Evaluation of fibrosis and fatty infiltration in minor salivary glands of patients with systemic autoimmune disease by computer assisted image analysis

PhD thesis

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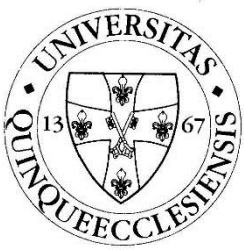

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Introduction

Anatomy, histology and physiology of minor salivary gland

Several hundreds of minor salivary glands (MSGs) can be found under the mucosa in the oral cavity. Similarly to the major salivary glands these consist of parenchyma and stroma. Most of their acini are mucinous and parts of the secretory duct system are less developed than that of the major salivary glands. They are important in lubrication of the mucosa and in producing antimicrobial proteins and glycoproteins, and so they play a major role in oral defence.

The importance of MSGs in diagnostics

Contrary to the parotid gland, labial minor salivary glands are easier to access and the risk of complication associated with the biopsy procedure is reduced. Therefore their use in the diagnostics of Sjögren's syndrome (SS), systemic amyloidosis and sarcoidosis became a common practice.

Histopathological changes in MSGs and their presence in systemic autoimmune diseases

Grading of the inflammatory infiltration in the samples have been developed and published by Chisholm. The focus described as a minimum of 50 lymphocytes and/or

plasma cells within 4 mm^2 of salivary gland tissue remains the hallmark for the diagnosis of SS. Despite their common appearance, the role and importance of lobular fibrosis (LF) and fatty infiltration (FI) are rather controversial in the literature. These changes are generally associated with ageing while some authors found them more commonly in MSGs of patients with different systemic autoimmune diseases (SS, Rheumatoid Arthritis (RA), Systemic Lupus Erythematosus (SLE), Systemic Sclerosis (SSc). Acinar loss (also described as mucin loss or acinar „atrophy") is generally considered as a consequence of the aforementioned histological changes.

Systemic autoimmune diseases and autoantibodies

An autoimmune reaction is characterised by an inflammatory response against self-structures (autoantigen). In case of systemic autoimmune diseases (SAD), this reaction affects more organs, organ systems. SS, RA, SSc, SLE are all classified as SADs. They are commonly characterised by female predominance and the onset is often over the age of 50 years. Both genetic and environmental factors are proven to play a crucial role in their development. Overlaps between different entities of the group are common thereby patients may present symptoms of multiple SADs. Diagnostics of these diseases rely on criteria which have been revised numerous times over the years and varied among

geographical areas. Both subjective and objective parameters are included in the systems. Detection of autoantibodies is one of the most crucial from the latter group. Screening for antinuclear antibodies (ANA) in systemic autoimmune disease suspected cases is a common practice. Diagnostic and prognostic markers such as rheumatoid factor (RF) and antibodies against cyclic-citrullinated proteins (anti-CCP) in RA, anti-SS-a and anti-SS-b in SS, anti-dsDNA and anti-nucleosome antibodies in SLE, anti-centromere antibodies (ACA) and anti-topoisomerase I (SCL-70) in SSc are essential in the clinical practice.

Aims

1. Evaluation of histopathological changes (focus forming inflammation (ffI), lobular fibrosis, fatty infiltration) and their combinations in MSGs of patients with different systemic autoimmune diseases and sicca controls.

2. Objective quantification of lobular fibrosis (LF) and fatty infiltration (FI) by image analysis.

3. To evaluate the association between histopathological changes and clinical parameters (gender, age, body mass index /BMI/, diagnosis of systemic autoimmune disease) and serum autoantibodies.

