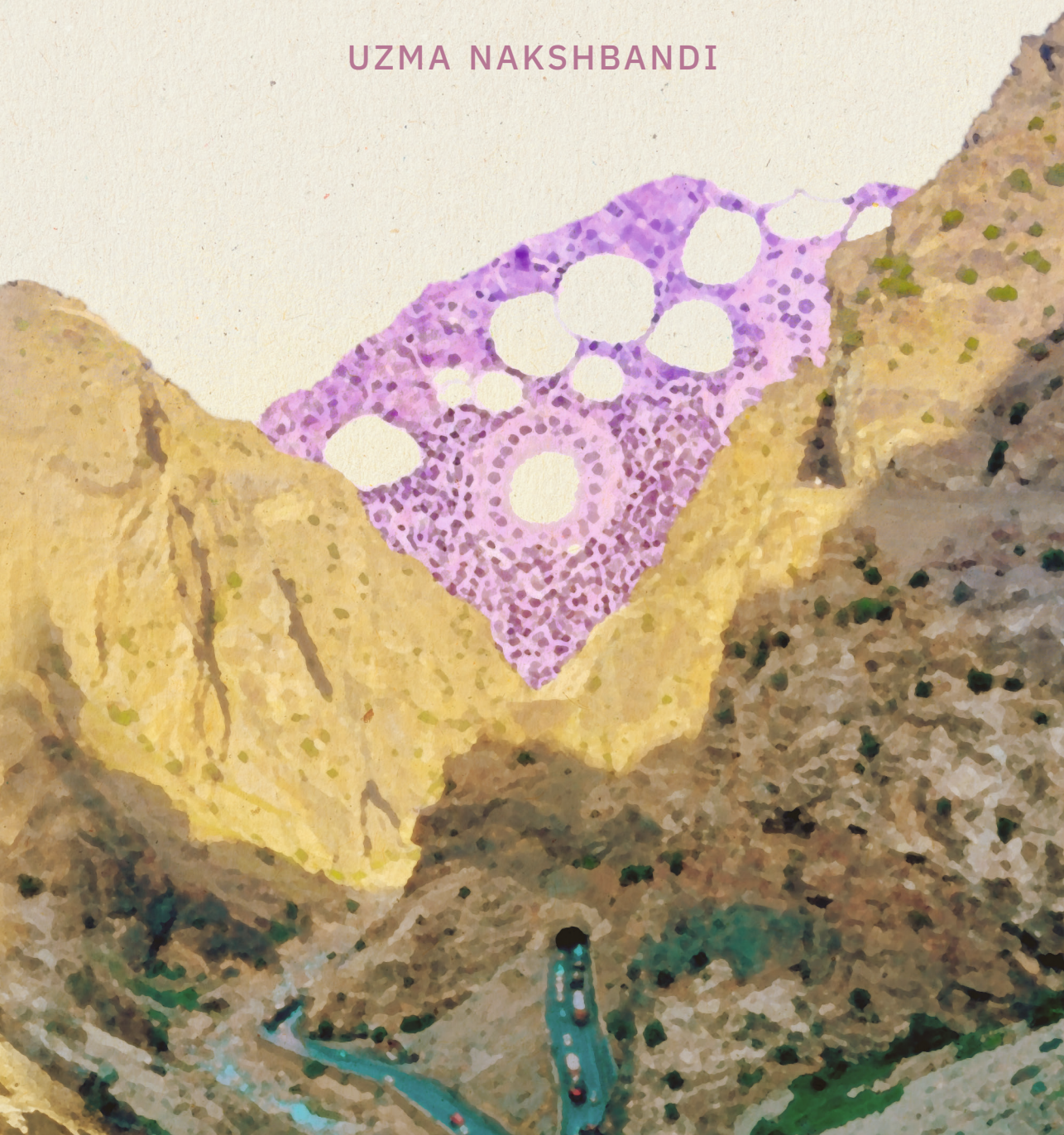
Unveiling the histopathological landscape of salivary glands in Sjögren's disease

UZMA NAKSHBANDI



Unveiling the histopathological landscape of salivary glands in Sjögren's disease

Uzma Nakshbandi

Cover & Lay-out Erwin Timmerman| Persoonlijk Proefschrift
Printing Ridderprint BV | www.ridderprint.nl

ISBN 978-94-6522-260-8

university of groningen

Unveiling the histopathological landscape of salivary glands in Sjögren's disease

PhD thesis

to obtain the degree of PhD at the
University of Groningen
on the authority of the
Rector Magnificus Prof. J.M.A. Scherpen
and in accordance with
the decision by the College of Deans.

This thesis will be defended in public on

Friday 20 June 2025 at 9.00 hours

by

Uzma Nakshbandi

born on 13 January 1995
in Peshawar, Pakistan

Supervisors
Prof. F.G.M. Kroese
Prof. A. Vissink

Co-supervisor
Dr. B. van der Vegt

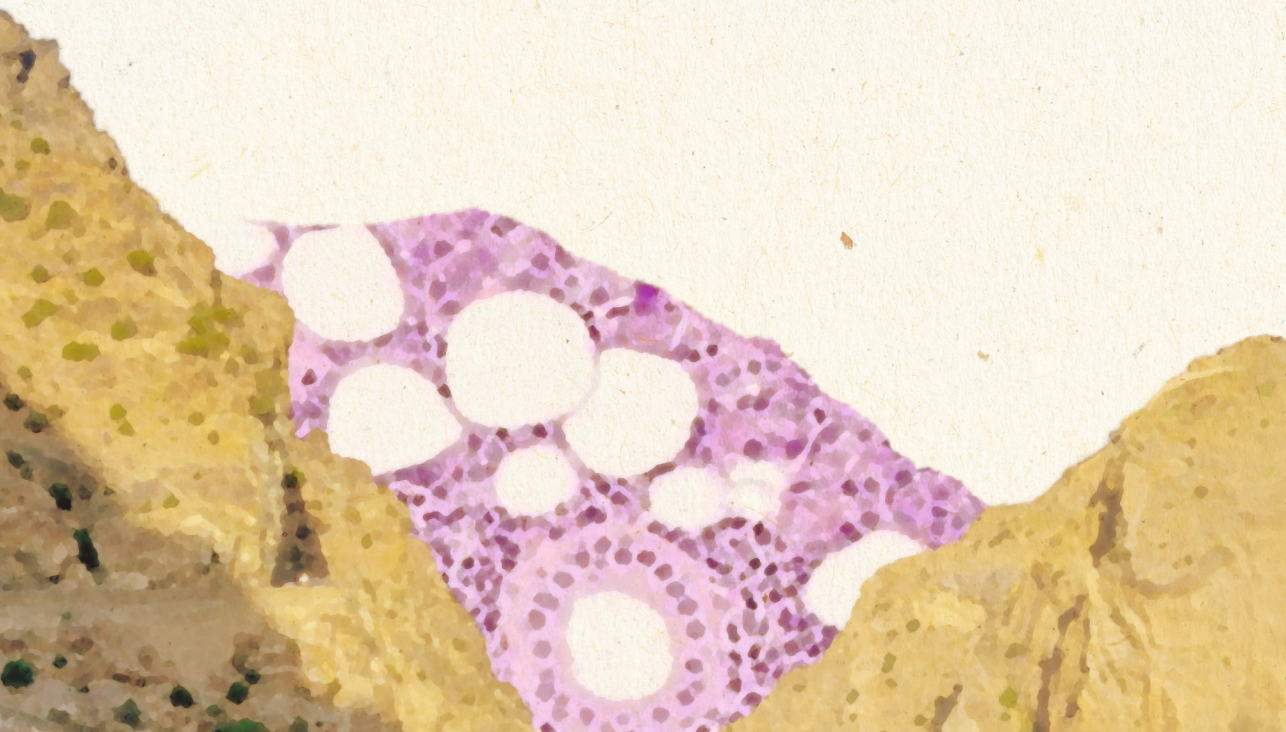
Assessment committee
Prof. I.M. Bajema
Prof. F.J. Bikker
Prof. B. Fisher

Paranimfen
Drs. G. Nakshbandi
Drs. S. Waziri

افتخارش برای والدینم

Voor mijn ouders

TABLE OF CONTENTS



CHAPTER 1

General introduction

Sjögren's disease (SjD), formerly known as Sjögren's syndrome, is a systemic autoimmune disease characterised by chronic inflammation of the exocrine glands, particularly the salivary and lacrimal glands. As a result, typical symptoms are hyposalivation and keratoconjunctivitis sicca often resulting in a dry feeling in the mouth (xerostomia) and dry eyes.[1,2] This condition is named after Dr. Henrik Sjögren, a Swedish ophthalmologist who described it in the early 20th century.[3] SjD predominantly afflicts adult women with a male to female ratio of 1:9 and an observed prevalence of 0.1%. In the general population, the peak incidence of SjD is around the mid-50s[1,4], but it can develop at any age. In addition to the characteristic ocular and oral dryness complaints, other organ systems are also often affected. Examples of extra-glandular manifestations are arthritis, vasculitis, interstitial lung diseases and polyneuropathy.[5]

The immunopathogenesis in SjD

The pathogenesis of SjD is not fully understood, but the aetiology seems to be multifactorial and involves a complex interplay of genetic factors, hormones and environmental triggers resulting in immune system dysregulation. Genetic predisposition is partially ascribed to genes involved in the Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, antigen presentation, and interferon- and lymphocyte signaling.[6] Environmental factors that have been associated with disease development are viruses, particularly the Epstein Bar virus and human T-cell leukaemia virus 1.[4]

While the immunopathogenesis remains to be elucidated, (T cell-dependent) B-cell hyperactivity has been acknowledged as a hallmark of disease. Serological signs of B-cell hyperactivity are the presence of anti-SSA/SSB autoantibodies, free light chains and B-cell associated cytokines and chemokines.[7] Indirect stimulation of B-cells is facilitated by T follicular helper (Tfh)-cells which produce interleukin-21 (IL-21). IL-21 serves as a potent inducer of plasma cell formation and is involved in somatic hypermutation, affinity maturation and, class switch recombination of B cells within germinal centres (GCs).[8] To regulate the effects of Tfh-cells, T follicular regulatory (Tfr)-cells are able to suppress both Tfh-cells and B-cells.[9]

B-cell hyperactivity is also reflected by the increased risk of non-Hodgkin lymphoma development in SjD patients. These lymphomas, which develop in 5-10% of SjD patients, are predominantly of the Mucosa Associated

Lymphoid Tissue (MALT) type and preferentially arise in the parotid gland.[10] These parotid MALT lymphomas are considered indolent neoplasms, but progression to aggressive diffuse large B-cell lymphoma (DLBCL) can occur.

