HHS Public Access

Author manuscript

Clin Exp Rheumatol. Author manuscript; available in PMC 2018 August 29.

Published in final edited form as:

Clin Exp Rheumatol. 2018 ; 36(Suppl 112): 80–88.

Minor salivary gland fibrosis in Sjögren's syndrome is elevated, associated with focus score and not solely a consequence of aging

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Abstract

Objective—Evaluate the presence of minor salivary gland (SG) fibrosis in primary Sjögren's syndrome (pSS) as a function of disease pathology or a consequence of ageing.

Methods—Subjects with sicca symptoms attending a Sjögren's research clinic were classified by American European Consensus Group (AECG) criteria as either pSS or non SS (nSS). Discovery (n=34 pSS, n=28 nSS) and replication (n=35 pSS, n=31 nSS) datasets were evaluated. Minor SG cross-sections from hematoxylin and eosin stained slides were imaged, digitally reconstructed and analyzed for percent area fibrosis. Relationships between SG fibrosis, age, and clinical measures were evaluated using Spearman correlations. Association with SS was assessed by: ROC curve, Variable Selection Using Random Forests (VSURF) and uni- and bi-variate regression analyses.

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Results—SS subjects had significantly more fibrotic tissue in their minor labial salivary glands (median 24.39%, range 5.12–51.67%) than nSS participants (median 16.7%, range 5.97–38.65%, $p<0.0001$); age did not differ between groups (average ± SD pSS 50.2 ±13.9 years, nSS 53.8±12.4 years). In both discovery and replication data sets, multiple regression models showed that the area of minor salivary gland fibrosis predicted pSS significantly better than age alone. Age-corrected linear regression revealed that the area of minor salivary gland fibrosis positively associated with vanBijsterveld score ($p=0.042$) and biopsy focus score ($p=0.002$). ROC curve and VSURF analyses ranked fibrosis as a significantly more important variable for subject discrimination than age.

Conclusion—SG fibrosis is an element of pSS pathology that is related to focus score and is not solely attributable to age.

Keywords

Sjögren's syndrome; minor salivary gland; fibrosis; focus score; aging

Sjögren's syndrome (SS) is a systemic rheumatic autoimmune disorder with cardinal features of chronic, severe dry eyes and mouth and focal lymphocytic infiltrates in salivary and lacrimal gland tissue [1, 2]. The etiology of SS includes genetic risk [3–5], epigenetic [6, 7], environmental [8] and stochastic factors. Causative pathogenic mechanisms remain unclear but involve dysregulation of innate and adaptive immunity [3, 9] and epithelial cell defects [10].

Fibrosis is a common consequence of tissue damage and inflammation [11] and often complicates rheumatic diseases. Several diseases genetically related to SS have well-described fibrotic components, including primary biliary cirrhosis, systemic sclerosis, ulcerative colitis, and systemic lupus erythematosus [12, 13]. The presence of autoantibodies [14–18], overactive innate immune pathways such as interferon and NF-κB [3, 9, 19–21] and tissue inflammation [13, 14, 16, 22–24] are also commonly shared amongst these disorders.

Fibrosis in salivary glands (SG) of SS patients has been noted [1, 25–28]. However, whether these fibrotic changes merely reflect ageing or are a feature of disease pathology is unclear. Establishment of age-related SG fibrosis in healthy subjects [29] has led to acceptance of SG fibrosis in SS as a consequence of aging and not disease [30–32]. Early studies were hampered by the lack of established SS classification criteria. Diagnosis and classification of SS patients relies on a constellation of objective exam results and subjective symptom reporting, yielding a heterogeneous cohort of patients whose individual courses of SS may be dissimilar. Complicating the assessment of fibrosis is the late age of onset of the disorder, usually in the fourth decade or later. A recent study involving 264 subjects with pSS found that fibrosis grade associated positively with dental damage and was inversely correlated with salivary flow [27]. Another study evaluating SG biopsy slides of 63 cases of pSS and 11 healthy controls reported an increased grade of fibrosis in pSS and an association between fibrosis and SS after adjusting for age [28]. While these studies have subjectively (using graded scales [25, 29, 33]) assessed fibrosis, no study has quantified fibrosis in the minor SGs in order to dissect the relationships between fibrosis, age and SS. Determining

whether fibrosis is a form of pathology in SS contributes to our overall understanding and may open new therapeutic avenues for SS patients.

This study was undertaken to determine if SS subjects have more, less, or similar fibrotic replacement as compared to subjects who have symptoms of dryness but do not meet established disease criteria (non SS) and to determine whether the presence of fibrosis is solely attributable to age. We report that SG fibrosis is a pathologic feature of SS related to focal SG inflammation and not solely a consequence of age. An age-related increase in minor SG fibrosis is confirmed and this study further establishes that minor SG fibrosis is an element of lymphocytic focus-associated SG pathology and is not solely an attribute of older subjects.

PARTICIPANTS AND METHODS

Participants

Biological samples, clinical and laboratory test values were obtained from the Sjögren's Research Clinic (SRC) (at Oklahoma Medical Research Foundation and University of Minnesota) as previously described [34]. Clinical measures including focus score, van Bijsterveld score, Schirmer's test, and collection of whole unstimulated salivary flow, were conducted as specified by the 2002 revised American European Consensus Groups [22]. Ro antibody titers were determined as described [35, 36]. Briefly, IgG antibody titers were determined by ELISA using bovine-derived Ro60 (Immunovision).

Participants were self- or physician-referred, underwent pre-clinical screening using questions pertaining to oral and ocular disease symptoms [35] and had at least one qualifying ocular and one qualifying oral dryness complaint. All participants gave fully informed consent in compliance with the Declaration of Helsinki, and the study was approved by both respective Institutional Review Boards. Participants were classified using the 2002 revised AECG criteria [22].

