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Image analysis of fibrosis in labial salivary glands of patients with systemic autoimmune diseases. Close correlation of lobular fibrosis to seropositive rheumatoid arthritis and increased anti-CCP and RF titres in the serum

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Summary

Lobular fibrosis in labial salivary glands of patients with systemic autoimmune disease is a rarely examined and rather neglected histological change. Its significance and disease association is poorly understood. Our aim was to explore the clinical correlations of fibrosis in labial salivary gland samples using objective methods and laboratory parameters. Labial salivary gland samples from more than 300 patients over a 3-year period were selected from the archives of the pathology department, histologically examined, digitised, image analysed and statistically evaluated to identify the presence and intensity of lobular fibrosis, its relation to age, clinical diagnoses of systemic autoimmune disease and the presence of rheumatoid factor (RF), anti-cyclic citrullinated peptide (CCP), antinuclear antibodies (ANAs), and anti-dsDNA serum markers. Significant correlation was found between lobular fibrosis and the presence of autoimmune disease ($p = 0.023$), mainly seropositive rheumatoid arthritis ($p < 0.001$). Also significant association was found between the fibrosis and the presence of serum anti-CCP ($p < 0.001$) and IgA/IgG/IgM-RF ($p < 0.001$, $p < 0.001$ and $p = 0.008$, respectively). Significant association was explored between the anti-dsDNA positivity and the negative histology groups ($p = 0.033$) and between the ANA positivity and the inflammation only group ($p = 0.021$). The results suggest that lobular fibrosis tends to associate to certain systemic autoimmune diseases, mainly seropositive rheumatoid arthritis, and seems to be rare in labial salivary gland biopsies of autoimmune diseases characterised by presence of anti-dsDNA. The close correlation of ANA positivity and the inflammation only histology was not surprising, since the majority of patients (62%) have Sjögren's syndrome, known for its inflammatory infiltrate. These findings emphasise that evaluation of lobular fibrosis and inflammation in histological samples of labial salivary gland biopsies are equally important.

Key words: Labial salivary gland; image analysis; fibrosis; systemic autoimmune disease; rheumatoid arthritis; RF; CCP.

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INTRODUCTION

Lobular fibrosis in the labial salivary glands is a well-known, but not extensively studied, rather underrated phenomenon. An excessive amount of collagen in biopsy samples of labial salivary glands in systemic autoimmune diseases such as Sjögren's syndrome (SS),[1–3] rheumatoid arthritis (RA),[4] systemic sclerosis (SSc)[5] and systemic lupus erythematosus (SLE)[5] has been observed and reported, but the distribution and extent in relation to these disease categories are controversial in the literature. Several histological studies on labial salivary gland biopsies have focused on multiple changes (inflammatory infiltrate, fibrosis, fatty infiltration, acinar atrophy, etc.) in different autoimmune diseases[1–8] or simply in the elderly[9–12] but the quantification of the pathological alterations, mainly that of fibrosis, was subjective.[5–8,10] Only one paper with planimetric quantification of the fibrosis was published on a RA disease population.[4] Diffuse fibrosis in labial salivary glands was also reported in a case in relation to a specific dietary habit.[13] Because of the overlaps in the clinical forms of autoimmune diseases, it is sometimes difficult to insert a particular case into a disease category; therefore, we decided to study the association of lobular fibrosis not just with the clinical syndromes but also the serum autoantibody titres of the patients, this way using the most objective parameters. Besides the presence of fibrosis, its extent may also be important, therefore an image analysis study was designed to measure the excess of collagen exactly and to compare it to the total salivary gland tissue.

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MATERIALS AND METHODS

Patients and biopsy samples

Lower lip minor salivary gland biopsy samples of 373 patients taken at the Department of Dentistry, Oral and Maxillofacial Surgery, Medical School and Clinical Center, Pécs University, Hungary, were examined. All the patients between 2012 and 2014 with suspected Sjögren's syndrome were referred from the Department of Rheumatology and Immunology, Medical School and Clinical Center, Pécs University. The indication of biopsy was presence of sicca symptoms. Age, gender, autoantibody levels and all the available parameters of the patients necessary for the study were acquired from the official clinical database (eMedsolution). Clinical diagnoses of patients involved in the study were also re-evaluated (SG) according to the then actual diagnostic criteria of SS[14] [American-European Consensus Group (AECG) criteria, 2002], RA[15] and other systemic autoimmune diseases.[16–18] Patients were grouped into five clinical categories according to their clinical diagnoses: (A) no systemic autoimmune disease; (B) SS syndrome and SS overlap (RA not included); (C) RA and RA overlap (SS not included); (D) SS-RA overlap; (E) any other systemic autoimmune disease (SLE, SSc, mixed connective tissue disease, etc.). Three patients were lost from the clinical follow up. Due to the lack of clinical data necessary for establishing the proper diagnosis they were excluded from those statistical analyses which involved clinical syndromes. Laboratory tests for autoantibodies (Supplementary Table 1, Appendix A) were performed in the Department of Immunology and Biotechnology, Medical School and Clinical Center, Pécs University. Standard formalin fixed, paraffin embedded blocks, 4 μm sections and routine haematoxylin-eosin (H&E) stains were prepared in conjunction with three ancillary histochemical stains: periodic acid-Schiff (PAS), picric acid-sirius red (PS) and Congo red stains for mucin-containing parenchyma, for the presence of fibrosis or amyloid, respectively. All were performed in the Department of Pathology, Medical School and Clinical Center, Pécs University.