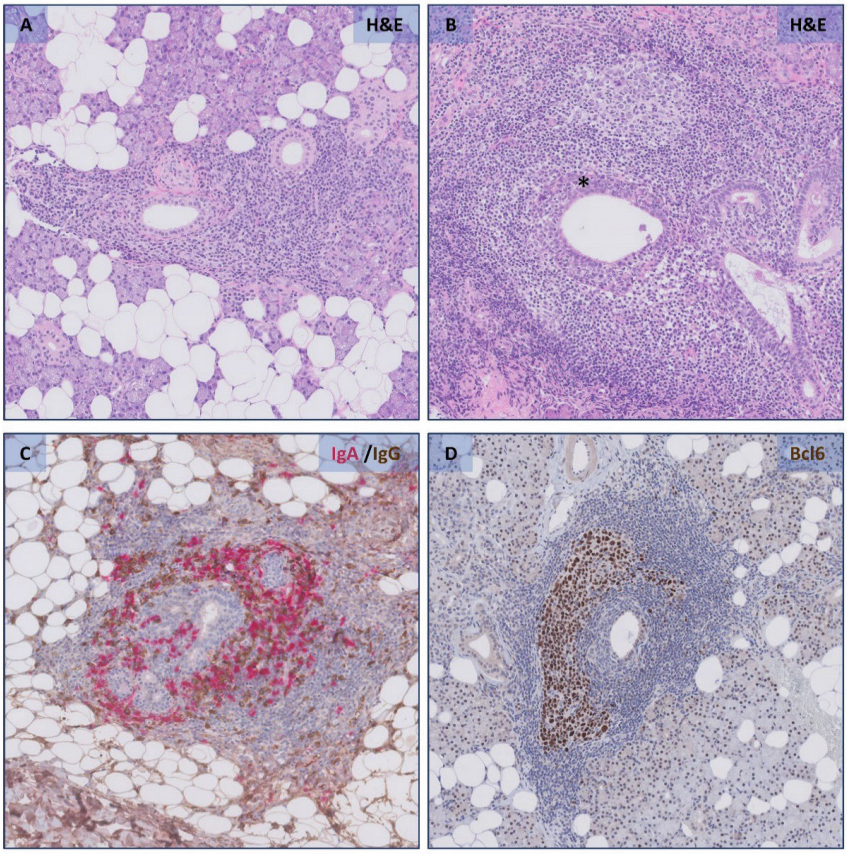


Figure 1. Histopathological key features in salivary gland biopsies of Sjögren's disease patients. A) Periductal lymphocytic infiltrate ≥50 lymphocytes. B) *Lymphoepithelial lesion (LEL) (striated duct with hyperplasia and infiltration of lymphocytes). C) Plasma cell shift, as shown by a relative increase in the number of IgG (brown) plasma cells, compared to IgA (pink) plasma cells. D) Germinal centre (GC) within a periductal focus.

Salivary glands are an important target organ in SjD. Salivary gland epithelial cells are likely contributing to the initiation and maintenance of SjD related glandular inflammation by producing cytokines including type 1 interferons

(IFNs), and chemokines. IFN type I enhances the production of CXCL10, a chemokine that recruits lymphoid cells to the glandular tissue.[11] Furthermore, the cytokines B-cell Activating Factor (BAFF) and A Proliferation-Inducing Ligand (APRIL), also driven by IFN, are upregulated resulting in the recruitment and activation of especially B cells.[12] Within salivary gland histopathological patterns linked to B-cell hyperactivity can be recognized by presence of lymphoepithelial lesions (LELs), GCs, and IgG secreting plasma cells (figure 1).

Salivary gland histopathology

In salivary glands of SjD patients, the chronic inflammation is a focal lymphocytic sialadenitis characterized by lymphocytic foci commonly associated with striated ducts.[13] Traditionally, a labial salivary gland biopsy is obtained for both the diagnosis and classification of disease. However, a parotid gland biopsy offers a comparable level of sensitivity and specificity as a labial gland biopsy.[14] Also, differences between patient-reported outcomes in terms of sensibility and post-operative pain are minor, when taking a parotid gland biopsy, making it a good alternative.[15] A recent development involves the ultrasound-guided core needle biopsy, which expands the applicability of parotid gland biopsies and brings a parotid gland biopsy available to more clinicians other than surgeons.[16] Parotid gland biopsies offer several advantages compared to labial gland biopsies, including the possibility to obtain repeated biopsies from the same gland, the higher likelihood of detecting a MALT lymphoma, and the opportunity to compare salivary gland histopathology with other clinical findings from the same gland (e.g., parotid salivary flow, ultrasound and saliva composition).[17]

In 2016 the American College of Rheumatology – European League Against Rheumatism (ACR-EULAR) classification criteria for SjD have been formulated (table 1).[18] Here, classification criteria are based on five objective items. According to these criteria, salivary gland biopsies continue to play a prominent role. Within the ACR-EULAR classification criteria, evaluation of the biopsy is restricted to the focus score (FS), which is defined as the number of lymphocytic foci per 4 mm^2.[19]

Table 1. ACR-EULAR classification criteria for Sjögren's disease (SjD)

Item	Weight/Score
Labial salivary gland with focal lymphocytic sialadenitis and focus score ≥ 1^	3
Anti-SSA (Ro) +	3
Ocular staining score ≥ 5 (or van Bijsterveld score ≥4) on at least one eye^^	1
Schirmer ≤ 5 mm/5 min on at least one eye	1
Unstimulated whole saliva flow rate ≤ 0.1 ml/min¥	1

The classification of SjD applies to any individual who meets the inclusion criteria*, does not have any condition listed as exclusion criteria**, and who has a score ≥4 when summing the weights from the following items:

* Inclusion criteria: these criteria are applicable to any patient with at least one symptom of ocular or oral dryness (defined as a positive response to at least one of the following questions:

1) Have you had daily, persistent, troublesome dry eyes for more than 3 months?
2) Do you have a recurrent sensation of sand or gravel in the eyes?
3) Do you use tear substitutes more than 3 times a day?
4) Have you had a daily feeling of dry mouth for more than 3 months?
5) Do you frequently drink liquids to aid in swallowing dry food?); or suspicion of SjD from ESSDAI questionnaire (at least one domain with positive item)

** Exclusion criteria: Prior diagnosis of any of the following conditions would exclude diagnosis of SjD and participation in SjD studies or therapeutic trials because of overlapping clinical features or interference with criteria tests:

- History of head and neck radiation treatment
- Active Hepatitis C infection (with positive PCR)
- Acquired immunodeficiency syndrome
- Sarcoidosis
- Amyloidosis
- Graft versus host disease
- IgG4-related disease

Note: Patients who are normally taking anticholinergic drugs should be evaluated for objective signs of salivary hypofunction and ocular dryness after a sufficient interval off these medications for these components to be a valid measure of oral and ocular dryness.

^ The histopathologic examination should be performed by a pathologist with expertise in the diagnosis of focal lymphocytic sialadenitis, and focus score count (based on number of foci per 4 mm^2) following a protocol described in Daniels et al.[24]

^^ Ocular staining score described in Whitcher et al.[25] van Bijsterveld score described in van Bijsterveld 1969[26]

¥ Unstimulated whole saliva described in Navazesh & Kumar, 2008[27]

A focus is defined as a periductal cluster of ≥50 lymphocytes. In salivary gland biopsies of suspected SjD patients a FS≥1 is considered as a positive biopsy. While the presence of these foci, is a hallmark of the disease, the FS has some important shortcomings. For instance, interpretation of FS is impossible when other types of sialadenitis are present. Also, FS is solely based on the number of foci and does not consider the size of infiltrates. Consequently, instances wherein multiple small foci are present may yield positive biopsy results, while biopsies containing a single large or confluent focus may not. As a result of B-cell hyperactivity, additional salivary gland histopathological features, beyond periductal infiltrates, are also associated with SjD. One of these features are LELs, which appear to be highly specific for SjD.[20] LELs are striated ducts, which are infiltrated by B-lymphocytes with concurrent hyperplasia of ductal basal cells. B-lymphocytes infiltrating the ductal epithelium may precede ductal hyperplasia and these lesions are therefore called pre-LELs.[21] Another characteristic histopathological feature found in the salivary gland tissue of SjD patients, is a relative increase in the number of IgG plasma cells with a concurrent relative decrease of IgA plasma cells, resulting in a so-called plasma cell shift.[22] Lymphocytic foci potentially evolve under influence of chemokines and cytokines into ectopic lymphoid structures that exhibit segregated T- and B-cell areas with follicular dendritic cell (FDC) networks. In approximately a quarter of patients even GCs may develop. Potentially, high-affinity memory B cells are generated here.[17,23]

Paediatric onset of SjD

Typically, SjD is diagnosed in the fifth decade of life. However 1.3% of SjD patients has a paediatric onset of disease.[28] The clinical manifestations of paediatric SjD patients differ from those observed in adults. Children with SjD often manifest primarily with non-specific extra-glandular symptoms, notable including fever and arthralgias.[28] In contrast to adults, paediatric SjD patients (pedSjD) less frequently report the typical sicca complaints such as keratoconjunctivitis sicca and xerostomia. Instead, their clinical profile is characterised by significant involvement of the major salivary glands such as recurring episodes of parotid gland swelling, diminished stimulated whole salivary (SWS) flow rates and higher salivary gland ultrasound scores compared to adult SjD

patients.[29] Importantly whether significant salivary gland involvement is also reflected by the underlying histopathology remains unclear.

Treatment and treatment outcomes

Unfortunately there are no approved biological Disease-Modifying Antirheumatic Drugs (DMARDs) yet and current treatment of SjD is predominantly focused on symptom alleviation. Most larger, phase III trials including treatments targeting B-cells or signalling pathways involved in formation, activation and expansion of B-cells, performed thus far have failed. Disappointing outcomes are partly due to the use of insufficient discriminatory outcome parameters.[30]

The EULAR-Sjögren's Syndrome Disease Activity Index (ESSDAI) is often used as primary endpoint in clinical trials.[30] However, due to the complex and heterogenous nature of disease, other clinically relevant disease features should be taken into account. Therefore, recently novel composite endpoints for assessing treatment efficacy in patients with SjD were formulated: the Composite of Relevant Endpoints for Sjögren's Syndrome (CRESS), and the Sjögren's Tool for Assessing Response (STAR). Both include a number of main disease features specific for SjD as reflected in objective measurements to assess systemic disease activity, patient reported symptoms, tear- and salivary gland involvement and serology.[31,32]

Next to the importance of selection of the appropriate endpoint, the right patients should be selected for the right trial. This is challenging as SjD patients exhibit significant diversity, and clinical trials reveal that specific subgroups within SjD respond more favourably to particular treatments. Patient stratification can be achieved by using for example patient-reported symptoms, salivary gland ultrasonography but also salivary gland histopathology[33–35]. Consequently, the emphasis should shift away from a generalised approach for SjD and toward a personalised medicine paradigm, aiming to identify which specific treatment is most beneficial for individual SjD patients.

