Objective: To analyze the distributions of collagen type I, collagen type III, and versican in the lamina propria of the human vocal fold.

Design: Cross-sectional analysis of cadaveric vocal folds of adult human larynges.

Setting: Academic tertiary referral center.

Subjects: Larynges harvested at autopsy from 10 adult men and 10 adult women.

Main Outcome Measures: Immunohistochemical reactions were performed using antihuman monoclonal antibodies to analyze the expression of collagen type I, collagen type III, and versican.

Results: Collagen type I density was lower in the intermediate layer compared with the superficial and deep layers of vocal folds. Collagen type III density was lower in the intermediate layer compared with the deep layer. Versican density was lower in the superficial layer compared with the intermediate and deep layers. Versican density was lower in the lamina propria of women compared with men; this difference was noted in the superficial layer only. There was a positive correlation between collagen type III and versican densities within the lamina propria.

Conclusion: Collagen type I, collagen type III, and versican are distributed differently within the lamina propria layers of the adult vocal folds.

present in fetal and adult human vocal folds, where it interacts with collagen deposition by regulating collagen fibrillogenesis.\textsuperscript{2,10,13} No quantitative studies to date have analyzed versican distribution within the 3 lamina propria layers.

It is plausible that the distribution of collagen types and versican may vary within the vocal fold lamina propria, as each layer may be submitted to different levels of mechanical stress during phonation. To better understand this subject, we analyzed the distribution of collagen type I, collagen type III, and versican within the lamina propria of female and male adult vocal folds.

**METHODS**

This study was approved by the Research Ethics Committee of the University of São Paulo School of Medicine, São Paulo, Brazil.

Human larynges from 20 autopsied adults (10 male and 10 female) were obtained from the São Paulo Autopsy Service in São Paulo within 24 hours after death. The mean (SD) age of the subjects was 67 (9.4) years (range, 50-85 years). The mean ages of male and female subjects were 66 and 70 years, respectively, with no statistically significant difference between sexes. Thirteen subjects were white, and 7 subjects were black. Subjects with a medical history of neck manipulations, such as oral or nasal intubation, tracheotomy, laryngeal surgery, or head and neck irradiation, were excluded from this study. Only larynges from nonsmoking subjects were included in the study.

Exeresis of the larynx was performed en bloc. None of the specimens showed macroscopic lesions. The right vocal fold was obtained from each larynx and fixed in a 10% formalin solution for 24 hours. Subsequently, 5-mm-thick coronal sections were obtained from the middle portion of the vocal fold membranous region. The sections were dehydrated in a graded alcohol series and embedded in paraffin. Tissue specimens were cut into 4-µm-thick histological sections and stained with hematoxylin-eosin for initial analysis.

For the analysis of collagen type I and collagen type III expression, immunohistochemical reactions were performed using an antihuman monoclonal antibody (C7510-12A, 1:250; United States Biological, Swampscott, Massachusetts) and another antihuman monoclonal antibody (CP191L, 1:500; Calbiochem-Novabiochem, San Diego, California). To analyze versican expression, a specific antihuman large proteoglycan (versican) antibody (Seikagaku America, Inc, Rockville, Maryland) was used. For the analysis of versican expression, the sections were pretreated for 1 hour at 37°C (chondroitinase ABC, 0.05 U/mL; Sigma-Aldrich, Oakville, Ontario, Canada).

For all antibodies used in the study, the sections were incubated overnight with the primary antibody in 1% bovine serum albumin in phosphate-buffered saline at 4 to 8°C. Secondary antibodies (LSAB+; Dako, Carpinteria, California; and Fast Red; Sigma, Steinheim, Germany) were used as chromogens. The slides were counterstained with Mayer hematoxylin. Incubation with phosphate-buffered saline supplemented with 1% bovine serum albumin instead of the primary antibody served as a negative control. As positive controls for collagens, skin sections were used. For versican, lung tissue was used as a positive control, as versican is part of the normal extracellular matrix composition of the lungs.\textsuperscript{14}