All participants with features of overlapping diseases (including rheumatoid arthritis, systemic lupus erythematosus, and systemic sclerosis) or with exclusion criteria for AECG classification (sarcoidosis, prior head and neck radiation, hepatitis C infection, acquired immunodeficiency syndrome, pre-existing lymphoma, and graft-versus-host-disease) were excluded from the study. Participants who failed to meet AECG criteria for pSS, but had dry eye and/or dry mouth complaints were designated as "non SS" (nSS). Participants in pSS and nSS categories were randomized into separate datasets, a discovery set (n=34 pSS, n=28 nSS) and a replication set (n=35 pSS, n=31 nSS). Randomization was performed using discrete uniform distribution sampling via the "sample" function in *R.* Imaging and fibrosis scoring of participants' biopsy tissue was performed with classification status blinded. Demographic and clinical data of participants are shown in Table 1. Dental data, including number of tooth restorations, was available for a subset of the subjects (n=44 pSS, n=36 nSS). Disease duration data was abstracted from patient questionnaires, and represent the most conservative estimation based on the date of diagnosis (age of study entry) and the calculated age of symptom onset (based on questions regarding subjective dryness).

Salivary gland biopsy and imaging

Four to six minor labial SGs per participant were formalin-fixed, paraffin-embedded, sectioned (4 μm), and stained with hematoxylin and eosin in either the University of Minnesota or University of Oklahoma Health Sciences Center oral pathology laboratories. Focus scores were determined by a board-certified oral pathologist. The slide used for focus scoring was imaged using a Zeiss 710 confocal microscope. Each glandular cross-section was imaged at 200× magnification in overlapping sections. These sections were digitally assembled using the Zeiss ZenBlue software package to yield reconstructions of entire glandular cross-sections (Figure 1).

Quantitative fibrosis assessment

Fibrotic changes were quantified by an observer blinded to disease classification status as described [36]. A standard grid of 2500 μm^2 was applied to SG cross-section images using ImageJ (National Institutes of Health, Bethesda, MD, USA). Each individual square of each section was scored using the following rubric: edges were accounted for by omitting any square where less than 50% of the area contained tissue (Figure 1). Areas of infiltration were included in the total area calculation but were assigned a value of '0'. Tissue positive squares containing ≥ 50% fibrotic tissue were assigned a value of 1. Tissue positive squares containing < 50% fibrotic tissue were assigned a value of 0. The number of fibrosis positive squares in each cross-section was multiplied by 2500μm^2 (area of each grid square). This value was divided by the total section area to generate the percent area fibrosis for each glandular cross-section for each participant:

$$\left\{\frac{(n * 2500um^2)}{section\ area}\right\} \times 100 = \%\ area\ fibrosis\ (where\ n = number\ of\ fibrosis\ positive\ grid\ squares).$$

The individual cross-section percent areas were then averaged to yield a per-participant mean percent-area fibrosis of minor labial SG tissue:

$$\left\{\frac{(\sec 1\ \%\ area) + (\sec 2\ \%\ area)\ldots(\sec x\%\ area)}{total\ section\ number}\right\} = average\ \%\ area\ fibrosis.$$

Fibrosis Severity Scores

A board-certified oral pathologist was provided with SG slides from a selected subset (pSS = 20, nSS n= 15) of subjects to independently assess degree of fibrosis. The observer was blinded to disease classification of the subject samples. Distribution analysis of fibrotic area was used to select SG slides for independent evaluation; slides were selected from each 'bin' equal to: ≤5%, 6–20%, 21–30%, and ≥30% to cover the full data range. Slides were scored as follows: 0 = normal tissue up to very minor periductal fibrosis; 1 = significant periductal fibrosis only, 2 = acinar replacement by fibrotic tissue with periductal fibrosis, 3 = widespread fibrosis including acinar replacement, lobular dysmorphia and extensive gland disruption.

Statistical Analyses

All were executed in R [37] or Prism 6.0 (GraphPad Software, La Jolla California USA, www.graphpad.com). Normality tests: two-tailed Shapiro-Wilks tests and where necessary, non-parametric tests. Both bivariate and univariate logistic regression generalized linear models (GLMs) were performed to assess association of fibrosis with disease. Simple linear regressions as well as linear regressions with variable correction (to assess association of fibrosis with clinical measures) utilized Box-Cox transformed data (`powerTransform` and `bcPower` functions in the ‘ `car`’ R package [38]). ROC curves were generated using the R package ‘ `pROC`’ [39]; DeLong’s test was used to measure likeness of ROC curves for fibrosis, focus score and age. Maximal Youden’s index, as determined by the ‘ `OptimalCutpoints`’ package [40] was used to determine the optimal threshold for fibrosis in predicting diagnosis. For random forest analysis, the default ‘ `VSURF`’[41] package was used to test the importance of average percent area fibrosis and age as well as categorical variables of sex, race, and AECG-determined diagnostic cutoffs for positivity of the following parameters: AECG questions on oral symptoms (yes/no), AECG questions on ocular symptoms (yes/no), vanBijsterveld score ≥ 4, Schirmer’s score ≤5mm/minute, whole unstimulated saliva flow ≤1.5mL in 15 minutes, focus score ≥1, presence of IgG anti-Ro/SSA or IgG anti-La/SSB.

