen area (LA) was the area bounded by the respiratory epithelium. (B) LA of the 6-, 18-, and 24-month-old mice was significantly greater (at most P = 0.020) than in mice at 2 months of age (*). (C) Lumen perimeter (LP, dotted line) was calculated using the formula for the perimeter of an ellipse, $P = 2\pi\sqrt{(a'^2 + b'^2)/2}$ where a' and b' are one-half of the short (a) and long (b) axes of the ellipse. (D) LP of the 6-, 18-, and 24-month-old mice was significantly greater (at most P = 0.006) than in mice at 2 months of age (*). In (A and C) scale bar 10 μm. Tissue sections were stained with Masson trichrome technique. In (B and D) values are expressed as means ± 1 standard error. Data were analyzed by the least significant difference (LSD) test. Results were considered statistically significant at $P < 0.05$.

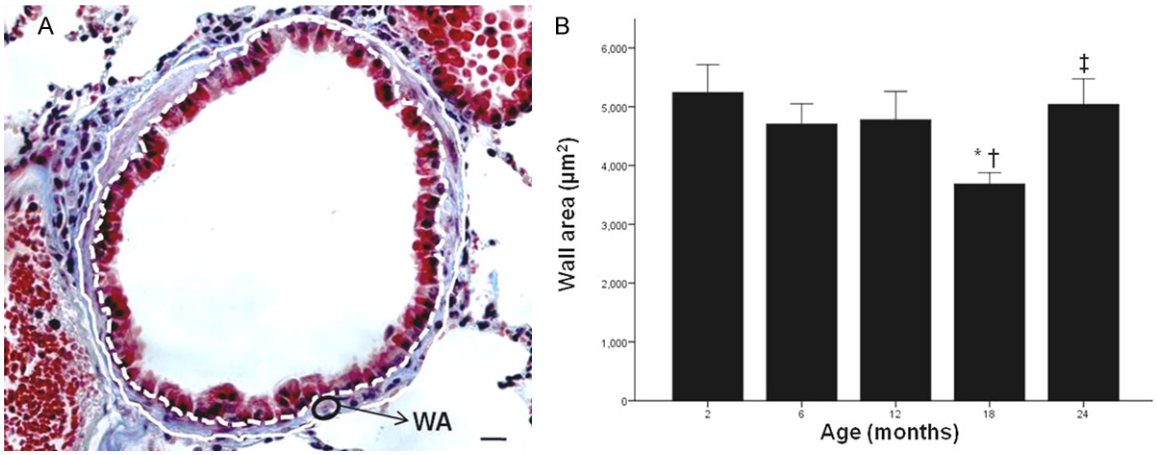

Figure 3. Morphometric analysis of the wall area of bronchioles from healthy 2-, 6-, 12-, 18-, and 24-month-old mice. A. Wall area (WA) represents the area of the bronchiolar wall from the base of the epithelium (dotted line) to the outer adventitia border (solid line). Scale bar 10 μm. Tissue sections were stained with Masson trichrome technique. B. WA in mice at 18 months of age was significantly smaller than in mice at 2 (*) and 6 (†) months of age (*p* values of 0.002 and 0.039, respectively). The WA in mice at 24 months of age was significantly greater than in the 18-month-old mice (‡; P = 0.009). Values are expressed as means ± 1 standard error. Data were analyzed by the least significant difference (LSD) test. Results were considered statistically significant at P < 0.05.

