Review

The role of salivary gland histopathology in primary Sjögren's syndrome: promises and pitfalls

F.G.M. Kroese[1], E.A. Haacke[1,2], M. Bombardieri[3]

[1]Department of Rheumatology and Clinical Immunology, University of Groningen, University Medical Centre Groningen, The Netherlands;
[2]Department of Pathology and Medical Biology, University of Groningen, University Medical Centre Groningen, The Netherlands;
[3]Centre for Experimental Medicine and Rheumatology, William Harvey Research Institute, Queen Mary University of London, UK.

Frans G.M. Kroese
Erlin A. Haacke
Michele Bombardieri

Please address correspondence to:
Dr Michele Bombardieri,
Centre for Experimental Medicine and Rheumatology,
William Harvey Research Institute,
Queen Mary University of London,
Charterhouse Square,
EC1M 6BQ, London, UK.
E-mail: m.bombardieri@qmul.ac.uk
or
Dr Frans G.M. Kroese,
Department of Rheumatology and Clinical Immunology,
University Medical Center Groningen,
University of Groningen, HPC AA21,
Hanzeplein 1,
9713 GZ Groningen, The Netherlands.
E-mail: f.g.m.kroese@umcg.nl

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ABSTRACT

The formation of lymphomononuclear cell infiltrates organising as periductal infiltrates in the salivary glands of patients with primary Sjögren's syndrome (pSS) is one of the hallmarks of the disease. Historically, the clinical role of salivary gland histopathology, most commonly performed on labial salivary gland biopsies, has been confined to the clinical classification and diagnosis of pSS whereby according to the ACR-EULAR a positive histopathology finding is a requirement for the diagnosis of pSS in the absence of anti-Ro/SSA antibodies. In recent years, further understanding of the heterogeneity of the immune cell infiltration and organisation within the salivary glands of pSS patients and its correlation with clinical manifestations of the disease has led to propose salivary gland histopathology as a novel tool able to identify patients at higher risk of developing more severe extraglandular manifestations and lymphoma. Furthermore, recent clinical developments in ongoing randomised clinical trials with novel biologics in pSS have focused on salivary glands histopathology to inform on patients stratification based on target validation, proof of drug efficacy and mechanisms of response/resistance to therapy. However, lack of standardisation of methodology and analysis has hindered the reproducibility of data from different groups and no definitive evidence in support of the use of salivary glands histopathology to inform clinical management of patients with pSS has been provided. In this review, we summarise recent evidence highlighting the promises and pitfalls of salivary glands histopathology in pSS emphasising the need for an international consensus on standardisation of methodology with validation in large prospective multicentre initiatives.

Introduction

In this review, following a brief introduction on the cellular and molecular mechanisms underlying the formation of the periductal inflammatory infiltrates in the target tissue of pSS, we will focus on three critical aspects of salivary glands histopathology which are of important clinical relevance in patients with pSS: i) the performance of histopathology as diagnostic tool; ii) the potential role of salivary gland biopsies as predictors of disease severity and progression towards lymphoma and iii) the emerging role of salivary gland histopathology in informing clinical response in clinical trials. For each of the above points we will review the current supporting evidence and we will critically review the existing limitations.

Formation of the periductal lymphoid infiltrate

The salivary glands of pSS patients are characterised by chronic inflammation as witnessed by the presence of lymphocytic infiltrates (or lymphocytic foci) located around the striated ducts (Fig. 1A). These so-called periductal foci can be seen in both minor and major salivary glands and are present in most pSS patients. The foci are mainly composed of T- and B-lymphocytes with few other mononuclear cells, including macrophages, myeloid and plasmacytoid dendritic cells, and follicular dendritic cells (1). They may develop to organised ectopic lymphoid structures resembling secondary lymphoid organs with segregated T- and B-cell areas, and high endothelial venules (HEVs) (2). These structures contain all components to carry out effectively immune responses. This is reflected by the presence of germinal centres (GCs) in the B-cell areas of the organised periductal infiltrate in roughly a quarter of the pSS patients (3) and the presence of

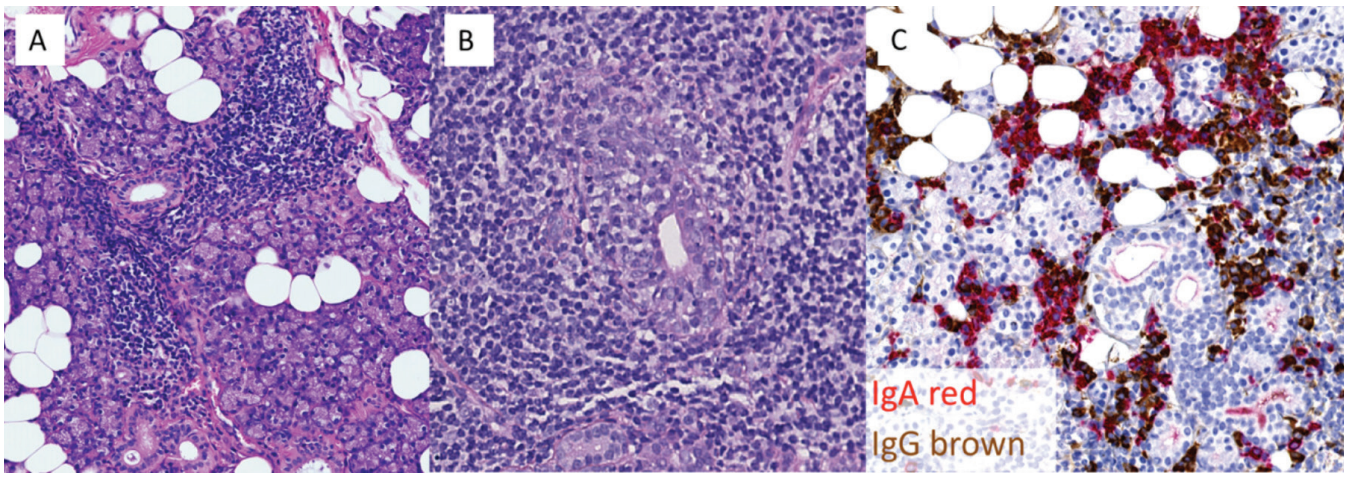

Fig. 1. Histopathological changes in parotid gland biopsies of pSS patients.
The examples are all from parotid gland tissue. **A**) Periductal focus surrounded by unaffected parenchyma. **B**) Centrally located duct forming a lymphoepithelial lesion within a focus. **C**) Dual staining for IgA and IgG expressing plasma cells, showing a decrease of <70%IgA expressing plasma cells.