RESULTS

Fibrosis is elevated in the minor labial salivary glands of subjects with primary Sjögren’s syndrome

To assess the presence and extent of fibrosis in minor salivary glands, a precise method of assessing fibrosis in hematoxylin and eosin stained SG biopsy tissue sections was implemented. Percent area fibrosis values are reported as the average of multiple (4 to 6) glandular cross-sections per subject (n=128 subjects). Average percent area fibrosis in the discovery and replication sets yielded similar results, with SG fibrosis being significantly greater in subjects with pSS compared to those in the nSS group (Figure 2A, 2C). In the combined dataset, pSS participants had a greater median percent area SG fibrosis (24.39%, range 5.12–51.67%) than nSS participants (16.7%, range 5.97–38.65%, $p<0.0001$). Importantly, there was no significant difference in participant age between pSS and nSS groups (Figure 2B, 2D). As expected, the pSS group exhibited higher incidence of positivity for measures utilized to classify individuals as having SS, including positivity for IgG anti-Ro/SS-A and anti-La/SS-B antibodies, vanBijsterveld score (ocular damage) and biopsy focus score (focal lymphocytic infiltration) (Table 1). In contrast, the percentage of pSS versus nSS subjects with a positive Schirmer’s test, indicating reduced tear flow, was not different in either the discovery or replication sets, while the fraction of subjects with a positive whole unstimulated salivary flow (WUSF) test, indicating reduced salivary flow, was only significantly different in the replication set.

To compare the percent area fibrosis measurement with pathologist-determined severity of SG fibrosis, an oral pathologist evaluated tissue slides from a sample of the subjects. This analysis utilized slides from a subset (n=35 subjects) of subjects evaluated for average percent area SG fibrosis measurements covering the range of quantitative fibrosis observed.

The pathology scores, which took into account proximity of fibrosis to ducts or acini and related acinar cell destruction, correlated significantly (r=0.6, p=0.0002) with the quantitative data (Figure 3), indicating that the quantitative percent area fibrosis measurement captured the severity of fibrotic changes. Additionally, a Mann-Whitney U test comparing the pathologist-assigned scores showed significantly greater fibrosis severity in pSS as compared to nSS groups (p= 0.04). Thus, the subjective fibrosis severity score correlates positively with our quantitative measure of fibrosis and recapitulates the elevated fibrosis phenotype observed in pSS subjects.

Fibrosis discriminates pSS from non SS sicca more effectively than age in regression models

In order to exclude age as a confounding covariate in the assessment of fibrosis in SS, a two variable logistic regression model was employed. SS disease status was the dependent variable, with age and percent area fibrosis as the predictive variables (Table 2). In both the discovery and replication analyses, fibrosis contributed to prediction of pSS classification (discovery set p=0.0009, OR = 1.16, accuracy = 68%; replication set p=0.0060, OR = 1.12, accuracy = 73%) while age did not (discovery set p=0.5720, OR = 0.99, replication set p=0.8346, OR = 1.00).

To compare the efficacy of age or fibrosis alone to distinguish pSS versus nSS, single variable models using only fibrosis or only age were constructed and compared to a multivariate model. ANOVA analysis revealed that only fibrosis significantly discriminated between pSS and nSS (discovery set p=0.0010, OR= 1.15, accuracy = 69.4%, replication set p=0.0021, OR = 1.12, accuracy = 73.0%). Finally, we compared the individual models to the full model, and found that the fibrosis-only model was not significantly different from the multivariate model in distinguishing pSS versus nSS status (discovery set p=0.5695, replication set p=0.8348), indicating that the addition of age does not significantly improve the fibrosis model. Within the multivariate models, age was not a significant contributor to model outcome (discovery set p=0.867, accuracy 54.8%, OR =1.1, replication set p=0.083, accuracy = 68%, OR =1.0,). Additionally, the age-only model was inferior to both the fibrosis-alone and multivariate models, indicating that the addition of the fibrosis measure significantly improves the age-only model (ANOVA versus full model discovery set p<0.0001, replication set p <0.0001). Taken together, these results indicate that the effect of fibrosis is separate from that of age. This supports the hypothesis that the elevated fibrosis observed in SS patients is not solely attributable to advanced patient age.

Regression modeling reveals that the addition of fibrosis to focus score more precisely discriminates between pSS and non SS sicca subjects

Biopsy focus score is a benchmark feature used to classify SS. To determine whether fibrosis can enhance the power of focus score in discriminating pSS from nSS subjects, regression modeling was employed. Separation of pSS from nSS was the dependent variable, while biopsy focus score and percent area fibrosis were the predictive variables (Table 3). In the discovery set, focus score significantly contributed to SS discrimination (p=0.011) while fibrosis exhibited a trend toward significant contribution to categorization (p=0.055). However, in the replication set both focus score (p=0.021) and fibrosis (p=0.0067) had the

capacity to distinguish between pSS and nSS. This multivariate model was compared to univariate logistic regression models containing only focus score or only fibrosis. In both sets, the multivariate model was significantly better at discriminating the disease groups than either univariate model, indicating that the inclusion of average percent area fibrosis enhances the power of focus score alone (discovery set $p<0.0391$, replication set $p<0.002$).

Minor salivary gland fibrosis associates with focus score, ocular damage and age

The relationship between percent area fibrosis, age and other clinical features was evaluated. Using simple linear regression, age ($p<0.0001$), biopsy focus score ($p<0.0001$), vanBijsterveld score ($p=0.028$), WUSF volume ($p=0.025$), and number of tooth restorations ($p=0.012$), were found to significantly associate with percent area fibrosis (Table 4). Anti-Ro autoantibody titer was evaluated within the pSS population for correlation with fibrosis, and no significant relationship was discovered ($p=0.30$, Spearman 2-tailed t test). We also compared the extent of fibrosis in Ro-positive and Ro-negative subjects, but found no significant difference between the groups (Ro^+ primary = 44, Ro^- primary = 24, $p=0.23$, Kolmogorov-Smirnov 2-tailed test.) Interestingly, we found no correlation between patient reported duration of disease and extent of fibrosis. A subset of primary patients (n=52) had disease duration data available, but no association with the degree of fibrosis was apparent ($p=0.67$, $r=0.06$). As age is a potentially confounding factor in these analyses, we age-corrected the linear regressions comparing fibrosis and clinical SS signs. Only vanBijsterveld score ($p=0.042$) and biopsy focus score ($p=0.002$) associated with degree of SG fibrosis (Table 4). Thus, while age and fibrosis are correlated, fibrosis is correlated with vanBijsterveld score and focus score beyond the contribution of age.