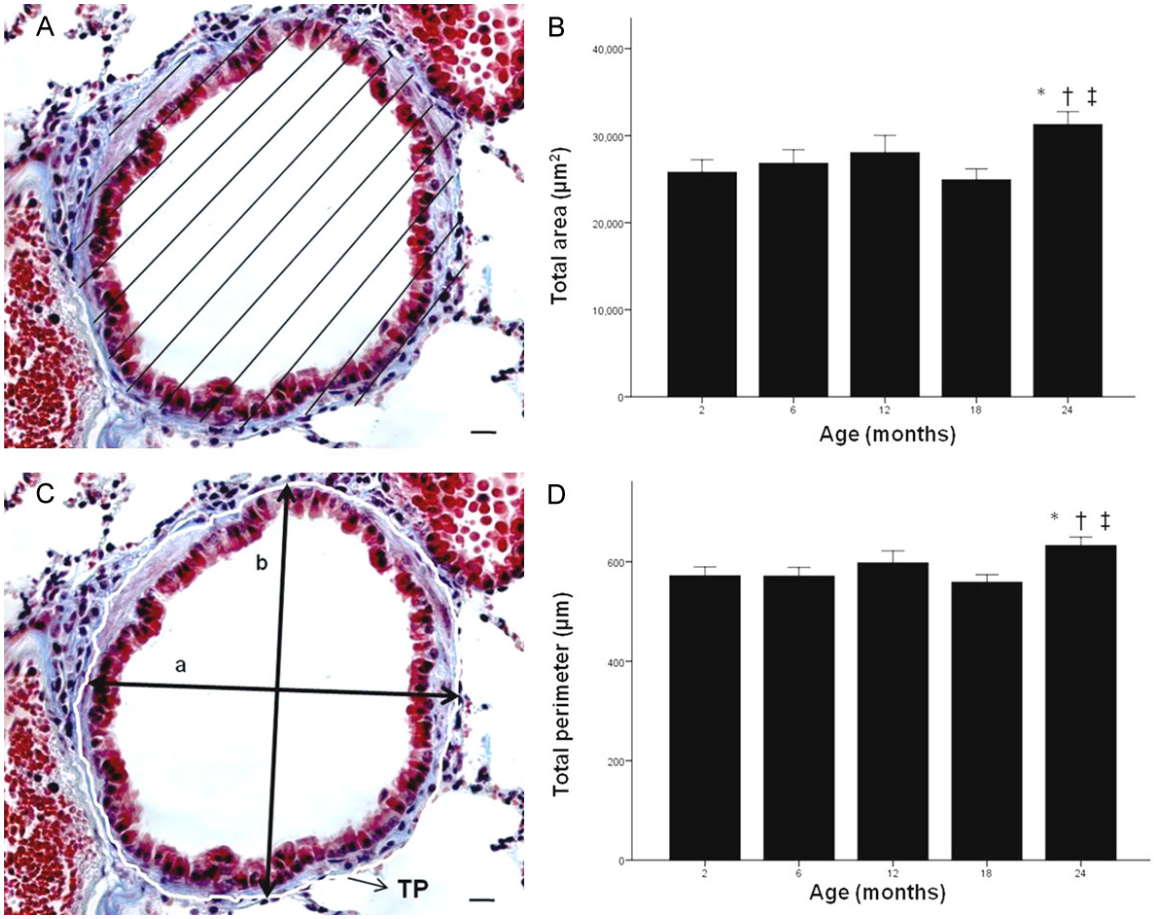

Figure 4. Morphometric analysis of the total area and the total perimeter of bronchioles from healthy 2-, 6-, 12-, 18-, and 24-month-old mice. (A) Total area (TA) was the area (diagonal lines) surrounded by the outer limit of the adventitia. (B) TA in mice at 24 months of age was significantly greater than in mice at 2 (*), 6 (†), and 18 (‡) months of age (at most P = 0.034). (C) Total perimeter (TP, line) was calculated using the formula for the perimeter of an ellipse, $P = 2\pi\sqrt{(a'^2 + b'^2)/2}$ where a' and b' are one-half of the short (a) and long (b) axes of the ellipse. (D) TP in mice at 24 months of age was significantly greater than in mice at 2 (*), 6 (†), and 18 (‡) months of age (at most P = 0.015). In (A and C) scale bar 10 μm. Tissue sections were stained with Masson trichrome technique. In (B and D) values are expressed as means ± 1 standard error. Data were analyzed by the least significant difference (LSD) test. Results were considered statistically significant at P < 0.05.

17.2, and 559.2 ± 14.8 μm, respectively; *p* values of 0.015, 0.014, and 0.002, respectively) (**Figure 4C**, **4D**).

Discussion

The aging lung exhibits structural alterations that lead to a decrease in its function. Although there is an almost general consensus that bronchioles dilate through aging process, there are actually scarce and inexact data, either experimental or descriptive, to support this notion. Verbeken et al. [3] measured only the internal diameter of bronchioles from "normal" (49.1 ± 20.3 years) and "senile" (69.9 ± 4.8 years) subjects and found no statistically significant difference between them. Dyer [1] in humans, Yamamoto et al. [15] in rats, and Elliott et al. [16] in mice, qualitatively compar-

ed bronchiole images of young and old individuals, and stated that the bronchioles dilate in the latters.