SCOPE OF THIS THESIS

The aim of this thesis was to get more insight in the histopathology of salivary glands of SjD patients and to assess its role in diagnosis, classification and patient stratification. Histopathological features additional to the FS were analysed in several patient cohorts and (histopathological) effects of immunomodulatory therapy versus placebo were assessed.

Part 1: salivary gland histopathology in Sjögren's disease

Lymphocytic foci potentially evolve under influence of chemo- and cytokines into ectopic lymphoid structures and possibly even GCs. The recognition of GCs can be difficult in H&E stained sections as small GCs can be overlooked and LELs can be mistaken as GCs. Currently there is no consensus guideline for the identification of GCs in salivary glands of suspected SjD patients. As a result, there is great discrepancy in identification of GCs in SjD since multiple staining methods are in use. In **chapter 2** we investigated whether additional immunohistochemical markers can aid in GC identification.

Traditionally, a labial salivary gland biopsy is obtained for diagnosis and classification of SjD. A parotid gland biopsy can serve as a safe and suitable alternative. However, to what extent the two biopsies are similar in terms of all main histopathological features, i.e., FS, (pre-)LELs, plasma cell shift, GCs was not known. Therefore, in **chapter 3** we compared FS and other histopathological features in paired labial and parotid salivary gland biopsies of SjD and non-SjD sicca patients in order to assess comparability between major and minor salivary gland histopathology in SjD.

PedSjD present more often with major salivary gland involvement compared to patients with adult-onset SjD. However, whether this difference in clinical presentation is also reflected by its histopathological pattern is unknown. Therefore, in **chapter 4** histopathological features of parotid salivary glands of pedSjD patients were compared with both a diagnostic cohort of adult patients with sicca complaints suggestive for SjD and an adult SjD cohort of patients participating in a clinical trial.

Tfh cells play a critical role in the enhanced activation of B-cells in SjD. The effects of Tfh cells are regulated by Tfr cells which express cytotoxic T-lym-

phocyte associated protein 4 (CTLA-4), a key inhibitory receptor. In SjD, there is an elevated ratio of circulating Tfr to Tfh cells when compared to healthy controls. Typically, a higher proportion of Tfr cells might be expected to lead to reduced B-cell activation. However, in SjD Tfr cells express lower levels of CTLA-4. This downregulation of CTLA-4 in Tfr cells may compromise their suppressive functions, potentially influencing the balance of immune responses and contributing to the dysregulated immune activity characteristic of SjD. In **chapter 5** we commented on an article about the potential role of Tfr/Tfh ratio and frequency of activated Tfh cells in blood as a biomarker for ectopic lymphoid structure formation in SjD patients.

Part 2: diagnosis, classification and patient stratification

Salivary gland biopsies have a prominent role in the ACR-EULAR classification criteria. Currently, positivity of a salivary gland biopsy is solely based on the FS. As the FS holds important limitations, in **chapters 6 and 7** we investigated whether improvement of the diagnostic accuracy of the labial and parotid gland biopsy in SjD can be achieved by taking additional histopathological features into account (pre-)LELs, plasma cell shift, GCs). We also assessed the impact of these additional histopathological features on the performance of the ACR-EULAR classification. These studies aimed to improve the diagnostic sensitivity and specificity of salivary gland biopsies and improve accuracy of classification criteria in SjD.

In **chapter 8** we analysed the agreement between the different histopathological key features in labial gland biopsies of suspected SjD patients. Moreover, the association between the presence of histopathological key features and SjD related serological/clinical parameters and expert diagnosis was assessed.

Given that the salivary glands are one of the most important targets of the autoimmune inflammatory process in SjD, evaluating changes within the glandular tissue is essential in assessing treatment efficacy. Abatacept, a biological DMARD, blocks CD28-mediated co-stimulation of T-cells.[36] Histopathological analysis of labial salivary gland biopsies of a phase II study showed limited effects of abatacept on glandular tissue.[37] However, the limited number of patients, lack of a placebo group and use of only minor salivary gland sections were limitations of the study. Therefore, in **chapter 9** the effect

of blocking T-cell dependent B-cell activation by abatacept treatment and placebo on the labial and parotid gland histopathology in SjD patients was assessed. Also, the capability of histopathological markers to predict response to abatacept treatment was investigated.
Finally, the results presented in this thesis are summarised and discussed in **chapter 10**.

REFERENCES

1. Mariette X, Criswell LA. Primary Sjögren's Syndrome. N Engl J Med. 2018 Mar;378(10):931–9.
2. Baer AN, Hammitt KM. Sjögren's Disease, Not Syndrome. Arthritis & rheumatology. 2021 Jul;73(7):1347–8.
3. Ghafoor M. Sjögren's Before Sjögren: Did Henrik Sjögren (1899-1986) Really Discover Sjögren's Disease? J Maxillofac Oral Surg. 2012 Sep;11(3):373–4.
4. Fox RI. Sjögren's syndrome. The Lancet. 2005;366(9482):321–31.
5. Fox RI. Extraglandular Manifestations of Sjögren's Syndrome (SS): Dermatologic, Arthritic, Endocrine, Pulmonary, Cardiovascular, Gastroenterology, Renal, Urology, and Gynecologic Manifestations. In: Sjögren's Syndrome. New York, NY: Springer New York; 2011. p. 285–316.
6. Reksten TR, Lessard CJ, Sivils KL. Genetics in Sjögren Syndrome. Rheum Dis Clin North Am. 2016 Aug;42(3):435–47.
7. Kroese FGM, Abdulahad WH, Haacke E, Bos NA, Vissink A, Bootsma H. B-cell hyperactivity in primary Sjögren's syndrome. Expert Rev Clin Immunol. 2014;10(4):483–99.
8. Crotty S. T follicular helper cell differentiation, function, and roles in disease. Immunity. 2014 Oct;41(4):529–42.
9. Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, et al. Foxp3+ follicular regulatory T cells control the germinal center response. Nat Med. 2011 Jul;17(8):975–82.
10. Spijkervet FKL, Haacke E, Kroese FGM, Bootsma H, Vissink A. Parotid Gland Biopsy, the Alternative Way to Diagnose Sjögren Syndrome. Rheumatic Disease Clinics of North America. 2016;42(3):485–99.
11. Verstappen GM, Pringle S, Bootsma H, Kroese FGM. Epithelial-immune cell interplay in primary Sjögren syndrome salivary gland pathogenesis. Nat Rev Rheumatol. 2021 Jun;17(6):333–48.
12. Jonsson M V., Szodoray P, Jellestad S, Jonsson R, Skarstein K. Association between circulating levels of the novel TNF family members APRIL and BAFF and lymphoid organization in primary Sjögren's syndrome. J Clin Immunol. 2005;25(3):189–201.
13. Chisholm DM, Mason DK. Labial salivary gland biopsy in Sjögren's disease. J Clin Pathol. 1968 Sep;21(5):656–60.
14. Pijpe J, Kalk WWI, van der Wal JE, Vissink A, Kluin PhM, Roodenburg JLN, et al. Parotid gland biopsy compared with labial biopsy in the diagnosis of patients with primary Sjogren's syndrome. Rheumatology. 2006 Jul 28;46(2):335–41.
15. Delli K, Dagal EF, Bootsma H, Vissink A, Spijkervet FKL. Patient-reported change of sensibility and pain after parotid and labial gland biopsy applied for primary Sjögren's syndrome diagnostics: one-year follow-up study. Clin Exp Rheumatol. 2018;36 Suppl 1(3):173–6.
16. Deroo L, Genbrugge E, Dochy F, Creytens D, Achten H, De Boeck K, et al. Ultrasound-guided core needle biopsy and incisional biopsy of the parotid gland are comparable in diagnosis of primary Sjögren's syndrome. Rheumatology. 2023 Aug 1;62(8):2765–72.
17. Kroese FGM, Haacke EA, Bombardieri M. The role of salivary gland histopathology in primary Sjögren's syndrome: promises and pitfalls. Clin Exp Rheumatol. 2018;36 Suppl 1(3):222–33.
18. Shiboski CH, Shiboski SC, Seror R, Criswell LA, Labetoulle M, Lietman TM, et al. 2016 American College of Rheumatology/European League Against Rheumatism Classification Criteria for Primary Sjögren's Syndrome: A Consensus and Data-Driven Methodology Involving Three International Patient Cohorts. Arthritis & rheumatology. 2017 Jan;69(1):35–45.
19. Christodoulou MI, Kapsogeorgou EK, Moutsopoulos HM. Characteristics of the minor salivary gland infiltrates in Sjögren's syndrome. J Autoimmun. 2010;34(4):400–7.

20. Ihrler S, Zietz C, Sendelhofert A, Riederer A, Löhrs U. Lymphoepithelial duct lesions in Sjögren-type sialadenitis. Virchows Arch. 1999 Apr;434(4):315–23.

21. van Ginkel MS, Haacke EA, Bootsma H, Arends S, van Nimwegen JF, Verstappen GM, et al. Presence of intraepithelial B-lymphocytes is associated with the formation of lymphoepithelial lesions in salivary glands of primary Sjögren's syndrome patients. Clin Exp Rheumatol. 2019;37 Suppl 118(3):42–8.

22. de Wilde PC, Vooys GP, Baak JP, van Houwelingen JC, Slootweg PJ, Hené RJ, et al. Quantitative immunohistologic and histomorphometric diagnostic criteria for Sjögren's syndrome. Pathol Res Pract. 1989 Nov;185(5):778–80.

23. Risselada AP, Looije MF, Kruize AA, Bijlsma JWJ, Van Roon JAG. The Role of Ectopic Germinal Centers in the Immunopathology of Primary Sjögren's Syndrome: A Systematic Review. Semin Arthritis Rheum. 2013;42(4):368–76.

24. Daniels TE, Cox D, Shiboski CH, Schiødt M, Wu A, Lanfranchi H, et al. Associations between salivary gland histopathologic diagnoses and phenotypic features of Sjögren's syndrome among 1,726 registry participants. Arthritis Rheum. 2011 Jul;63(7):2021–30.

25. Whitcher JP, Shiboski CH, Shiboski SC, Heidenreich AM, Kitagawa K, Zhang S, et al. A Simplified Quantitative Method for Assessing Keratoconjunctivitis Sicca From the Sjögren's Syndrome International Registry. Am J Ophthalmol. 2010;149(3):405–15.

26. Van Bijsterveld OP. Diagnostic Tests in the Sicca Syndrome. Archives of Ophthalmology. 1969;82(1):10–4.

27. Navazesh M, Kumar SKS, University of Southern California School of Dentistry. Measuring salivary flow: challenges and opportunities. J Am Dent Assoc. 2008 May;139(-Suppl):35S-40S.