Fibrosis discriminates pSS from non SS sicca more effectively than age by receiver operating characteristic (ROC) and random forest analyses

As univariate regression analyses showed that focus score and age are most closely associated with fibrosis, we directly compared the capacity of these factors to distinguish between the pSS and nSS groups by ROC analysis. Areas under the ROC curves (AUC) for age, average percent area fibrosis, and biopsy focus score were 57.58, 75.84, and 77.73%, respectively (combined dataset). DeLong's test for two correlated ROC curves showed a significant difference between fibrosis and age ($p=0.0012$), but not between fibrosis and focus score ($p=0.7292$) (Figure 4). We chose a threshold of 20.69% fibrosis to classify subjects because it optimizes both specificity (0.780 (0.653–0.877 95% CI)) and sensitivity (0.710 (0.588–0.813 95% CI)).

To compare the capacity of SG fibrosis to distinguish pSS from nSS subjects with other tests used in pSS classification, we used a non-parametric method of variable ranking and selection (by way of random forests). Via an iterative process, variables that do not contribute to the output are eliminated. Continuous variables were limited to average percent area fibrosis and subject age. Categorical variables included sex, race, AECG-questions regarding dry eye and mouth (at the time of clinic visit), as well as the results of objective pSS classification tests including highest vanBijsterveld score, lowest Schirmer's value, WUSF test, anti-Ro/SS-A and anti-La status/SS-B, and lip biopsy focus score. Only five of these twelve variables passed the importance threshold; they are, in decreasing order of

importance, anti-Ro/SS-A positivity (importance 0.1639), biopsy focus score ≥1.0 (0.0838), anti-La/SS-B positivity (0.0587), average percent area fibrosis (0.0284), and WUSF ≤ 1.5 ml/min (0.0144). Thus, degree of SG fibrosis selectively associates with the SS disease state, whereas subject age exerts no influence on SS disease state by random forest analysis.

DISCUSSION

This study is the first to quantitatively evaluate minor salivary gland fibrosis in subjects with pSS compared to those with sicca symptoms alone. In our analyses, fibrosis distinguishes pSS from those with sicca symptoms who do not meet criteria for SS and performs comparably to biopsy focus score in this regard. As our analyses did not include individuals meeting criteria for other rheumatic diseases, we have not evaluated the extent of salivary gland fibrosis as a tool for disease classification or diagnosis; rather, we offer compelling evidence that fibrosis is part of the SS disease process and not only a consequence of aging.

Our method considered and assessed all fibrotic tissue without prior knowledge of subject classification. High positive correlation of our quantitative measurements with the pathologist-assigned fibrosis severity scores demonstrates that the quantitative method captures changes considered to be of pathologic importance. We report here that subjects classified as pSS have higher average percent area fibrosis than those who do not fulfill pSS classification criteria. Notably, there was no significant difference in age between pSS and non SS sicca groups in either dataset. Across the entirety of data available from our research center, however, we observe a significant difference in age between pSS (pSS n= 635, median = 56 and nSS groups (median = 52, n= 765, $p<0.0001$) (unpublished data). In light of this difference, seen in the larger sample set, we treated age as an independent variable in our analyses, to avoid its confounding effects.

We tested whether fibrosis would associate with other clinical features of SS and detected positive relationships between biopsy focus score and SG fibrosis, and between ocular surface damage (vanBijsterveld score) and fibrosis. The inverse relationships between fibrosis and tooth restorations and fibrosis and WUSF were explained by age, and showed no significant association after age correction. Using multiple approaches, we dissected the individual contributions of age, fibrosis and biopsy focus score in separating subjects with pSS from those in the nSS group. We compared the ability of quantified fibrosis to discriminate between pSS and non-SS nSS groups, as compared to that of age and focus score. We took a threefold statistical approach to better elucidate potential relationships between these three variables: multivariate regression modeling, ROC curve comparison, and random forest variable ranking.

The results showed that, although age and fibrosis correlate, age alone could not explain the extent of fibrosis in pSS subjects as compared to their similarly-aged nSS counterparts. By comparing uni-to bi-variate regression models, we demonstrated that the addition of fibrosis significantly improves the age-alone model and increases the sensitivity and accuracy of the focus score model. These data strongly suggest an intimate relationship between lymphocytic infiltration and fibrotic tissue replacement. The present study undoubtedly

underestimates this relationship since SG foci by definition [42] and according to current classification criteria [22] must not be adjacent to fibrotic tissue.