(IgG) plasma blasts and plasma cells at the border of the infiltrate (2). Initially, (activated) lymphocytes and (IgG expressing) plasma blasts are thought to be attracted towards the salivary gland tissue by the combined action of pro-inflammatory cytokines and chemokines. The triggers for this early event are not known, but viruses might well be implicated (4). A major pro-inflammatory chemokine likely involved in the early formation of the periductal infiltrate is the chemokine CXCL10 (IP-10) which is highly expressed by the ductal epithelium (5, 6). Increased levels of CXCL10 are found in saliva, tears and serum from pSS patients. Early pSS patients express the highest levels of CXCL10 in their saliva, emphasising the importance of this chemokine in the initial phases of the disease (7, 8). CXCL10 binds to its receptor CXCR3, which is highly expressed by $CD4^+$ Th1 cells (9). Several other cells, including (activated and memory) B-cells, IgG secreting plasmablasts and some plasmacytoid dendritic cells express this receptor and can also be attracted to the salivary glands (10). The relatively unorganised infiltrates may transform towards more organised ectopic lymphoid structures. Triggers essential for this transition in human salivary glands are only poorly understood, but a role for lymphoid tissue inducer cells and Th17 cells have been suggested (11-13). These cells produce cytokines such as lymphotoxin β, RANKL, IL-17 and IL-22 which, in turn, promote production of the homeostatic lymphoid chemokines CXCL13, CCL19, CCL21 and CXCL12 by stromal and other cells (2, 12, 13). All these chemokines (protein and mRNA) are strongly upregulated in salivary glands of pSS patients (14-17). Collectively they are involved in attraction of naïve and memory T- and B-lymphocytes, plasmablasts, and other immune cells to the inflamed sites, and formation and maintenance of the organised lymphoid structures (2). Prolonged activation of the glands leads to development of follicular dendritic cell (FDC) networks and segregation of the T- and B-cells (18). The FDCs, which characteristically express the long isoform of CD21 (complement receptor 2), as well as other stromal cells produce CXCL13 and are instrumental in the formation of B-cell rich areas, due to the binding of this chemokine to its receptor CXCR5. In conjunction with CXCL12, CXCL13 also supports formation and function of GCs (15, 19, 20). This important role of CXCL13 in pSS histopathogenesis is illustrated by the fact that elevated levels of this chemokine are detected in the saliva and blood of pSS patients with the highest saliva levels seen in patients with xerostomia (21). Furthermore, high expression levels of CXCL13 in the labial gland biopsy are associated with higher number of periductal foci and higher disease activity (22). This might indicate that with progression of the disease, as indicated by more severe inflammatory lesions in the glandular tissue, the number of infiltrating B-lymphocytes increases (1). CXCL12 is also produced in healthy salivary glands and is important for migration and survival of (largely IgA^+) plasma cells in the proximity of the acini (23, 24). In pSS patients the production of CXCL12 by acinar and ductal cells is strongly enhanced (15, 16). Together with IL-6 and APRIL it forms survival niches for long-lived plasma cells within the salivary gland tissue (25). Thus, the initial infiltrate, which is rather unorganised, evolves under the influence of homeostatic, lymphoid chemokines towards ectopic lymphoid structures characterised by segregated T- and B-cell areas (with FDC networks), in which GCs may develop. Whether progression of the disease in individual pSS patients is always associated with these sequential stages of histopathology remains to be shown.

Focus score and its diagnostic role

The presence of foci is thus a hallmark of the disease. For this reason histopathological analysis of the salivary gland is an important item in diagnosis and classification. A biopsy can be taken from either the labial or the parotid salivary gland. Although in most centres a labial gland biopsy is taken, the parotid gland biopsy is a good alternative and is a well-tolerated and safe procedure in experienced hands (26, 27). For diagnosis and classification of

pSS the presence of focal lymphocytic sialadenitis (FLS) in glandular biopsies (either labial or parotid) is assessed. The term FLS refers to the histopathological pattern of the presence of one or more foci in the biopsies, while the tissue surrounding the foci is composed mainly of unaffected parenchyma (28). A focus is defined as an aggregate of ≥50 lymphocytes and the focus score (FS) is the total number of foci per 4mm^2 salivary gland tissue (29, 30). In both labial and parotid gland a FS of ≥1 is considered as a positive biopsy and used for the classification of pSS. With a high FS of about 10, the foci become confluent and an arbitrary maximal score of 12 is given (30, 31).