28. Ramos-Casals M, Acar-Denizli N, Vissink A, Brito-Zerón P, Li X, Carubbi F, et al. Childhood-onset of primary Sjögren's syndrome: phenotypic characterization at diagnosis of 158 children. Rheumatology (Oxford). 2021 Oct;60(10):4558–67.

29. Legger GE, Erdtsieck MB, de Wolff L, Stel AJ, Los LI, Verstappen GM, et al. Differences in presentation between paediatric- and adult-onset primary Sjögren's syndrome patients. Clin Exp Rheumatol. 2021;39 Suppl 1(6):85–92.

30. Arends S, de Wolff L, Deroo L, Verstappen GM, Vissink A, Kroese FGM, et al. Selection of study endpoints and patients for clinical trials in primary Sjögren's syndrome. Clin Exp Rheumatol. 2022 Jun 20;40(12):2225–32.

31. Arends S, de Wolff L, van Nimwegen JF, Verstappen GMPJ, Vehof J, Bombardieri M, et al. Composite of Relevant Endpoints for Sjögren's Syndrome (CRESS): development and validation of a novel outcome measure. Lancet Rheumatol. 2021 Aug;3(8):e553–62.

32. Seror R, Baron G, Camus M, Cornec D, Perrodeau E, Bowman SJ, et al. Development and preliminary validation of the Sjögren's Tool for Assessing Response (STAR): a consensual composite score for assessing treatment effect in primary Sjögren's syndrome. Ann Rheum Dis. 2022 Jul;81(7):979–89.

33. Tarn JR, Howard-Tripp N, Lendrem DW, Mariette X, Saraux A, Devauchelle-Pensec V, et al. Symptom-based stratification of patients with primary Sjögren's syndrome: multi-dimensional characterisation of international observational cohorts and reanalyses of randomised clinical trials. Lancet Rheumatol. 2019 Oct;1(2):e85–94.

34. Mossel E, van Nimwegen JF, Stel AJ, Wijnsma RF, Delli K, van Zuiden GS, et al. Clinical Phenotyping of Primary Sjögren Syndrome Patients Using Salivary Gland Ultrasonography: Data From the RESULT Cohort. J Rheumatol. 2021 May;48(5):717–27.

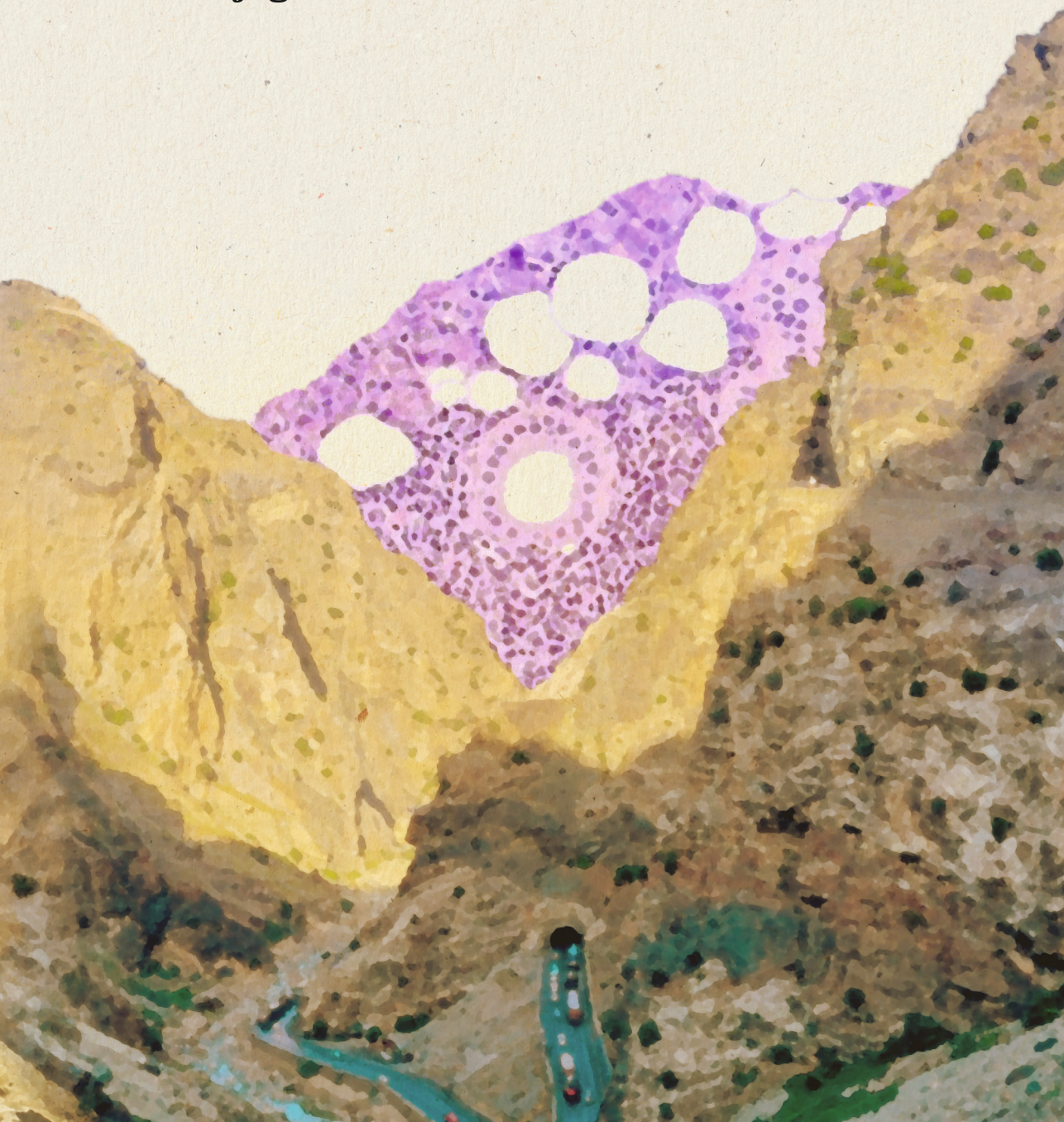
35. Delli K, Haacke EA, Kroese FGM, Pollard RP, Ihrler S, Van Der Vegt B, et al. Towards personalised treatment in primary Sjögren's syndrome: Baseline parotid histopathology predicts responsiveness to rituximab treatment. Ann Rheum Dis. 2016;

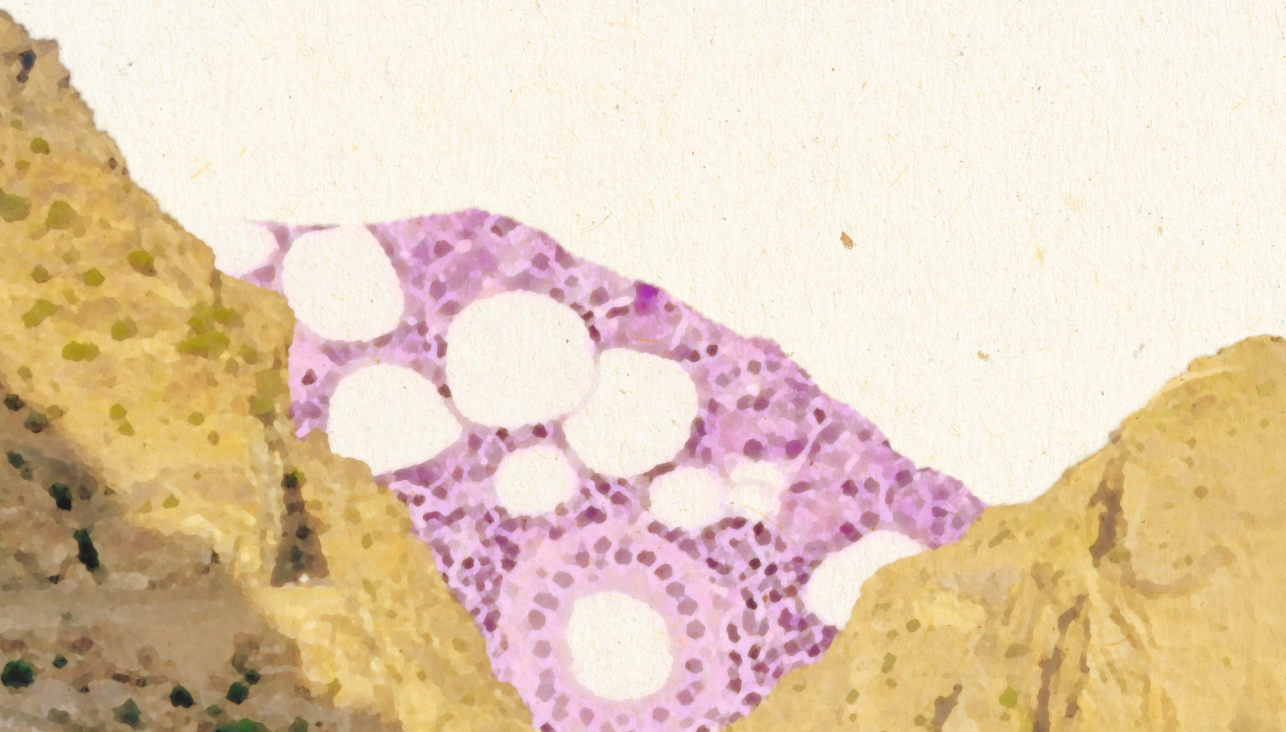
36. van Nimwegen JF, Mossel E, van Zuiden GS, Wijnsma RF, Delli K, Stel AJ, et al. Abatacept treatment for patients with early active primary Sjögren's syndrome: a single-centre, randomised, double-blind, placebo-controlled, phase 3 trial (ASAP-III study). Lancet Rheumatol. 2020 Mar;2(3):e153–63.

37. Haacke EA, van der Vegt B, Meiners PM, Vissink A, Spijkervet FKL, Bootsma H, et al. Abatacept treatment of patients with primary Sjögren's syndrome results in a decrease of germinal centres in salivary gland tissue. Clin Exp Rheumatol. 2017;35(2):317–20.