ROC analysis demonstrated that fibrosis out-performed age in predicting pSS versus nSS. In fact, fibrosis was comparable to the predictive power of biopsy focus score in this analysis. A relationship between degree of SG fibrosis and SG lymphocytic infiltration is further supported by our recent observation that the degree of SG $CD4^+$ T cell clonal expansion positively correlates with percent area SG fibrosis in pSS [36]. The random forest approach identified the five most important variables as anti-Ro/SS-A positivity > biopsy focus score > anti-La/SS-B positivity > extent of fibrosis > loss of saliva secretion (WUSF). The results of these analyses agree that fibrosis is a variable of importance in stratifying SS versus nSS subjects. While the exact cause of SG fibrosis and its role in SG dysfunction and SS disease remain unknown, the data presented here establish fibrosis as a pathologic feature of SS. The multi-model approach confirms that fibrosis makes a significant contribution to distinguishing non-SS sicca from SS, and that it does so above and independent of the contribution of age. These results are in agreement with those of Llamas-Gutierrez, et al [28], who observed an age-independent association between grade of fibrosis and pSS but included only 11 non-SS controls. Our results present strong, replicated evidence that quantified fibrosis is a feature of Sjögren's syndrome pathology and is not solely a feature of age.

Tissue fibrosis is a common consequence of chronic inflammation, suggesting that the theory of SG fibrosis in SS is plausible, if not probable. $CD4^+$ T cells, macrophages and epithelial cells all play roles in both normal homeostasis and pathological accumulation of collagen [43, 44] and are commonly found in glandular lesions in SS [25, 30, 31, 45–47]. Increased fibrotic change is correlated with the presence and degree of $CD4^+$ T cell clonal expansions in the minor salivary glands [36]. Moreover, diseases sharing genetic overlap with primary SS include inflammation-associated tissue fibrosis as a pathological feature [12, 48]. For example, in systemic sclerosis, genetic variants of *STAT4* and *IRF5*, (which associate with pSS [48]), demonstrated additive effects contributing to interstitial lung disease [49].

One of the confounding factors in SS disease research is the near-total lack of longitudinal data and the difficulty in precisely capturing theoretical disease duration from self-reported patient data. Disease duration, as it relates to SS, is a difficult parameter to capture, as 1) the onset of irritating dry eye and mouth is difficult to pinpoint in hindsight, and 2) when asked in different ways, patient's responses can be inconsistent. In our study, limited data on patient-reported disease duration was available, and no correlation between disease duration and the extent of salivary gland fibrosis was detected. Although fibrosis is widely considered to be a progressive process, it is possible that salivary gland fibrosis in SS is not progressive. Kapsogeorgou, et al. detected no fibrotic progression in labial salivary gland biopsies longitudinally collected a median of 4.5 years apart [50]. We also note that patient-reported disease duration is an imprecise measure.

Determining the disease chronology and sequence of events leading to glandular hypofunction in SS can only be accomplished by well-designed and comprehensive

longitudinal studies. Recognizing lymphocytic focus-associated SG fibrosis as a fundamental pathology in SS, however, furthers our understanding of the complexity of this disease and paves the way for future investigations evaluating the utility of this feature for sub-setting SS patients.

Acknowledgments

The authors gratefully acknowledge the contributions of Michael Brown, Ben Fowler, Justin Willige and the OMRF Imaging Core facility for digital reconstruction of tissue cross-sections, Dr. Rajaram Gopalakrishnan, Dr. Andrew J.W. Huang, Dr. Pamela J. Hughes, and Dr. Michael D. Rohrer for their contributions to the University of Minnesota SRC. Supported by NIH grants AR060804, AR050782, DE015223 and DE018209, COBRE GM110766, Phileona Foundation, and by an American Association of Dental Research Sjögren's Syndrome Foundation Student Fellowship to KML.

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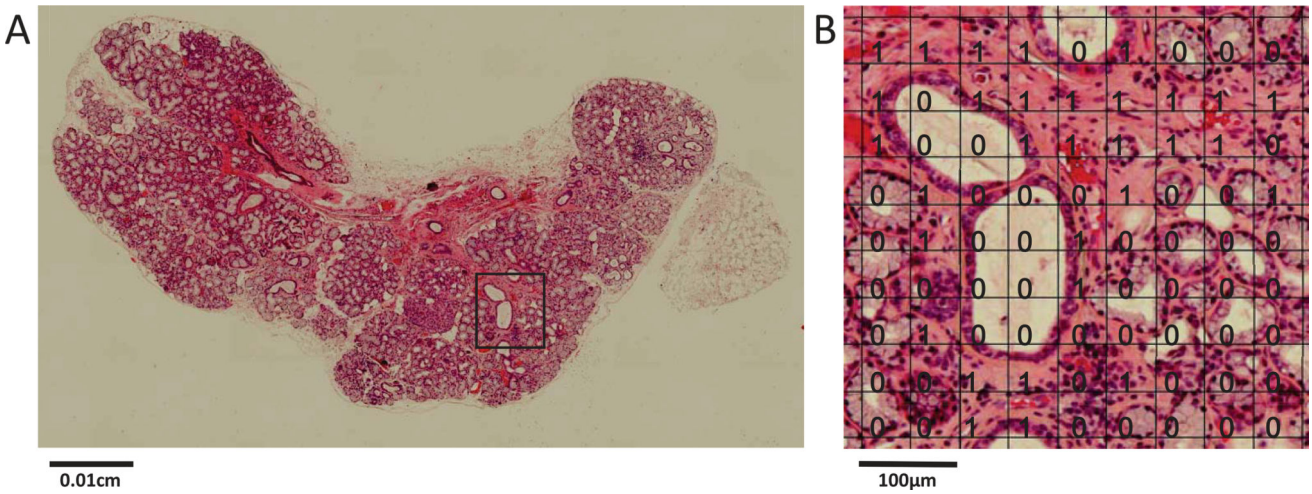

Figure 1.

Scoring of minor labial salivary gland fibrosis area. **A** Digital reconstruction of a minor labial salivary gland paraffin-embedded tissue section. Boxed region is magnified in B. **B** Representative scoring of labial SG tissue. “0” indicates squares where tissue is present, but is non-fibrotic. “1” indicates both tissue presence and fibrosis. Magnification = 200× Scale = 1.54 pixels/µm, grid square = 2500µm^2.