4. To clarify the factors contributing to the development of LF and FI in LSGs with a study of higher case number.

Materials and methods

Patients, biopsy samples and data collection

Five hundred seventy-six MSGs harvested at the Department of Dentistry, Oral and Maxillofacial Surgery, Medical School and Clinical Centre, Pécs in 5 consecutive years were collected from the archives of Pathology Department. All patients were referred to biopsy due to sicca symptoms. The samples were stained according to HE, PAS and Congo red. Acetic acid alcian blue picric-acid-red (APS) stained slides were made for digital image analysis. All cases were re-evaluated by light microscopy and the presence of focus forming inflammatory infiltration (ffI), LF and FI were registered. Clinical data of the patients were obtained from the database of the University (eMedsolution). Age, gender, BMI, the diagnosis of diabetes mellitus (DM), diagnosis of SAD and results of serological tests performed at the department of Immunology and Biotechnology were used. Regional Ethics Committee approved the present study.

Digitization and image analysis

APS stained slides of LF cases and PAS stained slides of FI cases and slides from 35 histologically negative cases were digitized. Three to six annotations were made on the digital slides according to the following criteria. In APS stained slides separate lobules or corresponding

areas, avoiding focus forming inflammation, central ducts and fatty infiltration were selected. In PAS stained slides whole minor salivary glands including multiple lobules (acini, ducts, inflammatory infiltration etc.) were selected. In some samples fibrosis was not present as a diffuse reaction, but as focal or partial change affecting only some distinct areas. In cases like this, annotations were made both in the affected and the spared areas. The software used for digital image analysis can differentiate based on colours and measure the percent of differently coloured areas of the slides. Collagen stained red (Sirius red) and mucin stained blue (alcian blue) in APS slides, therefore both could be measured on the same areas at the same time. Various shades of purple in PAS stained slides (glandular tissue) could be distinguished from white/blank areas (representing the dissolved fat from adipocytes due to sample processing). Fatty infiltration was calculated indirectly from these data.

Statistical analysis

The analysis was carried out by SPSS (IBM Corporation, New York, USA, v20.0). The result was assumed as significant, if "p value" was lower than 0.05. Normal minimal mucin and maximal collagen content of the control slides were determined. Gender, age, BMI values, BMI groups, diagnosis of DM, histologic diagnosis and groups and data acquired from the digital image analysis were used in the statistical analysis. Histologic changes

were analysed separately and in group classification as well. Histologic groups included: 0- no histologic changes, 1-focus forming inflammatory infiltration alone (ffI), 2- lobular fibrosis alone (LF), 3- fatty infiltration alone (FI), 4- combination of all three changes (ffI+LF+FI), 5- ffI+LF, 6-ffI+LF, and 7-LF+FI. All the statistical analysis were performed excluding the cases with partial and focal histologic changes as well. Cases classified according to systemic autoimmune disease (SAD) positive and negative as well as according to distinct diseases (SS, snRA and spRA, SSc, SLE, NDC and any other SAD) were analysed. Furthermore diagnostic groups: A-no SAD could be verified, B-SS and SS overlap (without RA), C-RA and RA overlap (without SS), D-SS-RA overlap, E- any other SAD (SLE, SSc, MCTD, NDC, etc.) were defined and applied in the statistics. Chi square tests were used for the evaluation of connection between categorical variable while T-tests and Analysis of Variance (one way ANOVA) with Bonferroni post hoc tests were used in cases of variables with normal distribution. In cases of non-normal variables for comparison between two samples Mann-Whitney tests were used, in cases of more samples Kruskal Wallis tests were performed. Linear regressions with Pearson correlation analysis were used to evaluate correlation between continuous variables. Binary logistic regression with forward selection was used in multivariable comparisons.

Results

Controls

We defined the normal maximal collagen content and blank area in percentage (correlates with fatty infiltration) and the normal minimal mucin content using the average of the measured values and applying twice the standard deviation. Normal values defined are 20% for collagen, 10% for blank area, and 50% for mucin.