PART 1

Salivary gland histopathology in Sjögren's disease





CHAPTER 2

Bcl6 for identification of germinal centres in salivary gland biopsies in primary Sjögren's syndrome

U. Nakshbandi[1], E.A. Haacke[2], H. Bootsma[1], A. Vissink[3], F.K.L. Spijkervet[3], B. van der Vegt[2], F.G.M. Kroese[1]

Departments of [1]Rheumatology and Clinical Immunology, [2]Pathology and Medical Biology, [3]Oral and Maxillofacial Surgery and, University of Groningen and University Medical Centre Groningen, Groningen, the Netherlands

Oral Diseases 2020;26:707-710

Histopathological assessment of salivary gland biopsies is an important element of the diagnostic workup of Sjögren's syndrome (SS).[1,2] Microscopic evaluation of salivary glands of primary SS (pSS) patients reveals characteristic periductal lymphocytic infiltrates (foci), which mainly consist of T and B lymphocytes, as well as a variety of non-lymphoid cells, including dendritic cells and macrophages. Over time, these infiltrates may become organised to ectopic lymphoid tissue with T/B cell compartmentalisation, presence of $CD21^+$ follicular dendritic cell (FDC) networks and high endothelial venules.[2–5] Germinal centres (GCs) are present within this ectopic lymphoid tissue in roughly one-quarter of the salivary gland biopsies of pSS patients and their presence is associated with more severe disease compared to GC-negative pSS patients.[6] These glandular ectopic GCs express mRNA encoding for activation-induced deaminase, an enzyme critical for the induction of somatic hypermutation and essential for the main function of GCs, the generation of high-affinity memory B cells.[7–9]

Presence of GCs in biopsies taken for the diagnosis of pSS has been suggested to be a risk factor for lymphoma development[10–12], a finding recently disputed by us.[13,14] Detection of GCs in routine haematoxylin and eosin (H&E)-stained sections can be challenging because small GCs may be overlooked and distinction between GCs and lymphoepithelial lesions may be difficult. Therefore, immunohistochemical identification using antibodies directed against CD21, expressed by FDCs (but also by B cells) or Bcl6, a transcription factor highly expressed by GC-B cells, has been used[7,14,15], but consensus criteria regarding identification of GCs are lacking.[16] Hence, the aim of this study was to asses which staining is most suitable to unequivocally identify GCs in diagnostic salivary gland biopsies of pSS patients by comparing H&E, CD21 and Bcl6 stainings. In our study, we restricted ourselves to these three markers, which can be easily applied in an automated fashion in diagnostic pathology laboratories. For this reason, we did not consider staining for other GC-associated markers, such as activation-induced deaminase, as potential candidates for identification of GCs in biopsies.

From 42 pSS patients, classified according to American College of Rheumatology (ACR)-European League Against Rheumatism (EULAR) classification criteria[17], both a labial salivary gland and a parotid salivary gland biopsy were obtained (see Table 1). Four-micrometre-thick serial sections of salivary gland

biopsies were stained with H&E and immunohistochemically for CD21 and Bcl6. For detailed ethical approval information, staining characteristics and statistical analysis see supplementary material.

Table 1. Demographic, clinical and histological parameters of patients with primary Sjögren's syndrome

	pSS patients (n=42)
Demographic characteristics	
Age, years	52 ± 13
Female, n (%)	41 (97.6)
Caucasian, n (%)	41 (97.6)
Serological parameters	
RF positive, n (%)	25 (59.5)
ANA positive, n (%)	10 (23.8)
Anti-SSA positive, n (%)	32 (76.2)
Anti-SSB positive, n (%)	15 (35.7)
IgG	15.4 [11.7–19.4]
ESR	23.0 [9.8–45.5]
CRP	2.8 [1.0–5.5]
Clinical parameters	
ESSDAI score	3.5 [2.0–9.0]
Schirmer, mm/5 min	2.5 [0.0–5.0]
UWS, ml/min	0.1 [0.0–0.2]
Histopathological parameters of the labial gland	
Focus score	1.3 [1.0–2.4]
≤ 70% IgA plasma cells, n (%)	19 (45.2)
Lymphoepithelial lesions, n (%)	16 (38.1)
Relative area of $CD45^+$ infiltrate*	9.1 [6.1–19.8]
Histopathological parameters of the parotid gland	
Focus score	1.0 [0.0–1.7]
≤ 70% IgA plasma cells, n (%)	12 (28.6)
Lymphoepithelial lesions, n (%)	18 (42.9)
Relative area of $CD45^+$ infiltrate*	4.5 [1.4–17.0]

Patients were classified according to the ACR-EULAR criteria. Data are represented as mean ± SD, median [95% CI] or number (%).

Abbreviations: ANA, antinuclear antibodies; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; ESSDAI, European League Against Rheumatism SS Disease Activity Index; n, number of patients; pSS, primary Sjögren's syndrome; RF, rheumatoid factor; SSA, Sjögren's syndrome antigen A; SSB, Sjögren's syndrome antigen B; UWS, unstimulated whole saliva.

*% of area of lymphocytic infiltrate in salivary gland parenchyma (Aperio ImageScope v12.0).

In H&E-stained sections, a GC was defined as a clearly visible lighter area in a lymphocytic infiltrate containing centrocytes, centroblasts, FDCs and macrophages. In CD21-stained sections, a network of positive staining within a

focus was classified as a FDC network. In Bcl6-stained sections, a cluster of ≥5 adjacent positive cells within a focus was classified as a GC[13]. Even though Bcl6 is also expressed by follicular helper T cells, this expression does not interfere with detection of GCs as these cells are not organised in clusters as GCs, but lie scattered throughout the tissue (Figure 1).

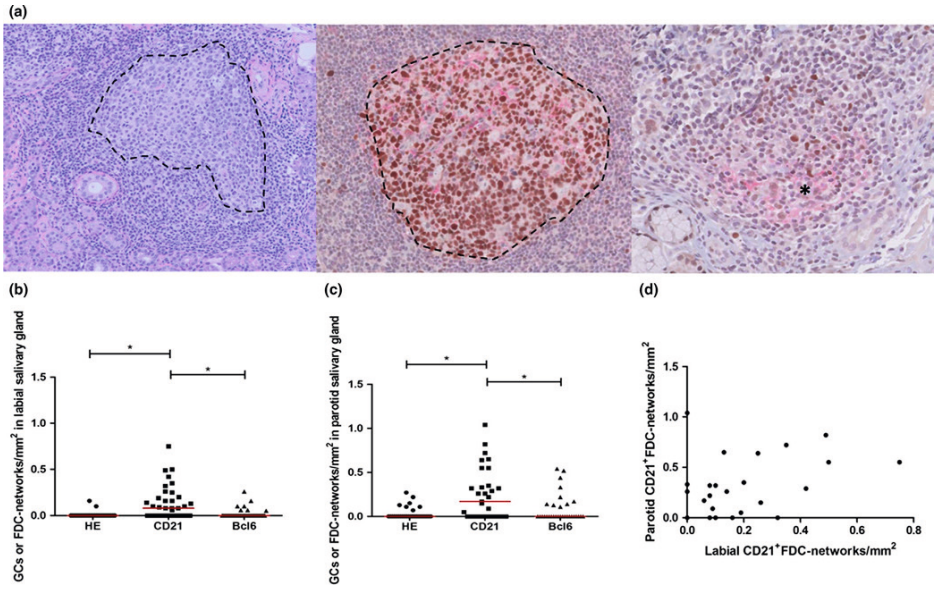


Figure 1. Presence of germinal centres and follicular dendritic cell networks in salivary gland biopsies of patients with primary Sjögren's syndrome. (a) Histopathological identification of germinal centres. Paraffin-embedded parotid gland biopsy of a patient with primary Sjögren's syndrome stained with H&E (left panel) and by double immunohistochemistry for CD21 (red) and Bcl6 (brown) (middle and right panel). The left panel shows a periductal focus with a H&E-stained GC (indicated by a dotted line, magnification 10×); the middle panel a $CD21^+$FDC network with a $Bcl6^+$GC (indicated by a dotted line, magnification 20×); and the right panel a $CD21^+$FDC network (indicated by an asterisk, magnification 20×) without a GC. (b) Number of GCs or FDC networks/mm^2 in labial (n = 36) salivary gland tissue after staining with H&E and immunohistochemically for Bcl6 or CD21. (c) Number of GCs or FDC networks/mm^2 parotid (n = 31) salivary gland tissue after staining with H&E and immunohistochemically for Bcl6 or CD21. (d) Spearman's rank-order correlation revealed a significant positive association between $CD21^+$FDC networks/mm^2 in parotid and labial salivary gland biopsies (r = .60, p = .001). Red lines indicate median values, *p < .05.

Six labial and eleven parotid salivary gland biopsies did not contain any H&E-defined periductal foci. For the remaining biopsies, 36 labial and 31 parotid glands, all individual H&E-defined foci (210 labial and 141 parotid glands) were analysed on serial sections. This staining revealed that 1% (3/210) of labial gland foci and 6% (9/141) of parotid gland foci contained H&E-defined GCs. Immunohistochemical staining for CD21 revealed that 24% (50/210) of the foci in the labial gland and 49% (69/141) of the foci in the parotid gland contained $CD21^+$FDC networks (Table 2). Importantly, after staining for Bcl6, we showed that only 18% (9/50) of the labial gland foci with $CD21^+$FDC networks and 32% (22/69) of the parotid gland foci with $CD21^+$FDC networks also comprised $Bcl6^+$GCs. Apparently, not all foci contain $CD21^+$FDC networks and not all foci with $CD21^+$FDC networks also harbour $Bcl6^+$GCs. This was confirmed by dual CD21/Bcl6 staining (Figure 1a). Consequently, the number of $CD21^+$FDC networks/mm^2 was significantly higher than the number of $H\&E^+$- and $Bcl6^+$-defined GCs/mm^2 in both labial and parotid salivary glands (Figure 1b and c). We observed a significant correlation between $CD21^+$FDC networks/mm^2 in parotid and labial salivary gland biopsies (Figure 1d, r=.60, p=.001), indicating comparability in lymphoid organisation at these two anatomical sites. Such a correlation was not seen for the presence of H&E- or Bcl6-defined GCs.

Table 2. Comparison between the number of germinal centres in labial and parotid salivary gland biopsies of primary Sjögren's syndrome patient

	Labial salivary gland	Parotid salivary gland
Number of biopsies with foci	36	31
Number of foci	210	141
% $H\&E^+$ GCs	1.4 (3/210)	6.4 (9/141)
% $Bcl6^+$ GCs	4.3 (9/210)	15.6 (22/141)
% $CD21^+$ FDC networks	23.8 (50/210)	48.6 (69/141)

Abbreviations: FDC, follicular dendritic cell; GCs, germinal centres; H&E, haematoxylin and eosin.

In a recent study, Carubbi et al. analysed the usage of CD3/CD20 as well as CD21 and Bcl6 as markers for the detection of GCs.[15] While they conclude that combination of CD3/CD20 and CD21 should be recommended for assessment of GCs, we clearly show here that usage of CD21 as surrogate marker for GCs

significantly overestimates GC counts. The reason for this is that formation of B-cell follicles and presence of $CD21^{+}$FDC networks (which are also present in primary B-cell follicles) do not also imply presence of GCs.[18] On the other hand, staining with H&E revealed fewer GCs compared to staining for Bcl6, most likely because small GCs can easily be overlooked on H&E.