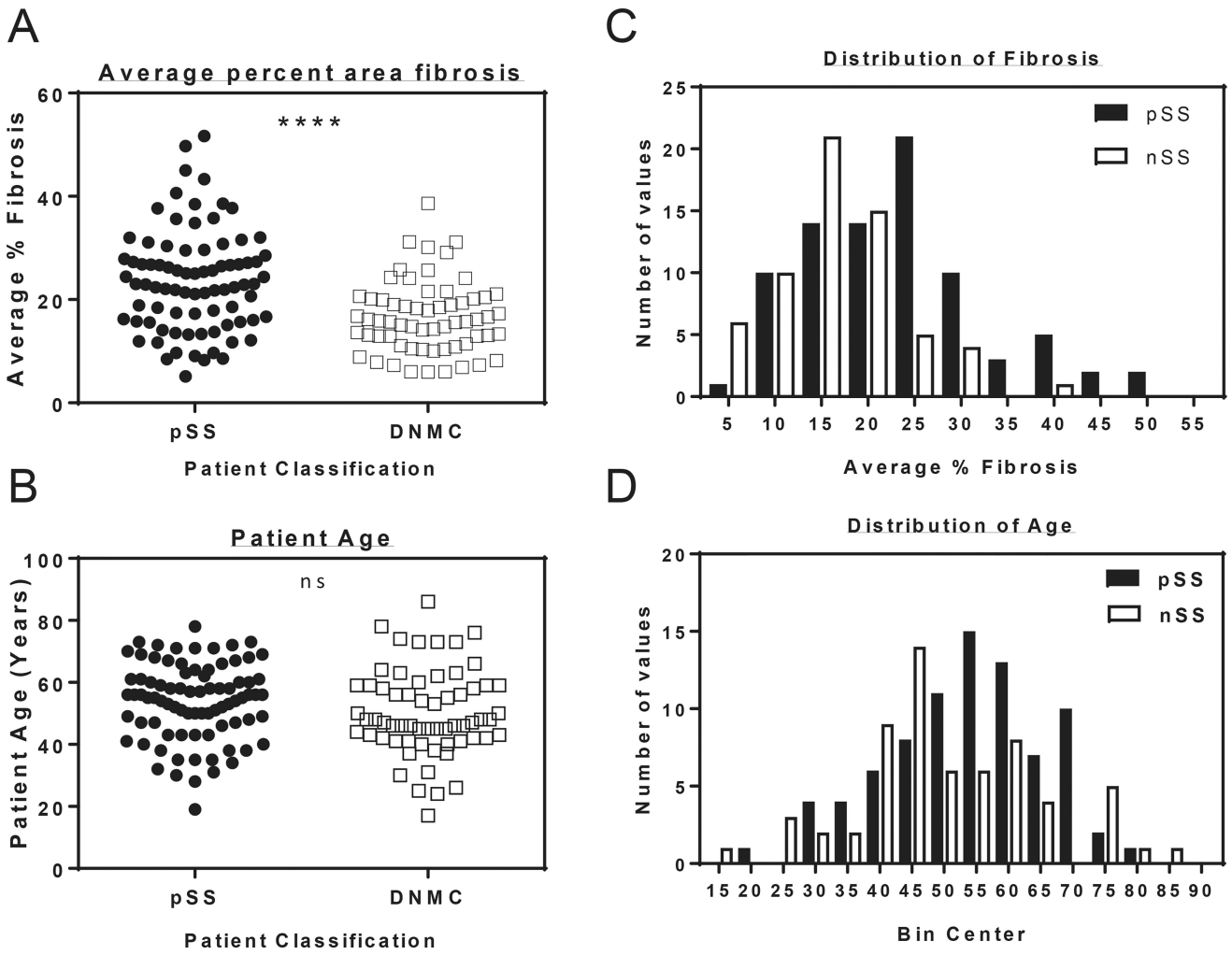

Figure 2.

Salivary gland fibrosis but not age is increased in pSS compared to nSS subject groups. **A** Average percent area fibrosis is significantly greater in pSS subjects (23.6% ± 1.1; mean ± SEM) as compared to nSS subjects (16.6% ± 0.90). Mann-Whitney t-test. **B** pSS subjects (53.8 ± 1.37 years; mean ± SD) are not significantly different in age as compared to nSS subjects (50.2 ± 1.76 years). Student's two-tailed unpaired t-test. **C, D** Distribution of percent area fibrosis and subject age are shown.