For quantitative analyses of collagen type I, collagen type III, and versican expression, we divided the lamina propria into superficial, intermediate, and deep layers according to the model proposed by Butler et al.\textsuperscript{13} Measurements of positively stained areas were performed by image analysis using a light microscope (Leica DMR; Leica Microsystems, Wetzlar, Germany) connected to a computer through a video camera using a commercially available software program (Image Pro Plus, version 4.1; Media Cybernetics, Silver Spring, Maryland). For each lamina propria compartment, 3 nonoverlapping areas at ×400 magnification were analyzed, totaling 9 analyzed areas for each section. Results were expressed as stained area per total area (in micrometers squared). Data were expressed as medians and ranges. Comparison of stained areas within the 3 compartments was performed using analysis of variance and the Kruskal-Wallis test, followed by the post hoc Tukey B test and Bonferroni correction, respectively (depending on the data distribution). Data were log transformed before analyses, which were performed using commercially available software (SPSS, version 15.0; SPSS Inc, Chicago, Illinois). Correlation between extracellular matrix elements was assessed using Spearman rank correlation. \( P < .05 \) was considered significant.

Collagen and versican stained as fibrillar structures in the lamina propria and among the vocal muscle cells. Collagen type I density was lower in the intermediate layer compared with the superficial (\( P < .001 \)) and deep (\( P = .005 \)) layers (Figure 1A and B). Collagen type III had a more homogeneous distribution within the vocal fold layers, with a statistically lower collagen type III density in the intermediate layer compared with the deep layer (\( P = .001 \)) but without differences in the superficial layer (Figure 1C and D). Versican density was lower in the superficial layer compared with the intermediate (\( P = .04 \)) and deep (\( P = .01 \)) layers (Figure 1E and F).

When all layers were considered together, there was a positive correlation between collagen type III and versican densities (\( r = 0.57, P = .01 \)). These results are shown in Figure 2.

Morphometric analysis categorized by sex revealed that collagen type I, collagen type III, and versican differed significantly among layers within the lamina propria. Women had lower collagen type I density in the intermediate layer compared with the superficial layer and deep layer (density, 0.28 kg/m\(^3\); 95% confidence interval, 0.10-0.51 kg/m\(^3\); \( P = .02 \)) in men (Figure 3A). For women, there was no difference in collagen type III distribution among layers. In contrast, collagen type III density was higher in the deep layer compared with the superficial (\( P = .04 \)) and intermediate (\( P = .02 \)) layers in men (Figure 3B). In women, versican density was lower in the superficial layer compared with the intermediate (\( P = .03 \)) and deep (\( P = .02 \)) layers. In men, there was no statistical difference in versican density among layers (Figure 3C).

No statistical difference was noted between total density of collagen type I and collagen type III in male vs female lamina propria vocal folds. Versican density was lower in the lamina propria of women compared with men. This difference was noted in the superficial layer only (\( P = .049 \)) (Figure 4). There were no correlations between patient age and collagen or versican density in the lamina propria among men or women.

**RESULTS**

In this study, we described the distribution of collagen type I, collagen type III, and versican within the vocal

**COMMENT**
fold lamina propria of age-matched adult men and women, as shown in Figure 5. Our data demonstrated that the patterns of collagen and versican can vary within the layers of the vocal fold lamina propria based on sex.

The distribution of total collagen (as detected by Sirius Red staining) in human vocal folds has been shown to be similar across all age groups, although concentrations vary according to age and sex. In normal adult vocal folds, most collagen is present in a superficial band immediately subjacent to the epithelium and in the deep layer of the lamina propria adjacent to the vocal muscle, and collagen concentrations are known to be higher in adult and older men. We confirmed this distribution of collagen type I (ie, the predominance of this protein in the superficial and deep layers of the lamina propria). When categorized by sex, our data show that the differences in collagen type I distribution among the layers of the vocal fold lamina propria were more pronounced in women. However, we observed no significant sex-specific differences in collagen type I distribution.