Inflammation

Average age was significantly ($p<0.001$) higher in ffI positive cases (54.04 vs. 58.22 years). In addition LF and FI were significantly more common in ffI cases ($p<0.001$ and $p=0.002$ respectively, 65.60% vs. 44.80% and 68.20% vs. 56.00%)

The presence of ffI was significantly ($p<0.001$) different among clinical diagnosis groups. It was more common in SS, SS and SS overlap and in SS-RA overlap cases (~94%).

Negative histology group (0) was less common while all histology groups with ffI (1,4,5,6) were more common ($p<0.001$) in SS. We observed similar difference in SAD + cases compared to SAD- ones ($p<0.001$).

ANA positivity was significantly more common in ffI positive histology groups ($p=0.014$). Similar correlation

was found with anti-ENA and anti-nucleosome positivity (p=0.002 and p=0.029). Anti-SS-a positivity was also more common in histology groups with ffI except group 4 (ffI+LF+FI) (p<0,001).

Fibrosis

Collagen content (extent of fibrosis) in the samples showed positive correlation with acinar loss (p<0.001, r=0.669). Although the extent of fibrosis did not show significant correlation with the age, the average age in fibrosis positive cases was significantly higher (p<0.001; 62.03 vs. 48.93 years) Similar correlation was found with fatty infiltration (p<0.001; 42.2% vs 78.4%).

The prevalence of fibrosis showed positive correlation with the diagnosis of SS (p=0.002, 63.03% LF positive) and spRA (p<0.001, 78.33% LF+). Collagen content was significantly lower in SLE cases (p=0.01 SLE negative: N=287 median: 24.790% max.: 45.710% min.: 13.800%, SLE positive: N=6 median: 20.089%, max: 22.780% min: 16.615%). The collagen content did not show significant correlation with any other disease.

Significant correlation was found between the presence of LF and certain clinical diagnosis groups (p<0.001), LF and clinical diagnosis groups based on RA serology (p<0.001), and LF and SAD +/- cases (p=0.001). LF was more common in SS, SS-RA, spRA cases, and in SAD+ group (compared to SAD negative, 59.3% vs 44.3%).

In spRA cases negative histology was rare while all histology groups containing LF (3,4,5,7) were more common (p=0.028). In SAD+ cases (compared to SAD-) all histology groups with LF but without ffI (2,7) were less common (p<0.001).

Positive correlation was found between LF and RF IgA and IgG positivity (p=0.004; 68.47% LF+ and p=0.016; 66.12% LF+) anti-CCP positivity (p=0.001; 74.65% LF+) and negative correlation with anti-dsDNA (p=0.01; 37.50% LF+) and anti-SS-a positivity (p=0.007; 40.30% LF+).

Anti-CCP positivity was more common in all LF containing histology groups (2,4,5,7) and in group 1 (ffI alone) (p=0,006).

Fatty Infiltration

The extent of fatty infiltration (blank area %) showed positive correlation with BMI (p=0.02 r=0.186) and age (p<0.001 r=0.455). The prevalence of FI was also higher in higher BMI groups (p<0.001). Positive correlation was found between the presence of ffI and the diagnosis of DM (p=0.001). 77.78 % of DM positive cases were FI positive while this ratio in DM negative group was 60.04%. Both average age and average BMI were significantly (p<0.001 and p=0.05) higher in FI positive cases (61.13 vs. 48.06 years and 29.46 vs. 24.45 BMI value).

Clinical diagnosis of SS positively correlated with the presence of FI (p=0,003). FI was detected in 70.14% of SS cases while it appeared in 57.56% of non SS cases.

Significant correlation was found between the presence of FI and clinical diagnosis groups (p= 0.002), FI and clinical diagnosis groups based on RA serology (p=0.006) and FI and SAD +/- cases (p=0.034). It was less common in group A (no SAD) and group E (any other SAD). It was more common in SAD+ than in SAD- cases (65.2% vs 55.7%), but the extent of FI did not differ in these groups (p=0.869, p=0.968 and p=0.590).