Light microscopy evaluation

All the H&E stained slides from the cases included in the study were examined by light microscope. Inflammatory infiltration (grade and score), fibrosis (diffuse or partial), acinar atrophy with ductal epithelial changes, and the presence of amyloid deposits were evaluated by two independent observers (TT and KK). Samples considered showing fibrosis (n = 199) as well as a group of negative controls (n = 24) without histopathological changes were selected for further analysis. Fibrosis was diffuse lobular in the majority of cases (Fig. 1A), but in some samples it was restricted to only one or two lobules surrounded by intact glandular tissue. These cases were referred to as partial fibrosis (Fig. 1B). Alcian blue-picric acid-sirius red (APS) stained slides were used on the selected cases especially for image analysis (Fig. 2). APS staining was performed according to Krutsay.[19] Three samples of the fibrosis groups were excluded from the study as the remaining tissue in the paraffin block was not sufficient for further analysis.

Digitisation and image analysis

APS stained slides were digitised with a slide scanner (Pannoramic MIDI; 3DHistech, Hungary). Image analysis was carried out on virtual slides using the Histoquant module of CaseViewer (3DHistech). Digitisation and image analysis were performed similarly to our previous works.[20,21] The module, adjusted by eye control, can differentiate between the colours and measure the percent of differently coloured areas of the slides. APS staining was chosen particularly because the colour contrast of the mucin (blue area) and the collagen (red area) could easily be distinguished and measured simultaneously on the same slides and in the same annotations. The aim was to measure the intralobular fibrosis only. Collagen content of the interlobular septae and that around the central ducts were excluded by the annotations which were made manually in the CaseViewer. Three to six annotations on each slide (depending on the size of the biopsy sample) were made, attempting to avoid the central ducts, inflammatory foci and extralobular collagen (Fig. 2). Annotation and masking are seen on Fig. 3A–C. The size and shape of the annotations were different as the lobules varied. In seven cases the inflammatory focuses were extensive, therefore the proper annotations could not be made. Because fibrosis was the focus of our interest, image analysis of inflammation only cases (group 1 – see 'Statistical analysis' section below) was not performed and these were excluded from certain statistical analyses. All the annotations were analysed separately. Masking of the red (collagen) and blue area (mucin) were adjusted with eye control. The software calculated the percentage of collagen and mucin containing area (MA) according to the formula: masked area (MA) / field area (FA) ×100, where the FA is the total annotated area.

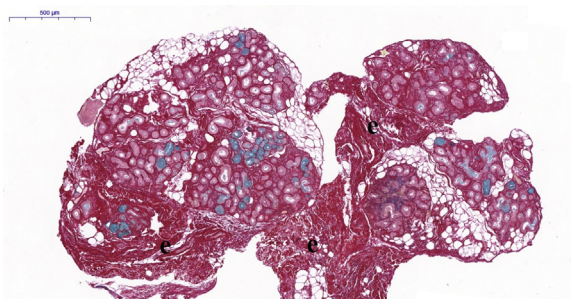

Fig. 2 Alcian blue-picric acid-sirius red (APS) staining of labial salivary gland. APS highlights the collagen increase (red) within the lobular interstitium. Mucinous glands (blue) disappear, duct epithelium predominates and a moderate increase of fat tissue is also visible. Extralobular collagen (e) is not measured.

Data collection and detection of autoantibodies

Age, gender and serum autoantibody titres (positivity) were acquired from the common clinical database of eMedsolution (T-Systems, Hungary). Various autoantibody titres were determined in the patients as only the relevant tests necessary for establishing the proper clinical diagnosis and treatment were requested. All the antibody tests were performed in the validated laboratory of the Department of Immunology and Biotechnology, Medical School and Clinical Center, Pécs University, using validated kits. Disease specific autoantibodies were measured using conventional ELISA tests. After positive ANA screening test (ANA-Ease ELISA Kit, GD74; Genesis, United Kingdom), anti-CenpB (ORG 633; Orgentec, Germany), anti-dsDNA (ORG 604, ORG 204; Orgentec) and ENA6 (SSA, SSB, Sm, RNP, Scl-70 and Jo-1) antibodies were measured using antigen-specific ELISA tests. All other

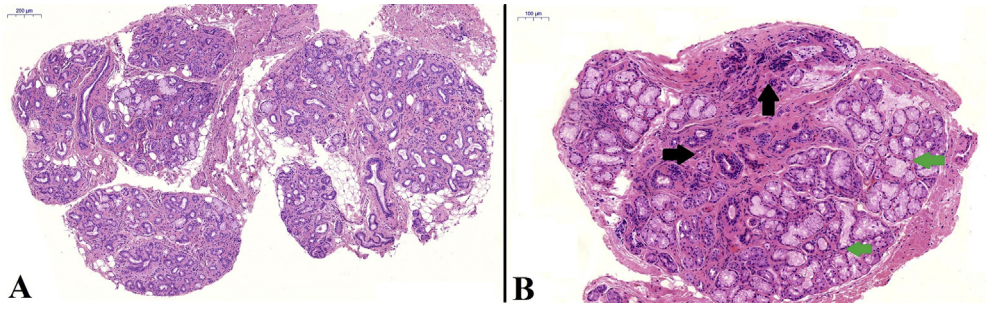

Fig. 1 (A) Diffuse and (B) focal fibrosis in H&E stained labial salivary glands. Diffuse, increased eosinophilic collagen deposition (diffuse lobular fibrosis) with duct ectasia and loss of mucinous glands (A) and focal fibrosis (black arrows) with duct ectasia in only two of the lobules (partial fibrosis) (B). Green arrows point to the intact lobules with mucinous glands.

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