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

K.M. Leehan1,2, N.P. Pezant1, A. Rasmussen1, K. Grundahl1, J.S. Moore1, L. Radfar3, D.M. Lewis3, D.U. Stone4, C.J. Lessard1,2, N.L. Rhodus5, B.M. Segal6, R.H. Scofield1,7,8, K.L. Sivils1,2, C. Montgomery1, A.D. Farris1,2

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 haematoxylin and eosin stained slides were imaged, digitally reconstructed and analysed 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.

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 the 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 vanBijsterfeld 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.

Introduction

Sjögren’s syndrome (SS) is a systemic rheumatoid 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 aetiology 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
participants were self- or physi-
36). Briefly, IgG antibody titres were
titres were determined as described (35,
and Consensus Groups (22). Ro antibody
by the 2002 revised American Europe
vary flow, were conducted as specified
in SS contributes to our overall under-
and may open new therapeutic
This study was undertaken to deter-
mine if SS subjects have more, less,
or similar fibrotic replacement as com-
pared to subjects who have symptoms
of dryness but do not meet established
disease criteria (non-SS) and to deter-
mine 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 fibro-
sis 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 labo-
rary test values were obtained from the
Sjögren’s Research Clinic (SRC)
(at Oklahoma Medical Research Foun-
dation and University of Minnesota)
as previously described (34). Clinical
measures including focus score, van
Bijsterveld score, Schirmer’s test, and
collection of whole unstimulated sali-
vary flow, were conducted as specified
by the 2002 revised American Europe-
an Consensus Groups (22). Ro antibody
titres were determined as described (35,
36). Briefly, IgG antibody titres were
determined by ELISA using bovine-
derived Ro60 (Innominovision).
The participants were self- or physi-
cian-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 com-
plaint. All participants gave fully in-
formed consent in compliance with the
Declaration of Helsinki, and the study
was approved by both respective Insti-
tutional Review Boards. Participants
were classified using the 2002 revised
AECG criteria (22).
All participants with features of over-
lapping diseases (including rheumatoid
arthritis, systemic lupus erythematosus,
and systemic sclerosis) or with exclu-
sion criteria for AECG classification
(sarcoidosis, prior head and neck radia-
tion, hepatitis C infection, acquired im-
munodeficiency 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 randomised
into separate datasets, a discovery set
(n=35 pSS, n=31 nSS) and a replication
set (n=34 pSS, n=28 nSS) and a replication
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 I. Dental data, including num-
ber 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 es-
timation based on the date of diagnosis
(age of study entry) and the calculated
age of symptom onset (based on ques-
tions regarding subjective dryness).

Salivary gland biopsy and imaging
Four to six minor labial SGs per partici-

ant were formalin-fixed, paraffin-embedded,
sectioned (4 μm), and stained with
haematoxylin and eosin in either the
University of Minnesota or Uni-
versity of Oklahoma Health Sciences
Center oral pathology laboratories. Fo-
cus 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
200x magnification in overlapping sec-
tions. These sections were digitally as-
sembled using the Zeiss ZenBlue soft-
ware package to yield reconstructions of
entire glandular cross-sections (Fig. 1).

Quantitative fibrosis assessment
Fibrotic changes were quantified by an
observer blinded to disease classifica-
tion status as described (36). A stan-
ard grid of 2500 μm² was applied to
SG cross-section images using ImageJ
(National Institutes of Health, Bethes-
da, MD, USA). Each individual square