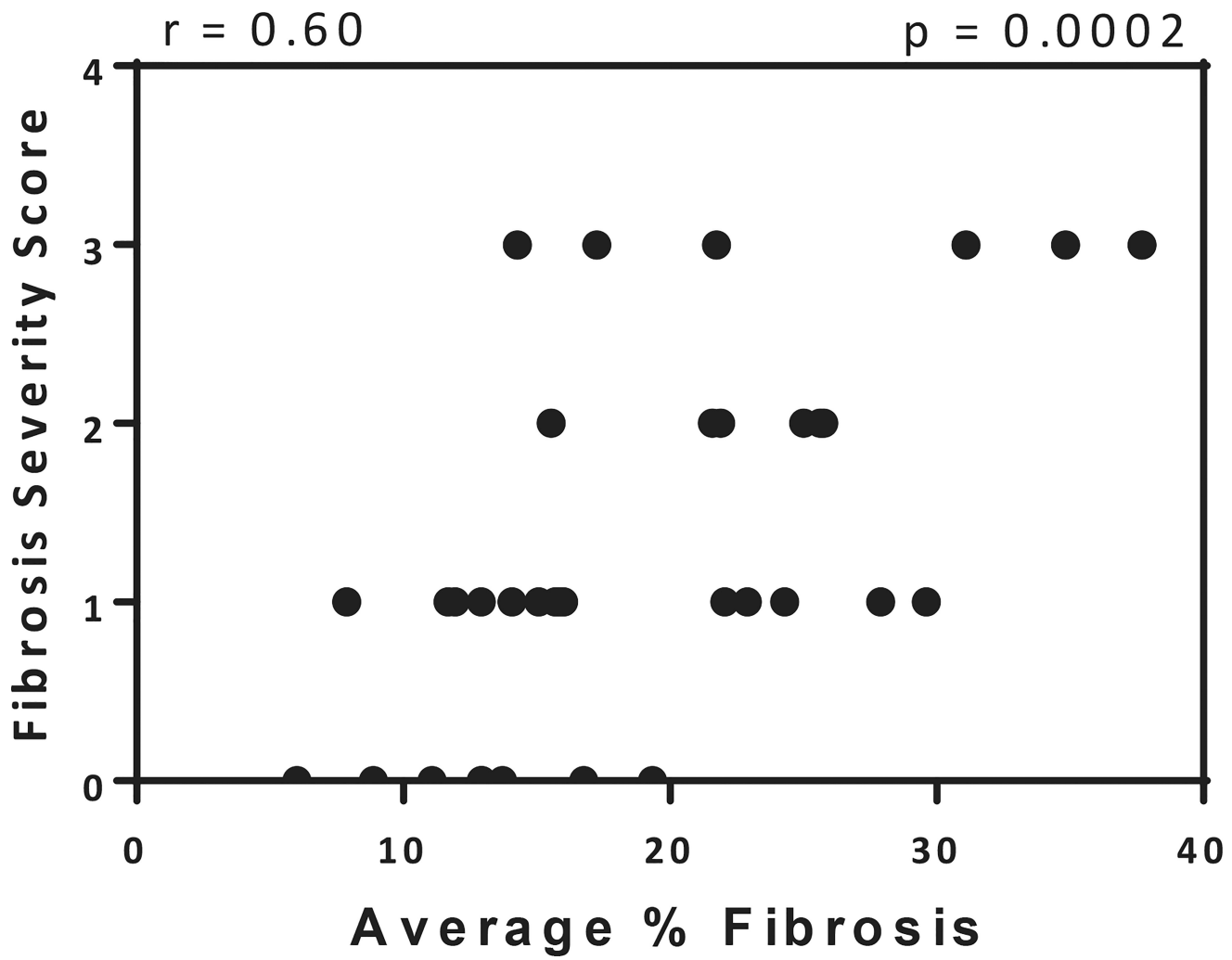

Figure 3.
Significant agreement between fibrosis severity scores and quantified percent fibrosis by area (pSS = 20, nSS n= 15), 2-tailed Spearman correlation.