Although staining for CD21 is thus less appropriate for detection of GCs, staining for CD21 is still valuable. FDCs play an essential role in the spatial orientation and B/T-cell compartmentalisation in ectopic lymphoid tissues due to their CXCL13 producing property. Presence of FDC networks suggests a more advanced stage of ectopic lymphoid development and may therefore be a useful marker for classification of the organisation of glandular tissue.[19]

In conclusion, we propose to use Bcl6 as a simple, sensitive and specific marker for unequivocal identification of GCs in salivary gland biopsies of (suspected) pSS patients. Large prospective studies are now needed to evaluate whether presence of GCs in diagnostic salivary gland biopsies for pSS is a risk factor for non-Hodgkin lymphomas or not, and whether it can be used for stratification of pSS patients for personalised medicine.[2,20]

REFERENCES

1. Fox RI. Standardisation of labial salivary gland biopsies in Sjogren's syndrome: importance for the practicing rheumatologist. Ann Rheum Dis. 2017 Jul;76(7):1159–60.
2. Kroese FGM, Haacke EA, Bombardieri M. The role of salivary gland histopathology in primary Sjögren's syndrome: promises and pitfalls. Clin Exp Rheumatol. 2018;36 Suppl 1(3):222–33.
3. Fisher BA, Jonsson R, Daniels T, Bombardieri M, Brown RM, Morgan P, et al. Standardisation of labial salivary gland histopathology in clinical trials in primary Sjögren's syndrome. Ann Rheum Dis. 2017;76(7):1161–8.
4. Kroese FG, Abdulahad WH, Haacke E, Bos NA, Vissink A, Bootsma H. B-cell hyperactivity in primary Sjögren's syndrome. Expert Rev Clin Immunol. 2014 Apr 1;10(4):483–99.
5. Salomonsson S, Rozell BL, Heimburger M, Wahren-Herlenius M. Minor salivary gland immunohistology in the diagnosis of primary Sjögren's syndrome. Journal of Oral Pathology & Medicine. 2009 Mar 23;38(3):282–8.
6. Risselada AP, Looije MF, Kruize AA, Bijlsma JWJ, Van Roon JAG. The Role of Ectopic Germinal Centers in the Immunopathology of Primary Sjögren's Syndrome: A Systematic Review. Semin Arthritis Rheum. 2013;42(4):368–76.
7. Bombardieri M, Barone F, Humby F, Kelly S, McGurk M, Morgan P, et al. Activation-induced cytidine deaminase expression in follicular dendritic cell networks and interfollicular large B cells supports functionality of ectopic lymphoid neogenesis in autoimmune sialoadenitis and MALT lymphoma in Sjögren's syndrome. J Immunol. 2007 Oct;179(7):4929–38.
8. Le Pottier L, Devauchelle V, Fautrel A, Daridon C, Saraux A, Youinou P, et al. Ectopic germinal centers are rare in Sjogren's syndrome salivary glands and do not exclude autoreactive B cells. J Immunol. 2009 Mar;182(6):3540–7.
9. Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell. 2000 Sep;102(5):553–63.
10. Nishishinya MB, Pereda CA, Muñoz-Fernández S, Pego-Reigosa JM, Rúa-Figueroa I, Andreu JL, et al. Identification of lymphoma predictors in patients with primary Sjögren's syndrome: a systematic literature review and meta-analysis. Rheumatol Int. 2015 Jan;35(1):17–26.
11. Sène D, Ismael S, Forien M, Charlotte F, Kaci R, Cacoub P, et al. Ectopic Germinal Center-Like Structures in Minor Salivary Gland Biopsy Tissue Predict Lymphoma Occurrence in Patients With Primary Sjögren's Syndrome. Arthritis Rheumatol. 2018 Sep;70(9):1481–8.
12. Theander E, Vasaitis L, Baecklund E, Nordmark G, Warfvinge G, Liedholm R, et al. Lymphoid organisation in labial salivary gland biopsies is a possible predictor for the development of malignant lymphoma in primary Sjögren's syndrome. Ann Rheum Dis. 2011 Aug;70(8):1363–8.
13. Haacke EA, Van Der Vegt B, Vissink A, Spijkervet FKL, Bootsma H, Kroese FGM. Germinal centres in diagnostic labial gland biopsies of patients with primary Sjogren's syndrome are not predictive for parotid MALT lymphoma development. Ann Rheum Dis. 2017;76(10):1783–6.
14. Haacke EA, van der Vegt B, Vissink A, Spijkervet FKL, Bootsma H, Kroese FGM. Germinal Centers in Diagnostic Biopsies of Patients With Primary Sjögren's Syndrome Are Not a Risk Factor for Non-Hodgkin's Lymphoma but a Reflection of High Disease Activity: Comment on the Article by Sène et al. Arthritis & Rheumatology. 2019 Jan 24;71(1):170–1.
15. Carubbi F, Alunno A, Cipriani P, Coletti G, Bigerna B, Manetti M, et al. Different operators and histologic techniques in the assessment of germinal center-like structures in primary Sjögren's syndrome minor salivary glands. PLoS One. 2019;14(1):e0211142.

16. Haacke EA, van der Vegt B, Vissink A, Spijkervet FKL, Bootsma H, Kroese FGM. Standardisation of the detection of germinal centres in salivary gland biopsies of patients with primary Sjögren's syndrome is needed to assess their clinical relevance. Ann Rheum Dis. 2018 Jun 22;77(6):e32.

17. Shiboski CH, Shiboski SC, Seror R, Criswell LA, Labetoulle M, Lietman TM, et al. 2016 American College of Rheumatology/European League Against Rheumatism Classification Criteria for Primary Sjögren's Syndrome: A Consensus and Data-Driven Methodology Involving Three International Patient Cohorts. Arthritis & Rheumatology. 2017 Jan 26;69(1):35–45.

18. MacLennan IC. Germinal centers. Annu Rev Immunol. 1994;12:117–39.

19. Jonsson M V, Skarstein K. Follicular dendritic cells confirm lymphoid organization in the minor salivary glands of primary Sjögren's syndrome. J Oral Pathol Med. 2008 Oct;37(9):515–21.

20. Delli K, Villa A, Farah CS, Celentano A, Ojeda D, Peterson DE, et al. World Workshop on Oral Medicine VII: Biomarkers predicting lymphoma in the salivary glands of patients with Sjögren's syndrome-A systematic review. Oral Dis. 2019 Jun;25 Suppl 1:49–63.

CONFLICT OF INTEREST

There are no competing interests for any author.

FUNDING INFORMATION

This study was financially supported by a grant from the National Institutes of Dental and Craniofacial Research (U01 DE017593 Salivary Biomarkers for Sjögren's Syndrome Detection-Definitive Validation) and by a grant from the Dutch Arthritis Foundation (Grant 2013-2-066). There was no involvement of these funding sources in study design, data collection, analysis and interpretation and writing of this report.

AUTHOR CONTRIBUTIONS

UN, EAH, FGMK, BvdV, HB and AV were involved in study concept and design. HB and AV recruited patients. FKLS performed all salivary gland biopsies. UN, EAH and BvdV collected data. UN, EAH, FGMK, BvdV, AV, FKLS and HB analysed and interpreted the data. All authors critically reviewed the manuscript and approved the final version to be published.

SUPPLEMENTARY MATERIAL

Ethical approval information

The study was approved by the Medical Research Ethics Committee of the UMCG, the Netherlands (METc2013.066). All participants gave consent according to the declaration of Helsinki.

Study information

Biopsies for this retrospective observational study were collected from 2014 until 2016. Inclusion and exclusion criteria are shown in supplementary table 1. All labial and parotid salivary gland biopsies were performed by one Oral and Maxillofacial surgeon (FKLS) at the University Medical Centre Groningen.

Immunohistological staining and histopathological assessment

CD21 staining: Sections were deparaffinised and antigen retrieval was performed (EDTA, pH 8). Endogenous peroxidase activity was blocked using H2O2 and PBS. Slides were incubated with CD21 antibodies (2G9; Cell Marque Corporation, USA) for 75 min. After rinsing, sections were treated with horse radish peroxidase (HRP) polymer (goat anti-mouse IgG) for 40 min. Staining was visualised with DAB and the sections were counterstained with haemotoxylin. This staining procedure was optimized for the detection of FDC networks.

Bcl6 staining and CD21/Bcl6 double staining: Bcl-6 (GI19E/A8; Ventana Medical systems, USA, prediluted by supplier) staining and CD21/Bcl6 double staining was performed after deparaffinisation, pre-treatment with Ultra CC1 (Ventana Medical Systems, USA), antigen retrieval and endogenous peroxidase blocking using the Benchmark automated staining platform (Ventana Medical Systems, USA). The double staining was performed serially.

All foci in labial and parotid salivary gland parenchyma were analysed for the presence of $CD21^{+}$FDC-networks and for $H\&E^{+}$- or $Bcl6^{+}$ GCs by a trained researcher (UN), an experienced pathology resident (EH) and a head and neck pathologist (BvdV). Discrepancies between observers were resolved in a consensus meeting.

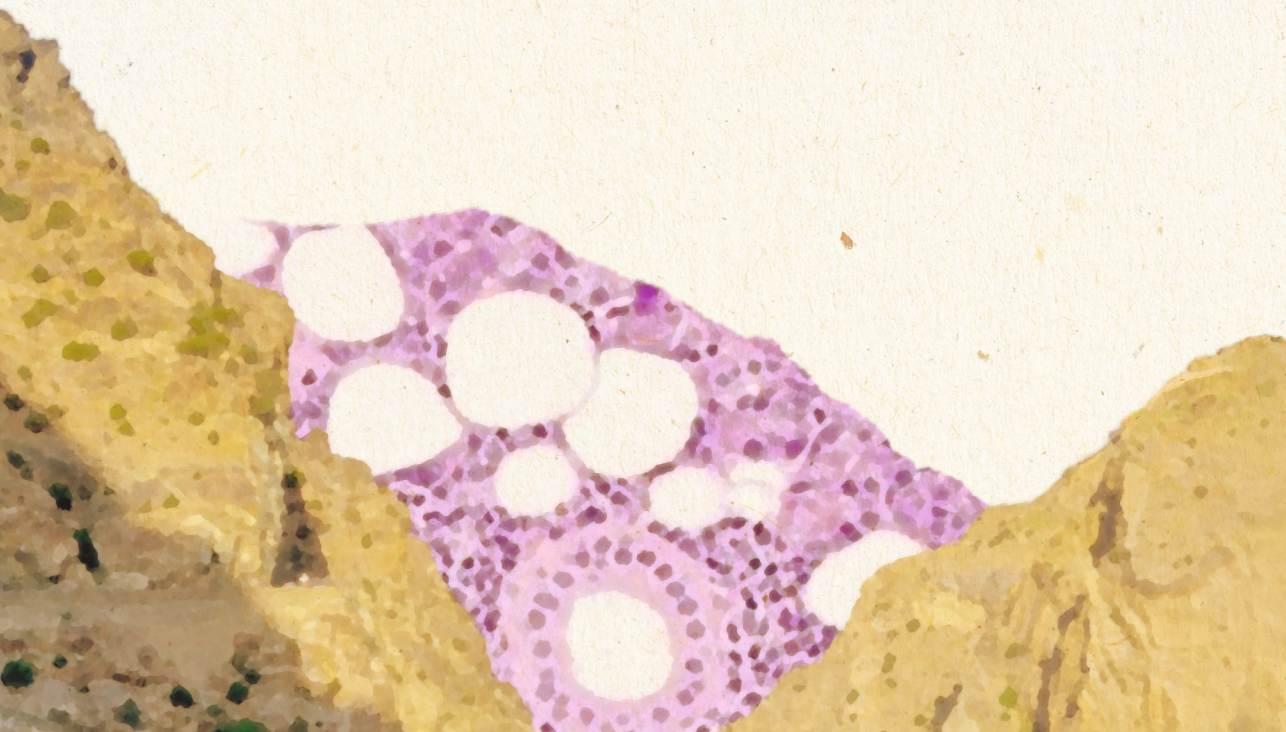
Statistical analysis

Data were analysed using SPSS version 23 statistical software (SPSS Inc., Chicago, IL). Differences between groups were tested with Mann-Whitney U test. Correlation analysis was performed using the Spearman's rank order correlation. P-values <0.05 were considered statistically significant.