Table I. Subject demographic characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Discovery Set</th>
<th></th>
<th>Replication Set</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pSS</td>
<td>DNMC</td>
<td>p-value*</td>
<td>pSS</td>
</tr>
<tr>
<td>Total participants (n)</td>
<td>34</td>
<td>28</td>
<td></td>
<td>35</td>
</tr>
<tr>
<td>Age mean (SD)</td>
<td>52.38</td>
<td>51.82</td>
<td>0.8713</td>
<td>56.20</td>
</tr>
<tr>
<td>(12.34)</td>
<td>(14.38)</td>
<td></td>
<td></td>
<td>(11.49)</td>
</tr>
<tr>
<td>Fibrosis mean (SD)</td>
<td>23.05</td>
<td>15.24</td>
<td>0.0002</td>
<td>26.85</td>
</tr>
<tr>
<td>(8.63)</td>
<td>(6.02)</td>
<td></td>
<td></td>
<td>(10.32)</td>
</tr>
<tr>
<td>Female (%)</td>
<td>82.35</td>
<td>85.71</td>
<td>1</td>
<td>88.57</td>
</tr>
<tr>
<td>Caucasian (%)</td>
<td>100</td>
<td>89.29</td>
<td>0.0866</td>
<td>85.71</td>
</tr>
<tr>
<td>Anti-Ro/SS-A positive (%)</td>
<td>64.7</td>
<td>0</td>
<td>&lt;0.0001</td>
<td>65.71</td>
</tr>
<tr>
<td>Anti-La/SS-B positive (%)</td>
<td>47.06</td>
<td>3.57</td>
<td>0.0001</td>
<td>34.26</td>
</tr>
<tr>
<td>WUSF positive (%)</td>
<td>64.71</td>
<td>46.43</td>
<td>0.2</td>
<td>62.85</td>
</tr>
<tr>
<td>Schirmer’s positive (%)</td>
<td>55.88</td>
<td>35.71</td>
<td>0.1323</td>
<td>45.71</td>
</tr>
<tr>
<td>VanBijsterveld positive (%)</td>
<td>61.76</td>
<td>35.71</td>
<td>0.0403</td>
<td>57.14</td>
</tr>
<tr>
<td>FSe1 (%)</td>
<td>73.53</td>
<td>17.24</td>
<td>&lt;0.0001</td>
<td>54.29</td>
</tr>
<tr>
<td>Anti-cholinergic drugs positive (%)</td>
<td>52.94</td>
<td>67.86</td>
<td>0.3012</td>
<td>62.86</td>
</tr>
</tbody>
</table>

*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).
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 (Fig. 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 2500 μm² (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:

\[
\text{fibrosis percent area} = \frac{(\text{area of each grid square})}{\text{section area}} \times 100 = \% \text{ area fibrosis}
\]

(\text{where } n = \text{number of fibrosis positive grid squares})

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

\[
\text{fibrosis area} = \frac{[(\text{sec 1% area}) + (\text{sec 2% area}) + \ldots + (\text{sec } x \% \text{ area})]}{\text{total section number}} = \text{average % area fibrosis}
\]

**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-Wilk tests and where necessary, non-parametric tests. Both bivariate and univariate logistic regression generalised 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) utilised 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 ≤5 mm/minute, whole unstimulated saliva flow ≤1.5 mL 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 haematoxylin 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 (Fig. 2A & C). 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 (Fig. 2B & D). As expected, the pSS group exhibited higher incidence of positivity for measures utilised to classify individuals as having SS, including positivity for IgG anti-Ro/SS-A and anti-La/SS-B antibodies, van-Bijsterveld score (ocular damage) and biopsy focus score (focal lymphocytic infiltration) (Table I). 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 utilised 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 (Fig. 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 the severity of 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,
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Fig. 3. Significant agreement between fibrosis severity scores and quantified percent fibrosis by area (pSS=20, nSS n=15), 2-tailed Spearman correlation.

Table II. Fibrosis contributes significantly more information to discrimination models than age.

<table>
<thead>
<tr>
<th>Set</th>
<th>Variables</th>
<th>Point estimate</th>
<th>p-value</th>
<th>OR</th>
<th>Accuracy</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS</td>
<td>2 Fibrosis</td>
<td>0.145</td>
<td>0.001</td>
<td>1.16</td>
<td>0.677</td>
<td>0.643</td>
<td>0.706</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>-0.013</td>
<td>0.572</td>
<td>0.99</td>
<td>0.694</td>
<td>0.679</td>
<td>0.706</td>
<td>0.570</td>
</tr>
<tr>
<td></td>
<td>1 Fibrosis</td>
<td>0.140</td>
<td>0.001</td>
<td>1.15</td>
<td>0.694</td>
<td>0.679</td>
<td>0.706</td>
<td>0.570</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>0.003</td>
<td>0.867</td>
<td>1.00</td>
<td>0.548</td>
<td>0.000</td>
<td>1.000</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>RS</td>
<td>2 Fibrosis</td>
<td>0.113</td>
<td>0.006</td>
<td>1.12</td>
<td>0.727</td>
<td>0.677</td>
<td>0.771</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>0.005</td>
<td>0.835</td>
<td>1.01</td>
<td>0.727</td>
<td>0.677</td>
<td>0.771</td>
<td>0.835</td>
</tr>
<tr>
<td></td>
<td>1 Fibrosis</td>
<td>0.116</td>
<td>0.002</td>
<td>1.12</td>
<td>0.727</td>
<td>0.677</td>
<td>0.771</td>
<td>0.835</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>0.035</td>
<td>0.083</td>
<td>1.04</td>
<td>0.682</td>
<td>0.581</td>
<td>0.771</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*DS: Discovery Set; RS: Replication Set. *ANOVA, as compared to multivariate model.