Here we report, for the first time, data of bronchiolar morphometry of normal mice through the aging process over a broad range of ages. We observed in the bronchioles an increase of the total area, an increase of the lumen area, and a decrease of the wall area.

Changes in bronchiolar dimensions were nonlinear through time (see 12-month-old mice in **Figure 2B** and **2D**, 24-month-old mice in **Figure 3B**, and 18-month-old mice in **Figure 4B** and **4D**). We and other authors previously found morphometric and functional changes in the lung that were nonlinear through time [1, 8, 16-18]. For now, there is no explanation for this phenomenon. However, perhaps the cause might be revealed by the analysis of differential gene expression profiles in lung during aging [19]. Also, novel approaches based on computational models that incorporate age-related changes in lung structure and function might provide more information in this area [20].

As mentioned above, pulmonary aging is associated with an increase in alveolar size. The mechanism underlying this finding is unclear. Alveolar enlargement has been attributed by several authors to a loss of elastin in lung parenchyma during aging. This modification decreases parenchymal elastic recoil and radially directed airway tethering [20-22]. The loss of tethering forces that maintains the shape of bronchioles might be the cause of the increases of LA, LP, TA, and TP observed in this study. In support of this assumption, Calvi et al. [18] observed a progressive reduction in the number of airway alveolar attachments with increasing age, which was accompanied by elastase activation and airspace enlargement. However, since other authors did not find significant differences in airways size and function between animals treated with elastase and controls [23, 24], and given that in several studies there was no observed loss of elastin in the senile lung [25, 26], more research is needed in this area. It is possible that in the aging process other factors contribute to the bronchiolar enlargement observed in this study.

On the other hand, the 2-month-old mice showed higher WA when compared with mice at all other age groups. Mice at 18 months of age had statistically significant smaller WA than the 2- and 6-month-old mice. Literature regarding changes in bronchiolar wall through the aging process is very scarce. Yamamoto et al. [15] and Huang et al. [21] found that the distribution pattern and density of elastin and collagen fibers of the distal airways were constant, irrespective of age. However, other authors claim that elastin fibers are disrupted and lost, and cross-linking of collagen and elastin is altered in bronchioles through the aging process [2]. With respect to the muscular layer, available data indicate that in human, airways muscle increases in absolute volume from infancy up to early adulthood with little change thereafter (for a review, see [27]). Yamamoto et al. [15] analyzed immunoreactivity for α-smooth muscle actin (ASMA), γ-smooth muscle actin (GSMA), desmin, and vimentin in bronchiolar muscle cells of rats of 9-36 months old. ASMA staining was positive at all age groups. Weak immunoreactivity for GSMA and desmin was detected at any age. Desmin staining was very weak in the rats aged ≥ 27 months. Vimentin immunoreactivity was not observed in any age group.

We were unable to measure individually each bronchiolar wall layer (i.e. submucosa, muscular, and adventitia) because muscle cells did not provide a complete wrap around most bronchioles, leaving large bronchiolar wall segments without any musculature, and giving the appearance of a wall formed by a single layer. In fact, in most of the bronchioles muscle appeared as thin bundles scattered into the other two layers of the bronchiolar wall (see left panels in **Figures 2**-**4**). Other authors have reported the same problem in mice [13], and it could be due to the fact that in the peripheral airways the muscle is arranged in a more longitudinal way than in the central airways, and in a helical or geodesic pattern [27].

Lung small airways are sites of dysfunction early in the course of chronic lung diseases [6, 7]. Alterations in bronchiolar structure reported here, along with alterations in bronchiolar epithelium previously reported by our group [8], might contribute to development of those diseases. Furthermore, bronchiolar and alveolar dilatation observed in the aging contributes to an increase in residual volume and decreased maximal expiratory airflow rates and vital capa-

city [28], which enhances the susceptibility of the lung to extrinsic insults in elderly subjects.

Conclusion

In conclusion, here we report, for the first time, data of bronchiolar morphometry of normal mice through the aging process. We observed in the bronchioles an increase of the total area, an increase of the lumen area, and a decrease of the wall area. These results establish a baseline of age-dependent bronchiolar parameters for use in future studies to detect pathologic changes. More research is needed to determine the molecular phenomena underlying the findings presented herein. Also, since there are gender differences in airway behavior during aging [29], similar analyses to those presented here must be performed in females.

Disclosure of conflict of interest

None.

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