Supplementary Table 1. Inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
• Ability to give informed consent. • Male or female patients 18 years of age or older. • Patients, classified according to ACR-EULAR classification criteria. • Must be willing to have a standard physical exam as part of standard clinical care and a complete diagnostic work-up according to the ACR criteria for ocular staining, labial salivary gland biopsy and serology. • Must be willing to have a standard physical exam and complete AECG diagnostic tests as part of standard clinical care (including eye exam, oral exam, salivary gland exam and biopsy). • Must be willing to donate 1ml of stimulated, whole saliva in 30 minutes or less. If a participant cannot produce 1ml during a 30 min collection period, subject will be unevaluable and will be considered a screen failure and withdrawn from the study. • Subjects must be willing to have a labial salivary gland biopsy in addition to a parotid biopsy. • Must be willing and able to give approximately 8ml of blood.	• Previous radiation to the head and neck. • Confirmed hepatitis C virus infection, which may cause SS-like signs and symptoms. • Known HIV infection, which can cause salivary gland infiltrates and enlargements similar to SS. • Sarcoidosis, which may cause alike signs and symptoms. • Graft-versus-host disease, which may cause SS-like signs and symptoms. • Oral cancer or history of oral cancer. • Presence of MALT lymphoma. • Pregnancy based on self-report. • Previously confirmed diagnosis of autoimmune disease known to be associated with sSS (RA, SLE, CREST, scleroderma, mixed connective tissue disease, polymyositis). • Insufficient biopsy material harvested from either labial or parotid salivary gland. • Absence of any focus in the labial and parotid gland biopsy.

Abbreviations: ACR, American College of Rheumatology; AECG, American-European Consensus Group; EULAR, European League Against Rheumatism; CREST, Calcinosis, Raynaud's syndrome, Esophageal dysmotility, Sclerodactylyl, Telangiectasia; MALT, Mucosa associated lymphoid tissue; RA, Rheumatoid arthritis; SLE, Systemic lupus erythematosus; sSS, secondary Sjögren's syndrome.



CHAPTER 3

Histopathological comparison of Sjögren related features in paired labial and parotid gland biopsies of sicca patients

U. Nakshbandi[1], M.S. van Ginkel[1], G.M.P.J. Verstappen[1], F.K.L. Spijkervet[2], S. Arends[1], E.A. Haacke[3], S.C. Liefers[1], A. Vissink[2], H. Bootsma[1], *F.G.M. Kroese[1], *B. van der Vegt[3]

*Shared last authorship

Departments of [1]Rheumatology and Clinical Immunology, [2]Oral and Maxillofacial Surgery and [3]Pathology and Medical Biology, University of Groningen and University Medical Center Groningen, Groningen, the Netherlands

Rheumatology 2024;63(10): 2670–2677

ABSTRACT

Objectives

To compare focus score (FS) and other histopathological features between paired labial and parotid salivary gland biopsies in a diagnostic cohort of suspected Sjögren's disease (SjD) patients.

Methods

Labial and parotid salivary gland biopsies were simultaneously obtained from patients with sicca complaints, suspected of having SjD. Biopsies were formalin fixed and paraffin embedded. Sections were stained with haematoxylin & eosin (H&E) and for CD3, CD20, CD45, cytokeratin, CD21, Bcl6, activation induced deaminase (AID), and IgA/IgG. FS and other histopathological features characteristic for SjD were analysed.

Results

Based on the expert opinion of three experienced rheumatologists, 36 patients were diagnosed as SjD and 63 as non-SjD sicca patients. When taking all patients together, absolute agreement of various histopathological features between labial and parotid biopsies was high and varied between 80% (FS) and 93% ((pre-)lymphoepithelial lesions (LELs)). More labial gland biopsies had a FS≥1 compared to their parotid counterpart. Accordingly, the area of infiltrate was larger in labial gland biopsies. When considering only SjD patients, labial glands contained significantly less B-lymphocytes, GCs/mm^2 and less severe LELs compared to parotid glands.

Conclusion

Labial and parotid glands from SjD patients contain similar histopathological key features, and thus both glands can be used for diagnosis and classification of SjD. However, parotid salivary glands reveal more evident B-lymphocyte related features, while labial glands exhibit more inflammation, which may be partially unrelated to SjD.

INTRODUCTION

Sjögren's disease (SjD) is a systemic auto-immune disease, characterized by chronic inflammation of salivary and lacrimal glands. Patients typically present with dryness complaints such as xerostomia and keratoconjunctivitis sicca.[1] In salivary glands of SjD patients, the chronic inflammation is a focal lymphocytic sialadenitis characterized by lymphocytic foci commonly associated with striated ducts.

Traditionally, a labial salivary gland biopsy is obtained for diagnosis and classification. A parotid biopsy has comparable sensitivity and specificity to a labial biopsy, making it a good alternative.[2] The patient-reported postoperative change in sensibility and pain in the area of the parotid and labial gland biopsy are minor and comparable.[3] The applicability of the parotid biopsy has increased since the introduction of ultrasound guided core needle biopsies, showing comparable results to incisional parotid gland biopsies.[4] Moreover, parotid gland biopsies have several advantages including the possibility to perform a repeated biopsy from the same gland and the higher likelihood of detecting a mucosa-associated lymphoid tissue (MALT) lymphoma compared to labial gland biopsies.

Salivary gland biopsies have a prominent position in the American College of Rheumatology – European League Against Rheumatism (ACR-EULAR) classification criteria for SjD.[5] In these criteria, histopathological classification of SjD is only based on the focus score (FS).[6,7] A focus is defined as a cluster of ≥50 lymphocytes and the FS is the number of foci per 4 mm^2 salivary gland tissue. In both labial and parotid gland biopsies, a FS≥1 is considered positive for SjD. While FS is used as a classification tool, it is only based on the number of foci and does not consider the size of the inflammatory infiltrates. Therefore, others have proposed to use the total size of these focal infiltrates as an alternative for the FS, thereby providing a better quantification of the extent of glandular inflammation.[8,9]

Although lymphocytic foci are characteristic for SjD, they are certainly not the only histopathological key-feature of the inflammatory infiltrate. Lymphocytic foci potentially evolve under influence of chemokines and cytokines into ectopic lymphoid structures that exhibit segregated T- and B-cell areas with follicular dendritic cell (FDC) networks and possibly even germi-

nal centres (GCs).[10,11] GCs typically express transcription factor Bcl-6 and the enzyme activation induced deaminase (AID), the enzyme responsible for somatic hypermutation and class switch recombination in the IgG genes of B-lymphocytes.[12] Other characteristic histopathological features found in salivary glands of SjD patients comprise a relative increase in the number of IgG producing plasma cells with a concomitant relative decrease in the number of IgA plasma cells (the so-called plasma cell immunoglobulin isotype shift), and the presence of lymphoepithelial lesions (LELs).[13,14] LELs are defined as striated ducts infiltrated by B-lymphocytes with concurrent hyperplasia of the ductal epithelium.[15] B-lymphocytes infiltrating the ductal epithelium may precede hyperplasia of the epithelium and ducts with intraepithelial B-lymphocytes, but without hyperplasia, are therefore called pre-LELs.[16] Presence of GCs, plasma cell shift and LELs reflect the hallmark finding of B-lymphocyte hyperactivity in SjD.[10,17] Recently, we have demonstrated that addition of two of these histopathological features to the FS increases diagnostic accuracy of the labial gland biopsy for SjD.[18]

While all these histopathological features can be seen in both minor (labial) and major (parotid) salivary glands, it is unclear whether all these features develop simultaneously in both gland types in an individual patient. This might be relevant for classification, diagnosis and prognosis, and also may increase our understanding of the pathogenesis of the disease. Therefore, the aim of this study was to compare FS and other histopathological features between paired labial and parotid salivary gland biopsies of SjD and non-SjD sicca patients.

METHODS

Patients

In this prospective study, consecutive patients with oral and/or ocular sicca complaints, suspected of having SjD, who underwent a full diagnostic workup at the University Medical Centre Groningen (UMCG), a tertiary referral centre and centre of expertise for SjD, were included between 2014 and 2017. Labial and parotid gland biopsies were simultaneously obtained under local infiltration anaesthesia by the same oral and maxillofacial surgeon (FKLS).[19] Ex-

clusion criteria for this study were: presence of another associated auto-immune disease, positive hepatitis C serology, salivary gland MALT lymphoma, sclerosing sialadenitis and insufficient biopsy material (total surface area of sections <1 mm^2). Participants gave written consent according to the declaration of Helsinki. The study was approved by the Medical Research Ethics Committee of the UMCG, the Netherlands (METc2013.066).