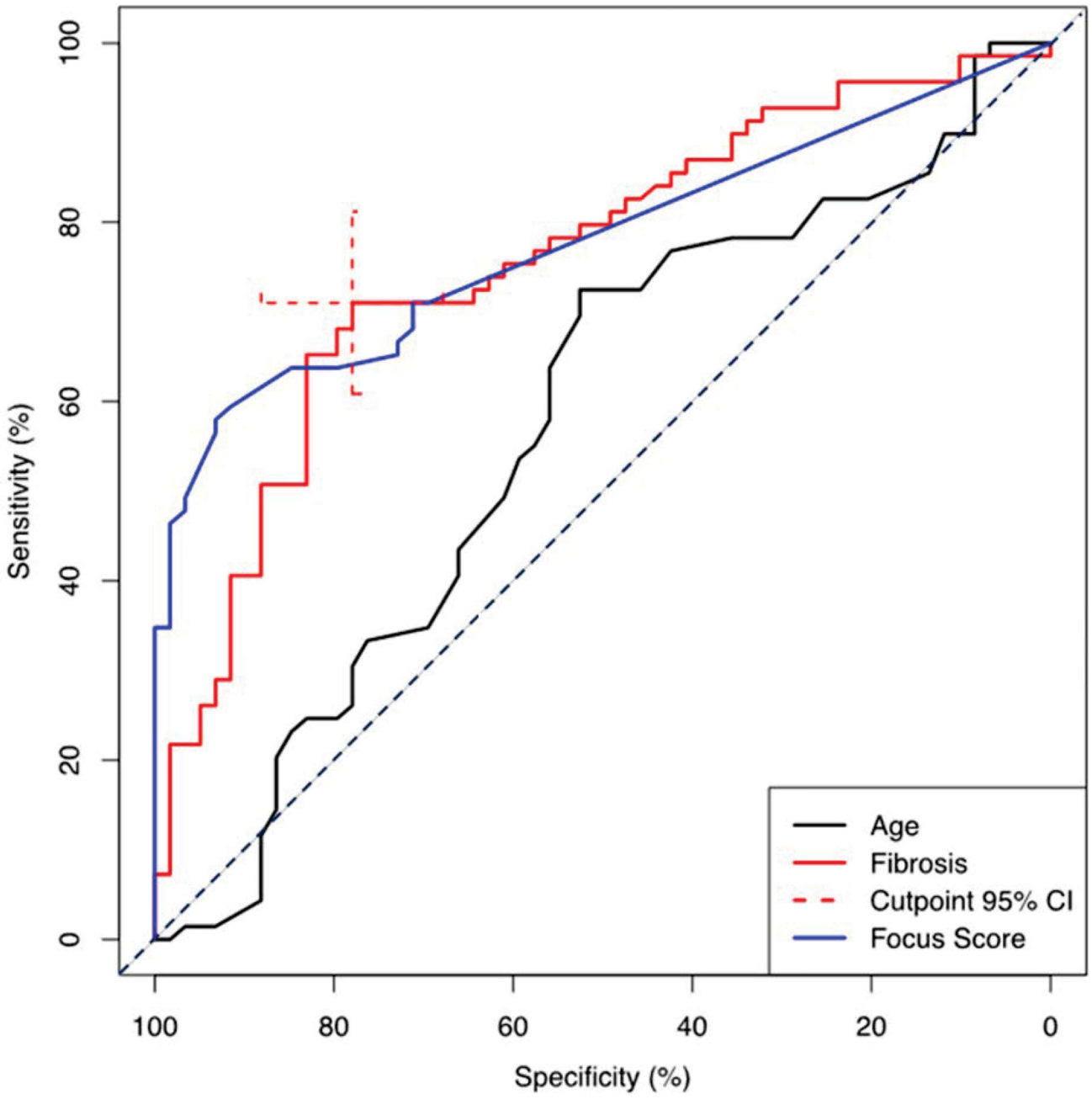

Figure 4.

Receiver operating characteristic (ROC) curves for age (black), average percent area fibrosis (red), and biopsy focus score (blue) are shown compared to the line of no-discrimination (dashed blue line). The optimal cut-point distinguishing pSS from DNMC is indicated by the intersection of the dashed red lines.

Table 1

Subject Demographic Characteristics

	Discovery Set			Replication Set		
	pSS	**DNMC**	**p-value***	**pSS**	**DNMC**	**p-value***
Total Participants (n)	34	28		35	31	
Age mean (SD)	52.38 (12.34)	51.82 (14.38)	0.8713	56.20 (11.49)	50.58 (14.08)	0.833
Fibrosis mean (SD)	23.05 (8.63)	15.24 (6.02)	**0.0002**	26.85 (10.32)	18.52 (7.28)	**0.0002**
Female (%)	82.35	85.71	1	88.57	90.32	1
Caucasian (%)	100	89.29	0.0866	85.71	64.52	0.0829
Anti-Ro/SS-A positive (%)	64.7	0	**<.0001**	65.71	3.22	**<.0001**
Anti-La/SS-B positive (%)	47.06	3.57	**0.0001**	34.26	6.45	**0.0068**
WUSF positive (%)	64.71	46.43	0.2	62.85	29.03	**0.0074**
Schirmer's positive (%)	55.88	35.71	0.1323	45.71	29.03	0.2073
VanBijsterveld positive (%)	61.76	35.71	**0.0403**	57.14	22.58	**0.0059**
FS≥1 (%)	73.53	17.24	**<.0001**	54.29	12.9	**0.0006**
Anti-cholinergic drugs positive (%)	52.94	67.86	0.3012	62.86	48.39	0.3212

*P-values were calculated by Fisher's exact test, except for age (unpaired 2-tailed t-test), and fibrosis mean (unpaired 2-tailed Mann-Whitney test).

Table 2

Fibrosis contributes significantly more information to discrimination models than age

Set*	#Variables		Point Estimate	p-value (z)	OR	Accuracy	Sensitivity	Specificity	p-value+
DS	2	Fibrosis	0.145	**0.001**	1.16	0.677	0.643	0.706	N/A
		Age	−0.013	0.572	0.99				
	1	Fibrosis	0.140	**0.001**	1.15	0.694	0.679	0.706	0.570
	1	Age	0.003	0.867	1.00	0.548	0.000	1.000	**<0.0001**
RS	2	Fibrosis	0.113	**0.006**	1.12	0.727	0.677	0.771	N/A
		Age	0.005	0.835	1.01				
	1	Fibrosis	0.116	**0.002**	1.12	0.727	0.677	0.771	0.835
	1	Age	0.035	0.083	1.04	0.682	0.581	0.771	**<0.0001**

*DS= Discovery Set, RS = Replication Set

+ANOVA, as compared to multivariate model

Table 3

Focus Score and Fibrosis combined enhance precision

Set*		#Variables	Point Estimate	p-value (z)	OR	Accuracy	Sensitivity	Specificity	p-value†
DS	2	Focus Score	1.287	**0.011**	3.62	0.790	0.857	0.735	N/A
		Fibrosis	0.093	0.055	1.10				
	1	Focus score	1.384	**0.003**	3.99	0.774	0.821	0.735	0.039
	1	fibrosis	0.140	**0.001**	1.15	0.694	0.679	0.706	**<0.0001**
RS	2	focus score	0.674	**0.021**	1.96	0.758	0.807	0.714	N/A
		fibrosis	0.112	**0.007**	1.12				
	1	focus score	0.869	**0.010**	2.38	0.682	0.839	0.543	**0.002**
	1	fibrosis	0.116	**0.002**	1.12	0.727	0.677	0.771	**0.0002**

*DS= Discovery Set, RS = Replication Set

†ANOVA, as compared to multivariate model

Table 4

Association of fibrosis with SS clinical features

	Linear regression		Age-corrected linear regression	
Clinical Variable	**Beta**	**p-value**	**Beta**	**p-value**
Age	0.034	**<0.0001**	NA	NA
vanBijsterveld Score	0.174	**0.028**	0.152	**0.042**
Schirmer's Score	−0.104	0.093	−0.054	0.366
WUSF	−0.426	**0.025**	−0.145	0.466
Biopsy Focus Score	0.836	**<0.0001**	0.713	**0.002**
Dental Restorations	0.083	**0.012**	0.042	0.245

WUSF: Whole unstimulated salivary flow