The importance of the calculation of the FS in the salivary gland biopsies is reflected by the prominent place of the biopsy in the current ACR-EULAR classification criteria. In these criteria a positive biopsy (*i.e.* FS≥1 per 4 mm^2) accounts for 3 points, similar to the presence of anti-SSA antibodies. In these criteria, 1 point is attributed to either reduced saliva production or reduced tear production (or ocular damage). A total of 4 or more points in suspected pSS patients (*i.e.* patients with at least one symptom of ocular or oral dryness or positive ESSDAI (European League Against Rheumatism Sjögren's Syndrome Disease Activity Index) leads to the classification of pSS (32, 33). Thus, for fulfillment of the criteria a positive biopsy and/or presence of anti-SSA antibodies is required. For a correct assessment of the FS, analysis of a glandular surface area of at least 8mm^2 is recommended (34). The specificity and diagnostic accuracy of the biopsy can be increased by multiple cutting levels, especially for pSS patients with a FS of 1.0 to 1.9 (35). For the diagnosis of pSS, the sensitivity and specificity of the parotid gland biopsy and labial gland biopsy are comparable. The sensitivity of the labial gland biopsy ranges from 63.5% to 93.7% and specificity from 61.2% to 100%. The parotid gland biopsy has a sensitivity of 78% and specificity of 86% (36, 37). Not all pSS patients have a positive salivary gland biopsy. In 18–40% of the pSS patients the labial gland biopsy is negative, *i.e.* have a FS<1.0 (37-39). These patients are at risk to be misclassified as non-pSS according to the ACR-EULAR criteria. The clinical diagnosis based upon expert opinion is in these cases important. Obviously, a FS<1.0 does not always imply that foci are completely lacking in the biopsies. It is not known whether the foci are evenly spread throughout the salivary glands, and there is always a risk for sampling errors, in particular in patients with low numbers of foci. Multiple cutting levels in an inconclusive biopsy might reduce the possibility of a falsely negative biopsy (34). Foci can also be found in healthy individuals: 6-9% of the healthy individuals have a FS of >1.0 in the labial gland biopsy and 5% in the parotid gland biopsy (37, 40, 41). In addition to the FS, two scoring systems for salivary glands are in use for diagnosis and classification of pSS (see Table I). Also these scoring systems are based on the presence of foci. Grading according to Tarpley takes the destruction of acinar tissue and fibrosis into account and Chisholm and Mason, also the presence of diffuse infiltrates, when the FS is lower than 1 (29, 42). Since all three scoring systems are in use, it is important to avoid any confusion, to indicate which scoring system has been used for the evaluation of the tissue.

Limitations of the focus score

The FS (and the other scoring systems based upon the number of foci) is a robust classification tool in defining biopsies as positive or negative, but it has certain shortcomings. Although the definition of the FS should not give rise *per se* to significant issues with interpretation, in reality failure to apply the FS or its miscalculation in clinical diagnostic setting is rather the norm. Vivino *et al.* reported that a second expert evaluation of 58 labial salivary glands re-analysed by a single centre led to revision of the initial diagnosis in a staggering 53% of the patients (43). Problems in assessing the FS also arise when other histopathological patterns such as non-specific chronic sialadenitis, sclerosing chronic sialadenitis are present (28). Furthermore, features such as acinar atrophy, interstitial fibrosis and increase in fat cells are frequently present in the biopsy. With increasing age of healthy individuals, acinar atrophy, fibrosis and increase of fat cells are commonly observed in the labial salivary gland tissue (44, 45). These age-associated changes result to a reduced acinar capacity and may consequently lead to a decrease in saliva production. Compared to healthy controls, the labial gland biopsies of pSS patients exhibit more acinar atrophy and fibrotic changes (46, 47). This is likely the result of the sustained chronic inflammation. There is no consensus whether there is an increase in the amount of adipose tissue in labial gland biopsies of pSS patients compared to age-matched healthy controls. The study of Skarstein *et al.* (48) reported a higher occurrence of fat infiltration in labial gland biopsies in pSS patients compared to non-pSS controls. In contrast, Leehan *et al.* (49) showed that the increased area of fat tissue in the labial gland biopsy is not specific associated with pSS but is a selective feature of aging. For the calculation of the FS, Fisher *et al.* (34) recommended to evaluate the whole section of the gland, including, fibrotic areas tissue, atrophic areas and adipose tissue. When fibrotic and atrophic changes in the biopsies progress, the inflammation is slowly extinguished leading to a "burnt-out" biopsy, even resulting in a negative FS (50). How many patients finally evolve to this stage is not known. Generally speaking, the FS is thought to be rather stable and only progresses slowly over time (50, 51). In the study of Shiboski *et al.* (52) repeated biopsies with a 2 to 3 year time interval were taken in 498 participants (Sjögren patients and non-SS sicca patients) from the SICCA-cohort. In these patients the labial gland biopsy changed in 7% of the patients from FS-negative to FS-positive, in 11% of the patients from positive to negative and in 82% of the patients the FS remained unchanged (52).

An important drawback of the FS is that it only based upon the number of foci and does not include the size of these foci. For a better estimation of the level of inflammation in the salivary gland biopsy, the area of infiltrate can be evaluated (34, 53). This gives a more pre-

cise indication of how much glandular tissue is involved in the inflammation. Likely, such an approach is also more sensitive to change, in case sequential biopsies are taken for evaluation of treatment effects, or for follow-up of disease progression. This is especially important when foci are confluent and should strictly be considered as a single focus. Measurement of the area of infiltrate can be done with a regular HE stain or after immunohistological staining for CD45 (staining all leucocytes) (34, 53). Currently, there is no cut-off level for the classification/diagnostic use of the area of infiltrate, but in clinical trials with pre- and post-treatment biopsies measurement of the infiltrated area can give important information (53).