Table III. Focus score and fibrosis combined enhance precision.

<table>
<thead>
<tr>
<th>Set</th>
<th>Variables</th>
<th>Point estimate</th>
<th>p-value</th>
<th>OR</th>
<th>Accuracy</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS</td>
<td>2 Focus score</td>
<td>1.287</td>
<td>0.011</td>
<td>3.62</td>
<td>0.790</td>
<td>0.857</td>
<td>0.735</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Fibrosis</td>
<td>0.093</td>
<td>0.055</td>
<td>1.10</td>
<td>0.737</td>
<td>0.821</td>
<td>0.735</td>
<td>0.039</td>
</tr>
<tr>
<td></td>
<td>1 Focus score</td>
<td>1.384</td>
<td>0.003</td>
<td>3.99</td>
<td>0.694</td>
<td>0.679</td>
<td>0.706</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Fibrosis</td>
<td>0.140</td>
<td>0.001</td>
<td>1.15</td>
<td>0.727</td>
<td>0.677</td>
<td>0.771</td>
<td>0.835</td>
</tr>
<tr>
<td>RS</td>
<td>2 Focus score</td>
<td>0.674</td>
<td>0.021</td>
<td>1.96</td>
<td>0.758</td>
<td>0.807</td>
<td>0.714</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Fibrosis</td>
<td>0.112</td>
<td>0.007</td>
<td>1.12</td>
<td>0.727</td>
<td>0.677</td>
<td>0.771</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>1 Focus score</td>
<td>0.869</td>
<td>0.010</td>
<td>2.38</td>
<td>0.682</td>
<td>0.839</td>
<td>0.543</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>Fibrosis</td>
<td>0.116</td>
<td>0.002</td>
<td>1.12</td>
<td>0.727</td>
<td>0.677</td>
<td>0.771</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

*DS: Discovery Set; RS: Replication Set. *ANOVA, as compared to multivariate model.

Table IV. Association of fibrosis with SS clinical features.

<table>
<thead>
<tr>
<th>Clinical variable</th>
<th>Linear regression</th>
<th>Age-corrected linear regression</th>
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<tbody>
<tr>
<td></td>
<td>Beta</td>
<td>p-value</td>
</tr>
<tr>
<td>Age</td>
<td>0.034</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>vanBijsterveld score</td>
<td>0.174</td>
<td>0.028</td>
</tr>
<tr>
<td>Schirmer’s score</td>
<td>-0.104</td>
<td>0.093</td>
</tr>
<tr>
<td>WUSF</td>
<td>-0.426</td>
<td>0.025</td>
</tr>
<tr>
<td>Biopsy focus score</td>
<td>0.836</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Dental restorations</td>
<td>0.083</td>
<td>0.012</td>
</tr>
</tbody>
</table>

WUSF: Whole unstimulated salivary flow.

Regression modelling 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 modelling was employed. Separation of pSS from nSS was the dependent variable, while biopsy focus score and percent area fibrosis were the predictive variables (Table III). In the discovery set, focus score significantly contributed to SS discrimination (p=0.011) while fibrosis exhibited a trend toward significant contribution to categorisation (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 IV). Anti-Ro autoantibody titre 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 IV). 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) (Fig. 4). We chose a threshold of 20.69% fibrosis to classify subjects because it optimises 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 centre, 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 modelling, 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 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 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. Recognising 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.

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