Negative correlation was found between the presence of FI and seropositivity for anti-SS-a (p=0.006), anti-SS-b (p=0.013) and anti-RNP (p=0.018). 53.73% of anti-SS-a positive cases were FI negative. This ratio was 55.56% in anti-SS-b positive and 75% in anti-RNP positive cases. The extent of FI was significantly lower in ANA positive cases (p=0.026; ANA+: N:77 median:16.988% max: 46.100% min:5.884%; ANA- :N=276 median= 20.992% max=67.767% min=6.840%). No correlation was found with other autoantibodies.

BMI, age, acinar loss, histology groups

Positive correlation was found between age and BMI value (p=0.001 r=0.178)

In group “any other SAD” (group E) distribution of histology groups without partial or focal changes showed significant difference (p=0.033). Negative histology (0) was less common while ffI alone (1) LF alone (2) and the combination of three histologic changes (ffI+LF+FI) were more common. RF IgA positivity was also more common in ffI alone, LF alone, ffI+LF+FI, and LF+FI groups and less common in FI alone, ffI+LF and ffI+FI groups, when histology groups without partial or focal changes were examined (p=0.016).

Average age was significantly different between BMI groups 1 and 2 (normal and overweight) (p=0.005 54.18 vs. 60.15 years). Average age was also significantly higher in SS cases (p=0.008, 57.89 vs. 54.86 years) but the average BMI did not differ significantly between SS and non SS cases. In anti-SS-a and anti-SS-b positive cases both average age (p=0.042 and p=0.003; 52.67 vs. 56.64 and 48.87 vs. 56.76 years) and BMI (p<0.001; 24.87 vs. 28.30 and 24.65 vs. 28.18) were significantly lower. In SAD positive group average age was also significantly higher (p=0.010; 56.98 vs. 53.75 years), but the average BMI did not differ between SAD positive and negative cases.

Discussion

LF in MSGs of SS patients was reported to be more common in several studies. We also observed similar correlation with SS but LF was even more common in spRA cases. In groups of SS-RA overlap and spRA and overlaps (except SS) LF was rather common (76.70% and 82.90% respectively). In SS and SS-overlap (except RA) and in snRA group LF was less frequent (59.90% and 52.90% respectively). Not surprisingly LF was also more frequent in SAD+ cases than in the negative ones (59.3% vs 44.3%). Age as a factor contributing to LF should not be dismissed either as the average age in LF positive cases was significantly higher than in the negative ones (62.03 vs. 48.93 years). However, the collagen content did not correlate with the age.

As expected in SS cases, all histology groups containing focus forming inflammatory infiltration were more common, whereas in SS-spRA overlaps, both the ffI characteristic for SS and the LF more typical in spRA were frequently present (92% and 72% positive respectively).

Friedman et al. observed higher frequency of LF in MSGs of patients with SSc (22 cases) and SLE (20 cases). We did not find LF more common in SLE or SSc cases, moreover in the former group the collagen content was significantly lower. However, it has to be mentioned

that a rather limited number of SLE cases (only 6) with available data of collagen measurement was accessible. These results would be less established (due to the case limitations) but in seropositive cases for markers commonly associated with SLE (anti-dsDNA and anti-SS-a positivity), fibrosis was significantly less common. These observations suggest that LF is less common and less severe in SLE cases. Due to the limitation of case numbers conclusions should not be drawn based on these pieces of information.

Markkanen et al. found positive correlation between LF and RA. Contrary to their work collagen content did not differ significantly in any of the SADs, but in spRA cases and SS-RA overlaps LF was more frequent. Furthermore serological markers associated with RA (RF and anti-CCP) also showed positive correlation with the presence of LF thus it certifies the strong link between spRA and LF. Anti-CCP positive cases showed the strongest correlation with LF as in all statistical comparisons the correlation remained significant.

We also reported strong, almost linear correlation between collagen increase (fibrosis) and mucin loss (acinar loss). This demonstrates the negative effect of fibrosis on the functionally active parenchyma of the gland and suggests a decreased function.