Other histopathological markers besides focus score

In addition to periductal infiltrates there are also other histopathological features in the glands that are associated with pSS and therefore might be indicative for this disease. Besides FS, lymphoepithelial lesions (LELs) and a relative decrease of IgA^+ plasma cells, appear to be characteristic for pSS (54-57). Both features can aid in assessment of the salivary gland biopsies for the diagnosis of pSS, especially when the FS in the biopsy is <1. LELs are striated ducts, which are infiltrated by lymphocytes with concurrent hyperplasia of the epithelial cells (Fig. 1B). LELs can be found both in labial and in parotid glands, albeit that they seem to be more pronounced within the parotid gland tissue (37). These structures are always associated with periductal infiltrates and solitary LELs are not present. In pSS patients LELs are present in 93% of the parotid gland biopsy compared to 33% of the labial gland biopsies (58). The sensitivity and specificity of LELs in the diagnosis of pSS is not known. The current hypothesis in development of LELs is that the infiltrated lymphocytes cause the hyperplasia of the epithelium (56, 58). This hyperplasia can ultimately result in complete occlusion of the striated duct. Based upon the proportion of the hyperplastic epithelium we proposed a classification system for the severity of the LELs: stage 1: a partial LEL (affecting <50% of the epithelium), stage 2: developed LEL (affecting 50% -100% of the epithelium), stage 3: occluded LEL (fully circumferentially affected epithelium without lumen) (53). The infiltrating lymphocytes comprise both B- and T-cells. With progression of the severity of the LELs the relative number of B-cells within the lesions increase, suggesting an important role for B-cells in epithelial proliferation. [Van Ginkel, unpublished data] The vast majority of the B-cells within the LELs of pSS patients express FcRL4 (58). Fewer $FcRL4^+$ B-cells are located outside the LELs, but within the foci. The $FcRL4^+$ B-cells represent a small subset of highly proliferative mucosal memory B-cells (59, 60). Most non-Hodgkin lymphomas associated with pSS are (salivary gland) mucosa-associated lymphoid tissue (MALT) B cell lymphomas and 93%-97% of these MALT lymphomas (MALT-L) express $FcRL4^+$ (58, 61). We have shown that also B-cells in the LELs are targeted with rituximab treatment (53, 62). This results into normalisation of the ductal epithelium, which strongly argues that factors derived from the $FcRL4^+$ B-cells are responsible for the epithelial hyperplasia. Although LELs are characteristic for pSS, it is not specific for the disease and for example HIV infections also could lead to cystic LELs within the glandular tissue (63). Whether and how LELs contribute to classification and/or diagnosis of pSS remains to be shown. But given its highly characteristic presence in pSS the role of LELs should further be explored.

Besides LELs the salivary gland of pSS patients also show a relative decrease in IgA^+ plasma cells, and relative increase in IgG^+ and IgM^+ plasma cells compared to control individuals (Fig. 1C) (55). This relative decrease is largely due to a marked increase in numbers of IgG^+ and in to a lower extent also IgM^+ plasma cells (25, 55). The absolute number of IgA^+ plasma cells remains more or less constant in pSS. The IgG^+ and IgM^+ plasma cells are mostly located in the periphery of the foci and unaffected parenchyma. The diagnostic threshold of <70% IgA was set by Bodeutsch *et al*. based upon labial gland biopsies pSS patients compared to the control groups consisting of healthy controls, keratoconjunctivitis sicca patients and RA patients (54). In the biopsies of pSS patients the relative decrease of <70% IgA has a specificity of 95.4% and a sensitivity of l00% (55). Several studies showed that a relative decrease of <70% IgA^+ plasma cells (and consequently increase in IgG^+ and IgM^+ plasma cells) was more sensitive and more disease specific than the FS (54, 57, 64). Even without the presence of foci a decrease of <70% IgA^+ plasma cells is possible (57). These robust data indicate that the combination of FS and <70% IgA can increase the accuracy of the biopsies as indicative for the diagnosis of pSS or not (57).

According to the ACR-EULAR classification criteria the salivary gland biopsy has to fulfill the criterion of FS≥1 to be considered positive (32, 33). In daily clinical practice, however, an evaluation of the salivary gland biopsy for the presence of LELs and <70% IgA^+ plasma cells besides the FS may aid in the correct diagnosis of pSS. For instance, if a biopsy with FS<1 but LELs and a decrease of >70% IgA^+ plasma cells are present, it is reasonable to interpret the biopsy as suggestive for pSS. Vice versa, if a biopsy has a FS just above 1 and lacks these additional features, the clinician should be aware that there might be a risk of a falsely positive biopsy.

Salivary gland histopathology as predictor of disease severity and lymphomagenesis: the case for and against ectopic GCs

The appreciation of the high heterogeneity of the degree of immune cell infiltration in the salivary glands of pSS patients ranging from sparse T cells to highly organised B cell-rich follicles has prompted the investigation of whether salivary gland histopathology could be associated with disease severity and predict disease evolution including lymphomagenesis. Approximately 5% of patients with pSS will develop a non-Hodgkin B-cell lymphoma, most commonly MALT-L, which predominantly arises in parotid glands as a low grade lymphoproliferative disorder, but can evolve into more aggressive large

Table I. Grading systems for salivary gland biopsies of Chisholm/Mason and Tarpley (29, 42).

Chisholm/Mason		Tarpley	
Grade	Lymphocytes per 4 mm^2 of salivary gland tissue	Grade	Description
0	Absent	0	Normal
1	Slight infiltrate	1	1 or 2 aggregates# (minimal infiltration)
2	Moderate infiltrate or less than one focus*	2	>3 aggregates#
3	One focus*	3	Diffuse infiltrate with partial destruction of acinar tissue with or without fibrosis
4	More than one focus*	4	Diffuse infiltrate with or without fibrosis destroying the lobular architecture completely

*Focus: a cluster of 50 or more lymphocytes and histiocytes.
#Aggregate: approximately 50 cells (lymphocytes, plasma cells, or histiocytes).

Table II. Guidelines for assessing salivary gland biopsies in general and for in clinical trials. Table from publication of Fisher *et al*. (34)

General assessment

1. The minimum number of minor salivary glands is suggested to be four (six if small), and should be surgically separated
2. The minimum surface area of gland sections examined should be 8 mm2
3. If the first cutting level is inconclusive, or in the context of a clinical trial, consideration should be given to including two additional cutting levels at 200 μm intervals (typical focus diameter is <50 μm) in order to increase the surface area
4. Care should be given to preparation of paraffin blocks, with smaller glands set higher to allow midspecimen sampling during cutting
5. Histological examination should determine whether there is FLS present. Attribution of FLS, or possible FLS, should be followed by calculation of a focus score
6. The extent (absent, mild, moderate, severe) of atrophy, fibrosis, duct dilatation and non-specific chronic sialadenitis, in addition to the presence or absence of FLS, should be reported
7. Calculation of the focus score should include the whole of the glandular surface area in the denominator, to avoid introduction of bias
8. The presence or absence of germinal centre-like structures and lymphoepithelial lesions should be reported

Guidance relevant to clinical trials

1. The Focus score should be recorded, and the area of individual foci should also be summed and divided by glandular area to give a more quantitative indication of the extent of glandular infiltration
2. Once FLS has been confirmed, all foci should be included in the Focus score and in area of foci calculations, even when adjacent to abnormal acini or ducts, to avoid introduction of bias
3. Staining for CD3, CD20 and CD21 should be included, and the presence of germinal centre-like structures should be reported as the proportion of foci with both T/B-cell segregation and follicular dendritic cell networks. Consideration should be given to reporting the mean B/T cell ratio in foci
4. Scoring should be undertaken by two trained observers who have reviewed a reference slide set, and with reporting of intraobserver and interobserver variability
5. Samples should be scored blind to subject and order
6. High-resolution image morphometry of each sample should be stored
7. Given the stable or slowly progressive nature of the histological features, baseline biopsies may be substituted with prior biopsies to reduce the number of biopsies required. However, given the limited evidence available, these should have been acquired no longer than 1 year prior to baseline
8. The optimal period of time for rebiopsy has not been established and will depend on the agent employed.