The analysis of collagen type III distribution also revealed differences, with the most notable being the higher collagen type III density in the deep layer of the lamina propria. Our data show that these layer-specific differ-
ences in the distribution of collagen type III were more pronounced in men, although the difference between sexes was not significant. Because collagen type III tends to be more highly concentrated in dynamic regions of elastic tissues, differences in the distribution of collagen type I and collagen type III might provide insight into the stress to which different lamina propria regions are submit-
ted. As suggested by Tateya et al, collagen type I provides the tensile strength around the basal membrane and vocal ligament that is required to maintain the shape of the vocal fold during vibration, whereas the more homogeneous distribution of collagen type III is important to maintain tissue elasticity.

Versican is a large aggregating proteoglycan that binds to hyaluronic acid and frequently interacts with elastic networks. The highly hydrated versican–hyaluronic acid complex has a significant role in inhibiting cell-matrix interactions, affecting hydrostatic pressure, as well as dissipating impact and compressive stresses in the vocal fold lamina propria. Versican has previously been shown to be present in adult and fetal vocal folds. However, no previous studies have assessed the distribution of versican within the layers of the vocal fold lamina propria in adults. Our data show that versican density was higher in the intermediate and deep layers of the lamina propria and that this difference was more pronounced in women. In addition, versican density in the superficial layer was higher in men than in women. Our data are in disagreement with
those reported by Hahn et al., who found no differences between men and women in terms of versican distribution. However, those authors examined a smaller population than that evaluated in the present study (5 subjects vs 20 subjects) and used only a semi-quantitative analysis.

A comparison between our results and those of previous studies shows that the distribution of versican within the human lamina propria varies according to age. In a human fetus, versican density has been shown to be highest in the superficial layer of the vocal fold lamina propria. However, in the present study, versican density was lowest in the superficial layer. The reasons for such differences are unclear. It has been shown that, in the presence of some proteoglycans, collagen synthesis yields thinner fibrils. This theory could explain the higher density of collagen type I (thick fibrils) and the lower density of versican in the superficial layer of the vocal fold lamina propria of adults. This is also consistent with our findings, which indicated a positive correlation between collagen type III (thinner fibrils) and versican densities.

Understanding sex-related differences in the composition of the extracellular matrix in vocal fold lamina propria is relevant because it can provide insights into the greater vulnerability of women to lamina propria scarring and to certain vocal fold disorders. Although the concentration of total collagen in the lamina propria might be higher in men, our findings and those by Hahn et al. show that the distribution of collagen type I and collagen type III is similar between sexes. However, we found versican density in the superficial layer to be higher in men than in women. This finding might explain why women have a greater predisposition to the occurrence of benign laryngeal lesions, such as vocal nodules. It is likely that versican has an important role in absorbing impact in the vocal fold during phonation. If versican density in the superficial layer of the vocal fold lamina propria is lower, the vocal fold might be more prone to mechanical damage during phonation.

Other authors have identified correlations between age and collagen expression, reporting increased collagen content in older populations. We were unable to identify any such correlations, as our study group consisted of an adult population of a narrow age range, which constitutes a limitation of the present study. It would have been relevant to study developmental changes of the extracellular matrix in the vocal folds of adolescents compared with adults. Unfortunately, we did not have access to a significant number of cases in this age range from our autopsies. We have not determined the extracellular matrix components within the macula flava, which is another limitation of this study.

In conclusion, our data show that there are layer-specific and sex-specific differences in the distribution of collagen type I, collagen type III, and versican within the lamina propria of adult vocal folds. Deeper knowledge of the extracellular matrix distribution of these proteins within the lamina propria is fundamental to understanding the mechanics of phonation and disease pathogenesis in the vocal folds.

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Author Contributions: Drs Bühlér, Sennes, Tsuji, Mauad, Ferraz da Silva, and Saldiva had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Bühlér, Ferraz da Silva, and Saldiva. Acquisition of data: Tsuji. Analysis and interpretation of data: Sennes and Mauad. Drafting of the manuscript: Bühlér, Ferraz da Silva, and Saldiva. Critical revision of the manuscript for important intellectual content: Sennes, Tsuji, and Mauad. Statistical analysis: Ferraz da Silva. Administrative, technical, and material support: Bühlér and Saldiva. Study supervision: Sennes, Tsuji, and Mauad.

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