Similarly to several reports we also noted the positive correlation between ageing and fatty infiltration of the MSGs. Furthermore significant correlation was found between the age and the extent of FI.

We could not reproduce results published by Skarstein et al. regarding the strong correlation between FI and ffI negative cases of SS. Significant correlation was observed between SS and the presence of FI, but the control group that the SS cases were compared to was different. In Skarsten's work the control group included age matched, SAD negative sicca controls whereas in the present study SS cases were compared to non SS cases (including SAD negative and positive cases as well). Furthermore the average age of SS patients were significantly higher compared to the non SS cases (57.89 vs. 54.86). Ageing is proved to be a significant factor contributing to the development of FI. Therefore FI should not be considered as histological manifestation of a specific SAD. It is strongly related to other parameters like ageing and obesity. Beside the well-known age-FI correlation it was also certified that BMI positively correlates with both the presence and extent of FI. Not only was the average BMI higher in FI positive cases (29.46 vs. 24.45) but FI positive cases were more frequent in higher BMI groups.

Our data showed that LF is closely linked to spRA and to its serological markers (mainly anti-CCP) while the

extent and presence of FI are mainly related to ageing and obesity. Sicca complaint is the most common indication of MSG biopsy, as not just the ffI, but all histological changes contributing to reduced saliva production should be investigated. Since both LF and FI could contribute to acinar loss and therefore to reduced saliva flow, they all should be mentioned as pathological findings. Furthermore LF may suggest SAD positivity providing useful information to the clinicians.

New results

1. We applied APS staining first the time to measure the mucin content and collagen content in MSGs by image analysis.

2. We used objective image analysis the first time in the literature to quantify the extent of FI in MSGs.

3. We proved that both the presence and extent of FI correlate with not only with ageing but also with obesity.

4. We also proved the rather logical, strong negative correlation between the extent of fibrosis and mucin content (preserved acini).

5. Our team was the first to prove the correlation between LF and spRA and we also confirmed its relation to SS and SS overlaps with a higher case number study.

6. Our data showed strong correlation between LF and anti-CCP positivity.

7. Our findings additionally confirmed that spRA and snRA are two different entities not only considering their pathogenesis but also their clinical presentation.

List of publications

Publications related to the thesis:

Katona K, Elekes E, Farkas N, Kneif M, Sütő G, Tornóczky T. Image analysis of fatty infiltration in labial salivary gland biopsies: extent and its correlation to age, obesity and diabetes. **J Oral Pathol Med. 2017 IF: 2,237**

Katona K, Farkas N, Sütő G, Tornóczky T. Adipose tissue infiltration in minor salivary glands of patients with Sjögren's syndrome: Lack of significant correlation with the disease. An image analysis of 174 cases. **Autoimmunity.2017. IF: 2,648**

Katona K, Farkas N, Kneif M, Sütő G, Berki T, Balatonyi B, Tornóczky T. 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 titers in the serum. **Pathology 2018 IF: 3,068** *(2017)*

Publications not related to the thesis:

Tóth E, Tornóczky T, Kneif J, Perkecz A, Katona K, Piski Z, Kemény Á, Gerlinger I, Szolcsányi J, Kun J, Pintér E. Upregulation of extraneuronal TRPV1 expression in chronic rhinosinusitis with nasal polyps. **Rhinology. 2018 IF: 2.931** *(2017)*

Oral presentations related to the thesis

72. Patológus Kongresszus, (2015) Hajdúszoboszló, Előadás "Kisnyálmirigy hisztopatológi-ai elváltozások szisztémás autoimmun betegségekben"

Fiatal Patológusok Találkozója (2016) Budapest. „A kisnyálmirigyekben előforduló zsíros infiltráció vizsgálata számítógépes image analysis-el: a jelenség kiterjedése és kapcsolata az életkorral és elhízással”