B cell lymphomas (65). Malignant B cell clones originate from precursors already infiltrating the pSS salivary glands during the polyclonal phase of the local humoral response and are progressively enriched during the progression from lymphoepithelial lesions to MALT-L (58, 66). Of interest, malignant B cells often display Ig variable heavy (IgVH) gene rearrangement observed in rheumatoid factor producing B cells suggesting that lymphomagenesis in pSS is an (auto)antigen-driven process taking place within the salivary gland tissue (67, 68). Antigen-driven B cell selection normally takes place in GCs within secondary lymphoid organs but there is definitive evidence that also ectopic GCs in the salivary glands of pSS allow affinity maturation of GC B cells with somatic Ig gene hypermutation (69). Somatic hypermutation, but also Ig isotype class switching, is dependent on the enzyme activation-induced cytidine deaminase (AID) encoded by the AICDA gene (70) which is expressed by GCs in the pSS salivary glands (71). Of interest, AICDA mRNA expression in MALT-L is strongly associated with the process of aberrant somatic hypermutation whereby off-target mutations in important genes regulating B cell proliferations promote genetic instability and malignancy (72). Thus, there is a strong immunological rationale whereby GCs might represent key early pathogenic players in the process of B cell lymphomagenesis and their histopathological detection in the salivary glands might help in predicting patients at increased risk of lymphomagenesis; however, conflicting evidence on this topic has emerged in the last 10 years, including respective data obtained independently from the Authors of this review. These studies are reviewed below in chronological order of publication.

In a first report in a small retrospective cohort of 8 pSS patients with MALT-L and matched parotid and labial salivary gland biopsies the prevalence of GCs in labial salivary gland tissue prior (average 3.3 years) to lymphoma was reported to be significantly higher (75% *vs*. 33%) in SS patients who later developed MALT-L (71). In this work,

GCs were defined by the presence of $CD21^+$ FDC networks containing AID^+ GC B cells. In a retrospective-prospective study of a large Swedish cohort of 174 pSS minor salivary gland biopsies Theander *et al*. reported the presence of ectopic GCs, determined using haematoxylin and eosin as focal lymphocytic foci with a densely packed dark zone and a light zone, in 32% of the patients. Linkage with the national cancer registry identified 7 pSS patients with NHL with a median time lapse between salivary gland biopsy and NHL diagnosis of 8 years. Similar to the previous report, the prevalence of GCs in pSS patients who later developed lymphoma was significantly increased (86%). The relative risk of developing NHL in pSS patients with GC formation calculated using Cox regression analysis and Kaplan Meier statistics/log rank test was 15.4-fold higher compared to the GC negative pSS subset with a negative predictive value of 99%. Additionally, the presence of GC correlated with anti-SSA/SSB autoantibodies, lymphadenopathy and peripheral neuropathy as defined in the relevant ESSDAI domains (73).

These promising data were not confirmed by Johnsen *et al*. who conducted a retrospective nationwide search in the Cancer Registry of Norway and identified 21 patients with lymphoma (~50% with salivary gland MALT-L) including 12 with previous, concomitant or subsequent matching labial salivary glands tissue blocks (74). These were analysed for GCs and compared with 28 labial salivary gland biopsies from patients with pSS without lymphoma matched for sex and age. GCs were defined by haematoxylin and eosin staining plus immunohistochemistry for IgD/CD21 and IgD/CD38. Although pSS patients with lymphoma displayed a significantly higher FS, no significant difference in the prevalence of GCs was observed. Somewhat surprisingly, in this study ectopic GCs were more frequent in pSS without than with lymphoma (46% *vs*. 33%) a result which was also mirrored by a higher prevalence of CD21+ FDC networks (71% *vs*. 58%).

In a large Greek cohort the presence of GC in the labial salivary gland biopsy were not an independent risk factor for NHL development (75). This cohort compromised 92 pSS patients with NHL (79% MALT-L) and 381 pSS patient without lymphoma. Of the 92 pSS patients with NHL, 49 minor salivary gland biopsies were available and 11 (22%) showed GC. In the pSS patients without lymphoma 12/101 (12%) showed GC in the minor salivary gland biopsy (p=0.15). In the biopsies of the pSS patients who did develop NHL a FS >1.6, Tarpley score ≥3 and the presence of monoclonality in the tissue were significantly more frequently observed. The manner in which GCs were assessed in the biopsies was not given in the article.

Similar negative results were reported in the University of Groningen Medical Center cohort where 11 labial salivary gland biopsies taken at diagnosis were available from patients who later developed MALT-L (median time lapse 4 years) (76). A population of 22 pSS patients with no lymphoma was taken as control. In this cohort, GC identification was based on haematoxylin and eosin plus staining for Bcl-6, a marker of GC B cells. No difference in the prevalence of GC was observed (18% in both groups), although pre-lymphomatous LEL were observed in 7 out of 11 of the GC+ pSS subset prior to lymphoma.

In a more recent multicentre French study of 115 minor salivary gland biopsies with 8 identified cases of subsequent NHL (median time lapse 51 months), Sene et al reported a significant increase in the prevalence of GCs comparing pSS patients with and without subsequent NHL (37.5% *vs*. 15% with an incidence of GCs of 16.5% in the total pSS cohort) (77). In this study the definition of the GC was based on haematoxylin and eosin staining together with presence of FDC and B cells with low activity of Bcl-2 and high activity of Bcl-6. Of relevance, after Cox multiple regression analysis, but not in univariate analysis, the presence of ectopic GCs in the salivary glands remained an independent positive predictor of lymphoma with a conferred increase hazard ratio of 7.8.

In addition to the above studies, indirect evidence of the potential clinical relevance of GCs on lymphomagenesis has been provided by Carubbi *et al*. (78) who showed that GC^+ pSS patients displayed increased prevalence of several clinical and laboratory risk factors previously associated with lymphoma including salivary gland swelling, hypergammaglobulinaemia, RF positivity. Additionally, they reported that pSS patients with GC had a higher prevalence of a salivary gland FS >3, which is of significant interest considering that a FS ≥3 has been reported in multivariable linear regression analysis as an independent risk factor for lymphomagenesis in pSS patients based on a meta-analysis by Risselada *et al*. (79).

Reconciling conflicting data on ectopic GCs in pSS: the need for longitudinal studies and consensus on standardisation

As emerged from the studies reviewed above salivary gland histopathology, with particular reference to the identification of ectopic GCs, has potential clinical utility in the management of pSS by identifying patients with an increased risk of severe manifestations and lymphoma. A critical review of the evident pitfalls of existing studies, including those from the Authors of this review, would help in further progressing the field thus paving the way for more conclusive studies. At least two major criticisms should be made regarding existing data; those related to study design and those related to the identification of GCs. Regarding the former, the major limitation is the retrospective nature of these studies, potentially introducing selection bias, particularly in the control population. Additionally, considering the relatively low prevalence of lymphoma in pSS, all the previous studies have largely underpowered cohorts with only a handful of cases with lymphoma available for analysis. Further confusion is dependent on the inclusion of different subtypes of NHL in the different studies, from selected populations of MALT-L to a mix of different lymphoproliferative malignancies underlined by diverse pathogenic mechanisms. Furthermore, high variability in the follow up time from labial biopsies to diagnosis of

Table III. Overview of studies assessing histopathological salivary gland tissue before and after treatment with biologicals in pSS patients.

Biological	Target	Trial	Study design	Number biopsies	Gland	Time of biopsy	Histopathological evaluation
Etanercept	TNF-α	Zandbelt[110]	Open label	N=10 Etanercept	Labial	Baseline + week 12	FS = , %IgA =
Abatacept	CD28-mediated co-stimulation	Adler [93]	Open label	N=10 Abatacept	Labial	Baseline + week 28	Foci/mm^2 =, Number of foci ↓, CD20 =, CD3 =, CD4 =, CD8 =, Foxp3+ regulatory T-cells ↓, IgA =, IgG =, IgM =, fibrosis =, atrophy =
		Haacke [58,94], Verstappen [95]	Open label	N=15 Abatacept (Verstappen N=14)	Parotid	Baseline + week 24	FS =, CD45 =, GCs (HE) ↓, LELs (HE) =, CD20 =, CD3 =, IgA =, IgG =, IgM ↑, CD21 =, FcRL4+ B-cells =, ICOS ↓, IL21+CD4+ cells =
Infliximab	TNF-α	Mariette[111]	RDBPCT	N=57 (Infliximab + controls)	Labial	Baseline + week 10	FS =
Rituximab	CD20$^+$ B-cells	Delli [53], Haacke[58]	RDBPCT	N=16 RTX, (Haacke N=18) N=9 placebo	Parotid	Baseline + week 12	FS =, CD45 ↓, GCs (HE) ↓, LELs (HE) ↓, CD20 ↓, CD3 =, FcRL4+ B-cells ↓
		Ciccia[97]	Open label	N=10 RTX	Labial	Baseline + week 48	Chisholm grading =to ↓, IL22+ infiltrating cells ↓, IL22+ myoepithelial cells ↓
		Cornec [98]	Open label and RDBPCT	N=14 low dose RTX (open label) N=17 high dose RTX (RDBPCT)	Labial	Low dose RTX: baseline + week 12 High dose RTX and placebo: baseline + week 24	Low dose RTX week 12: proportion B-cells ↓, high dose RTX week 24: proportion B-cells =. Note: patients with persistent blood B-cell depletion at week 24: ↓ or = of tissue B-cells. Patients with blood B-cell repopulation before week 24: ↑ proportion of tissue B-cells at week 24.[85]
		Pijpe [62]	Open label	N=14 placebo (RDBPCT) N= 5 RTX	Parotid	Baseline + week 12	%acinar parenchyma =, %lymphocytic infiltrate ↓, %fat =, germinal centres ↓, LELs ↓, proliferation of acinar parenchyma (%Ki-67) ↓, B:T lymphocyte ratio in the infiltrate ↓
		Pers[100]	Open label	N= 15 RTX	Labial	Baseline + 4 months (N=15), 12 months (N=3), 24 months (N=2)	B-cells ↓ (repopulation at 24 months), CD3 =, CD4 =, CD8 =
		Devauchelle-Pensec[103]	Open label	N=16 RTX	Labial	Baseline + week 12	FS =, B-cells ↓
		Carubbi[96]	Open label	N=19 RTX N=22 DMARDs	Labial	Baseline + week 120	RTX: FS ↓, GCs (CD21) ↓, CXCR4+ and CXCR5+ cells in the mononuclear cell infiltrate ↓
Belimumab	BAFF/BLyS	Seror[91]	Open label	N=15 Belimumab	Labial	Baseline + week 28	Chisholm grading ↓, FS =to↓, B-cell/T-cell ratio in the foci ↓, % BAFF-positive cells in foci ↓, NK infiltrate inside and outside the foci =
		De Vita[92]	Open label	N=12 Belimumab	Labial	Baseline + week 52	FS =

TNF-α: tumour necrosis factor- α, BAFF: B-cell activating factor, BlyS: B-lymphocyte stimulator, RDBPCT: randomised double-blind placebo controlled trial, RTX: rituximab; DMARDs: disease-modifying anti-rheumatic drugs; FS: focus score; GCs: germinal centres, IgA / IgG / IgM: IgA / IgG / IgM expressing plasma cells; HE: haematoxylin and eosin; LELs: lymphoepithelial lesions; ↑: increased; ↓: decreased; =: no significant change.

NHL, ranging from medians of few months to several years further complicates the comparative analysis in different studies. On top of the above limitations which are somewhat intrinsic to the complexity of studying a low prevalence clinical manifestation in a relatively low prevalence disease such as pSS, there are technical aspects related to the identification of GCs which could be overcome with a consensus on standardisation of the protocols used to define what an ectopic GC is. This aspect should move away from the identification of GCs in secondary lymphoid organs such as tonsils or lymph node as ectopic GCs in pSS salivary do not necessarily reflect the same physiopathological mechanisms. As reviewed above, virtually all existing studies on GCs in pSS salivary glands have used a different definition of GCs resulting in highly variable incidences of GCs in the different cohorts. Simple haematoxylin and eosin staining has intrinsic limitations as a clear separation of dark and light zone is frequently absent in ectopic GCs. Furthemore, LEL can sometimes be mistakenly identified as GCs. Addition of immunohistochemistry for the identification of GCs is certainly of help, however, there is no consensus on the markers required. Double staining for CD3/CD20 followed by CD21 can help identifying follicles with T/B cell segregation with differentiation of FDC networks; however, CD21 staining can potentially overestimate the prevalence of GCs as the non-long isoform of CD21 is also expressed by B cells. More specific staining for GC B cells such as Bcl-6 and AID display variable degrees of sensitivities and risk falling in the opposite direction of underestimating GC prevalence. Additionally, there are other critical aspects which need to be considered and cannot be critically evaluated in the present studies. GCs are "skip lesions" which are variably present in adjacent minor salivary glands, thus a minimal number of individual biopsies should be taken in order to cover minimal total area of 8 mm^2, as we recently suggested (34). Additionally, including multiple cutting levels would maximise the accuracy of the analysis (35). All these factors combined advocate for consensus guidelines to standardise the assessment of ectopic germinal centre-like structures in salivary gland tissue of pSS-patients (80, 81) which is currently work in progress as part of the EULAR-endorsed eSSential study group. Furthermore, design of prospective multicentre studies with centralised review of labial salivary gland biopsies would be required to provide definitive evidence in support of the use of salivary gland histopathology in general and ectopic GCs in particular as clinically meaningful predictors of disease progression to inform management of pSS patients. This could be achieved through large collaborations of academic partners, such as the Horizon2020 EU-funded HarmonicSS consortium (http://harmonicss.eu).

Role of salivary glands biopsies in clinical trials

Since xerostomia is one of the major complaints of pSS and salivary glands are a primary target of the autoimmune process, the histopathological changes in these glands are of utmost importance for evaluating (new) treatment options. Currently, most clinical trials take the change in ESSDAI scores as primary endpoint, implying that pre- and post-treatment biopsies are not required (82, 83). Nevertheless, post-treatment biopsy evaluation may give important information about efficacy of the treatment, mode of action of the compound, effect on the glandular tissue and the pathogenic mechanism of the disease.

The inclusion of pSS patients in clinical trials is usually based upon the latest classification criteria for pSS. Hence also patients without a salivary gland biopsy, or with a "negative" biopsy may enter these trials. In these negative biopsies, in which lymphoid infiltrates may even be complete absent, the possible effect of treatment on the lymphocytic inflammation cannot be assessed in their post-treatment biopsies. Only few clinical trials used a positive salivary gland biopsy as additional inclusion criterion (84, 85). The disadvantage is that fewer pSS patients qualify for inclusion into the study and that there might be a patient bias.

Although repeated biopsies can be taken from both minor and major salivary glands, taking biopsies from the parotid glands has certain advantages. The major advantage of parotid glands over labial glands is that post-treatment biopsies can be taken from exactly the same gland as the initial (diagnostic) biopsy to evaluate treatment efficacy (37, 86). Also, disease recurrence or progression can be monitored. Another advantage of the parotid biopsy is that histopathological results can be directly correlated with other clinical and laboratory findings from the same gland such as parotid salivary flow, composition of the saliva and ultrasound (27, 87). Fisher *et al*. published consensus guidelines for assessing salivary gland biopsies in clinical trials (Table II). The evaluation of the biopsies encompasses the same aspects as for diagnostic biopsies and includes FS, percentage of $CD45^+$ infiltrate presence of GCs and LELs. In addition, features like fibrosis, atrophy, fat infiltration and sialadenitis patterns besides FLS, should be reported (34). In addition to these general aspects, specific analysis should be performed, depending upon the drug used for treatment.

Response of salivary gland biopsies to biological DMARDs therapy

There are no approved biological DMARDs yet for the treatment of pSS, but there are a number or trials that have been performed or are currently running. In Table III the major findings have been summarised of the trials that also analysed the effect of treatment on the salivary gland biopsy. Timing of the post treatment biopsies varies between the different trials and the histopathological items that were assessed are diverse. Obviously there is a need for standardisation, in order to allow a proper comparison between the various studies.

Here we describe briefly the results of the histopathological evaluation in recent trials. BAFF (BLyS) plays an important role in the pathogenesis of pSS and elevated levels are present in the salivary glands (88). Belimumab blocks the binding of soluble BAFF (BLyS) to its receptors on B-cells. In this manner the survival of (autoreactive) B-cells,

their maturation and differentiation towards immunoglobulin secreting plasma cells are thought to be hampered (88, 89). The efficacy of belimumab was first shown in an open label (BELISS) study of Mariette *et al*. (90) in which 18/30 (60%) of the pSS patients responded based upon the SSRI-30 (Sjögren's Syndrome Responder Index-30) and 15/30 (50%) upon ESSDAI ≥3 points at week 28. Seror *et al*. (91) showed in a sub-study of 15 pSS patients of the BELISS trial that after 28 weeks of belimumab treatment the FS in the post-treatment labial gland biopsies tended to decline with a significant decrease in the Chisholm grading (see Table I). Furthermore, belimumab decreased the proportion of BAFF-positive cells and the B-/ T-cell ratio in the foci. Although this study was rather small, belimumab did seem to alter the composition of the infiltrates in the glandular tissue and a clinical response in ESSDAI of ≥3 was seen in 6/15 (40%) of pSS patients. In biopsies taken after continuous treatment up to 52 weeks, no significant difference in the FS was seen compared to baseline levels. Although FS and salivary function did not change after 52 weeks of treatment, the clinical response as measured by ESSDAI, continued to be lower than baseline levels (92).

Abatacept (CTLA4-Ig), which blocks the CD28-mediated co-stimulation of T-cells, has shown efficacy with respect to the ESSDAI in a phase II trial of pSS (85). Treatment with abatacept resulted in a significant decline in ESSDAI, whereas unstimulated and stimulated whole saliva flow did not increase. Abatacept treatment did not alter FS or the number of B-cells and T-cells in the parotid gland or the labial gland biopsy (93, 94). Nor did the amount of $CD45^+$ infiltrate and number or severity of LELs change in the parotid gland biopsy. However, the number of GCs declined, and there was an increase in the number of IgM^+ plasma cells (94). Likely this reduction of GC activity is the consequence of a reduced CD28-mediated activation of T_{fh} cells (95). In the labial gland biopsy, Adler *et al*. found decreased numbers of $Foxp3^+$ regulatory T-cells in the foci after abatacept treatment (93).

Rituximab is directed to CD20 and treatment of pSS patients leads to a near complete depletion of B-cells in the blood at 12 weeks. Rituximab is also able to affect the glandular infiltrates in both labial and parotid glands, as reflected by lower FS or reduced surface area of $CD45^+$ staining (53, 62, 96-100). This effect is the direct result of a decline in B-cell numbers within the infiltrate, since T-cell numbers were unaffected. In addition there is a decrease in the number of GCs, within the infiltrates (53, 96). Not only B-cells that constitute the infiltrate, but also intra-epithelial ($FcRL4^+$) B-cells within the LELs were depleted by rituximab (58). This depletion of the intra-epithelial B-cells of the striated ducts was associated with a normalisation/restoration of the ductal epithelium (53, 62), which illustrates the importance of the interaction between intra-epithelial B-cells and formation of LELs. These changes in parotid gland tissue after rituximab treatment, in particular the reduction in infiltrate size and normalisation of LELs, is partially reflected in the glandular function. Three studies reported that the salivary flow increased after rituximab treatment (53, 62, 84, 96). Other clinical studies showed no change in the salivary function upon rituximab treatment (99, 101-104). All together these studies indicate that the salivary gland function (measured by whole salivary flow) did not further deteriorate but remained stable or even improved after rituximab treatment.

Biopsies for stratification of patients

The various histopathological features might also be used for patient stratification for treatment, and precision medicine. For example, Delli *et al*. (53) showed that high absolute number of B-cells in the baseline parotid gland biopsy predicts better responsiveness of patients with pSS to rituximab treatment. Response to rituximab was in this study defined as a decline in ESSDAI of ≥3 points which is considered a clinically meaningful improvement (105). This seems to be in contrast with studies of Cornec *et al*. (98, 106) showing that pSS patients with a high proportion of B-cells and higher FS in the labial salivary gland predicted the absence of a clinical response to rituximab. In these studies responders were defined by the SSRI-30 a composite endpoint (107). As discussed extensively elsewhere (108, 109), differences in assessing B-cells in sections (numbers *vs*. proportions) and differences in how clinical response were measured (ESSDAI *vs*. SSRI-30), may contribute to explain the apparent differences in outcomes.

For belimumab, Seror *et al*. (91) showed that low numbers of NK-cells, mainly located in the periphery of the foci of the labial gland biopsy are associated with a better response to belimumab. Also in this study response was defined as an decrease in ESSDAI of ≥3 points. Remarkably, the baseline number of $BAFF^+$ cells in the biopsy could not predict response to belimumab.

Even when treatment with (biological) DMARDs leads to (complete) resolution of the inflammation of the glandular tissue, it is questionable whether this will result in a salivary gland that is fully restored and saliva production that has been returned to normal. The inflammation in the glandular tissue is likely to cause irreversible damage in the form of fibrosis and loss of acinar cells leading to sustained reduced saliva production (46, 47). Furthermore, in pSS patients the salivary gland stem cells responsible for the production of new epithelial and acinar cells are decreased in number and functional ability and exhausted as compared to healthy controls (112). This implies that after resolution of the inflammation, the gland cannot fully restore to the level of healthy individuals, and consequently saliva production might still be sub-optimal.

In summary, in clinical trials with pSS patients repeated biopsies give important information about treatment efficacy, the mode of action of the drug and the pathogenic mechanism of the inflammation within the salivary gland tissue. Treatment with biologicals such as rituximab and belimumab appear to have a positive effect on the salivary glands with at least stabilisation of the salivary flow. Results from studies combining these two biologicals are eagerly awaited. Further studies for

stratification of patients and studies to evaluate glandular function when the salivary infiltrates are fully diminished are needed.

Conclusions

There is little doubt that salivary gland histopathology is a cornerstone of the diagnostic algorithm in pSS, although the accuracy of the evaluation of FLS and FS calculations could be significantly improved and can lead to erroneous or delayed diagnosis of pSS. Also evaluation of LELs and IgG/IgA plasma cells may contribute to the diagnosis. In addition to its diagnostic role, there is emerging evidence that salivary gland biopsies may help in predicting disease evolution and progression to lymphoma. However, lack of consensus on the definition of the minimal requirements for an accurate interpretation of the degree of immune cell infiltration and organisation in the salivary glands has hindered progress in the field. Finally, with the new era of biological therapies coming to fruition in pSS, although currently limited to the clinical trial phase, there is hope that salivary glands histopathology may inform on patient stratification based on target validation and on the mechanisms of response, relapse and resistance to novel biologics.

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