Clinical evaluation

All patients were diagnosed as SjD or non-SjD sicca based on the expert opinion of three experienced rheumatologists (HB, AJS, LB). The expert panel had access to anonymized clinical vignettes including all signs and symptoms, medication use, lab tests clinical parameters and FS of labial and parotid gland biopsies. Disagreement was resolved during a consensus meeting.[18]

Histochemical- and immunohistochemical staining

Biopsy material was formalin fixed (4%), paraffin embedded and serially sectioned at 3 µm thickness. After deparaffinisation, sections were stained for haematoxylin & eosin (H&E) or by immunohistochemistry. Immunohistochemical staining was performed either manually or using an automated staining platform (Benchmark XT, Ventana Medical Systems, Inc.) (see supplementary table 1). For manual immunohistochemical staining antigen retrieval was carried out by incubating the tissue sections for 15 minutes with EDTA buffer, pH of 8.0. Endogenous peroxidase activity was blocked using H_2O_2. Hereafter, slides were incubated with primary antibodies for 75 min and a poly-HRP labelled secondary antibody (Thermo Fisher Scientific). Activation-Induced cytidine Deaminase (AID) staining was performed as follows: after deparaffinisation, antigen retrieval was performed overnight using Tris-HCl buffer with a pH of 9.0 at 80 °C. Endogenous peroxidase activity was blocked using H_2O_2. Hereafter, slides were incubated with primary antibody, rat anti-human AID, for 30 min. After incubation with a HRP-labelled rabbit anti-rat IgG secondary antibody (Invitrogen), a HRP-labelled goat anti-rabbit IgG tertiary antibody (Dako) and HRP-labelled rabbit anti-goat IgG quaternary antibody (Dako). Antibodies for all manual stainings were visualized by using DAB (3,3' diaminobenzidine) and slides were counterstained with haematoxylin. Automated staining was performed according to the manufacturer's protocols. All

stained slides were digitized using a Philips UFS slide scanner (Philips, Best, The Netherlands) and assessed using Philips IntelliSite Pathology Solution software. The FS was calculated on a whole H&E-stained salivary gland section (BvdV, EH). Discrepancies were resolved during a consensus meeting.

Quantitative digital image analyses (DIA) of salivary gland sections stained for $CD3^+$ T-lymphocytes, $CD20^+$ B-lymphocytes and $CD45^+$ lymphocytic infiltrates were performed using QuPath v0.1.2.[20] For each section, the total area of parenchyma was evaluated by defining regions of interest using the Simple Tissue Detection application (threshold 215), excluding extra and intra-parenchymal areas with adipose tissue. Hereafter, atrophic and extra-parenchymal fibrotic areas were manually excluded. All algorithms were verified by an experienced head and neck pathologist (BvdV).

Histopathological analyses

To assess the area of the parenchyma that was infiltrated by $CD45^+$ lymphocytic infiltrates, the so-called ''cytokeratin annotation'' function in QuPath was used to select DAB positive areas. The threshold between CD45 positive and negative areas was set to 0.15. The area of $CD45^+$ infiltrate was calculated as a percentage of the area of parenchymal tissue (supplementary figure 1A-B).

Positive Cell Detection algorithm was used to select DAB-positive cells and an Object Classifier was used to adjust the algorithm. Hereafter the number of CD3 and CD20 DAB-positive cells was calculated per mm^2 of parenchymal tissue (supplementary figure 1C-D).

FDC-networks were identified by positive CD21 staining, and the number of FDC-networks per mm^2 parenchymal tissue was manually counted. GCs were identified by positive Bcl6 staining. GCs were defined as a cluster of ≥5 Bcl6-positive cells.[11] The number of GCs/mm^2 was manually assessed. All sections with a $CD21^+$ FDC-network were stained for AID as an alternative functional marker for GCs. GCs were defined as a cluster of ≥5 AID positive cells. As a negative control, 10 sections with foci but without a $CD21^+$ FDC-network were also stained.

In order to estimate the percentages of IgA^+ and IgG^+ plasma cells, biopsies were double stained for IgA and IgG and manually evaluated. A percentage of >30% IgG^+ plasma cells of all IgA and IgG plasma cells was considered as a threshold for an IgA/IgG shift.[13]

The grade of organisation of the lymphocytic infiltrate was assessed for all individual foci and the highest grade was noted per section. Grade of organisation was defined as follows (supplementary figure 2): Grade 1: lymphocytic foci were present, but a clear T/B-lymphocyte segregation was lacking based on the CD3 and CD20 stainings, and FDC-networks and GCs were absent. Grade 2: either T/B cell segregation within a focus and/or an FDC-network was present, but without presence of a GC. Grade 3: grade 2 features accompanied by the presence of a GC.

For the assessment of pre-LELs and LELs, high molecular weight cytokeratin (hmwCK) and CD20 stainings were performed on consecutive sections. After alignment of sections, a DIA algorithm in Visiopharm Integrator System (Hørsholm, Denmark), was used to identify pre-LELs and LELs, as previously described.[16] Presence of intraepithelial $CD20^+$ B-lymphocytes was assessed manually when hmwCK and CD20-stained sections could not be aligned. The number of (pre-) LEL/mm^2 and the maximum severity of LELs was scored as previously described[16] with the addition of pre-LELs. LEL-stages were defined as follows: Stage 0 LEL (i.e. pre-LEL): presence of intraepithelial B-lymphocytes without ductal hyperplasia. Stage 1 LEL: lymphocytic ductal infiltration and ductal hyperplasia affecting <50% of the epithelium. Stage 2 LEL: lymphocytic ductal infiltration and ductal hyperplasia affecting between 50-100% of the epithelium. Stage 3 LEL: lymphocytic ductal infiltration and fully circumferentially hyperplastic epithelium without lumen.

Sections were analysed by trained researchers (UN, MvG, SCL, EAH) under supervision of an experienced head and neck pathologist (BvdV). Disagreements were resolved during a consensus meeting.

Statistical analysis

Data were analysed using SPSS version 28 statistical software (SPSS Inc., Chicago, IL). Results were expressed as number of patients (%), mean ± SD, or median (IQR) for categorical, normally distributed, and non-normally distributed data, respectively. Differences in clinical and histopathological parameters between SjD and non-SjD sicca patients were tested with Chi-Square or Fisher's Exact test, Independent Samples t-test and Mann-Whitney U test when appropriate. Histopathological features of paired parotid and labial salivary gland biopsies were compared with McNemar's test, Wilcoxon Signed-

Rank test and by calculating the absolute agreement. The association between histopathological features in the parotid and labial glands were analysed using Spearman correlation coefficient (ρ), and interpreted as poor (0.0–0.2), fair (0.2–0.4), moderate (0.4–0.6), good (0.6–0.8) or excellent (0.8–1.0). P-values <0.05 were considered statistically significant.

RESULTS

Patients

From a diagnostic cohort, 99 out of 111 consecutive patients with sicca complaints were included in the analyses. Patients were excluded from this study due to presence of another associated auto-immune disease (n=7), hepatitis C infection (n=1), parotid MALT lymphoma (n=2), sclerosing sialadenitis (n=1) or insufficient biopsy material (n=1). Of the 99 included patients, 36 patients were categorised as SjD and 63 patients as non-SjD sicca by the expert panel. Demographic, serological and clinical characteristics of SjD patients and non-SjD sicca patients are shown in table 1. As expected, clinical and serological disease characteristics were more frequently present in SjD patients compared to non-SjD sicca patients.

Among the patients diagnosed with SjD by the experts, nearly all patients were also classified as SjD, according to the ACR-EULAR criteria. However, two of these patients did not meet the ACR-EULAR classification criteria. Both patients presented with a positive parotid gland biopsy, and one out of two also had a positive labial gland biopsy (see supplementary table 2). The rationale for SjD diagnosis by the expert for patient number 1, in addition to a positive parotid gland biopsy, was based on high disease activity reflected by an ESSDAI score of 18. For patient number 2, indications for SjD included a positive family history and the presence of Raynaud's phenomenon.

Among the non-SjD sicca patients, nine individuals did meet the ACR-EULAR classification criteria. The total ACR-EULAR points of these patients ranged from 4-6. Five out of these nine patients exhibited a FS≥1 in their labial gland biopsy along with one of the minor criteria (Schirmer's test ≤5mm/min or UWS ≤0.1 ml/min) but without a positive parotid gland biopsy or the presence of anti-SSA autoantibodies. One patient had both a positive labial

gland and parotid gland biopsy and officially tested positive for anti-SSA autoantibodies. Despite meeting the classification criteria, experts opted not to diagnose SjD as the SSA titer was only 17, and sicca complaints were attributed to comorbidities such as diabetes mellitus type 2. Three non-SjD sicca patients fulfilled the classification criteria based on anti-SSA positivity in combination with minor items but lacked a positive labial or parotid gland biopsy.

Table 1. Clinical and serological parameters of SjD patients and non-SjD sicca patients.

	SjD patients (n=36)	Non-SjD sicca patients (n=63)	p-value
Clinical parameters			
Age, years	51 ± 14	50 ± 13	0.38
Female, n (%)	35 (97.2)	54 (85.6)	0.09
Caucasian, n (%)	33 (91.7)	59 (93.7)	0.33
ACR/EULAR+	34 (94.4)	9 (14.3)	*<0.001*
ESSDAI score	4 [2-12]	1 [0-3]	*<0.001*
ESSDAI glandular domain	0 [0-2]	0 [0-0]	*0.001*
Schirmer ≤5mm, n (%)	36 (57.1)	29 (80.6)	*0.032*
OSS ≥5, n (%)	15 (44.1)	8 (12.7)	*<0.001*
UWS <0.10ml/min, n (%)	20 (55.6)	24 (38.1)	0.10
Serological parameters			
Anti-SSA positive, n (%)	29 (80.6)	6 (9.5)	*<0.001*
Anti-SSB positive, n (%)	16 (44.4)	0 (0)	*<0.001*
RF positive, n (%)	25 (69.4)	3 (4.8)	*<0.001*
IgG g/L	17.1 [12.6-20.0]	10.4 [8.7-12.3]	*<0.001*
ESR mm/hour	25.0 [15.0-46.5]	9.5 [4.0-17.0]	*<0.001*
CRP mg/L	2.5 [1.0-5.0]	1.1 [0.5-4.0]	0.06

Data are represented as mean ± SD, median [IQR] or number (%). Abbreviations: ESSDAI, European League Against Rheumatism SS Disease Activity Index; OSS, Oscular Staining Score; UWS, unstimulated whole saliva; SWS, stimulated whole saliva; RF, rheumatoid factor; ANA, antinuclear antibodies; SSA, Sjögren's syndrome antigen A; SSB, Sjögren's syndrome antigen B; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.

Comparison of histopathological features between paired labial and parotid gland biopsies

Total group of sicca patients suspected of having SjD

First, we performed a pairwise analysis of all labial and parotid biopsies from SjD patients and non-SjD sicca patients together. Absolute agreement between labial and parotid glands was high, being 80% for the FS, 89% for GCs, 84% for the IgA/IgG plasma cell shift and 93% for (pre-)LELs (see table 2). In the total study population, a FS≥1 was more often observed in labial glands compared to parotid glands (p=0.012). The presence of other histopathological key features, namely presence of GCs, IgA/IgG plasma cell shift and (pre-)LELs, did not differ significantly between the paired biopsies (table 2).

Table 2. Presence of histopathological key features in paired salivary gland sections of the total study population (non-SjD sicca and SjD patients, n=99).

		Labial salivary gland	
Parotid salivary gland		**FS≥1**	**FS<1**
	FS≥1	19 (19.2)	4 (4.0)
	FS<1	16 (16.2)	60 (60.6)
		GC	**No GC**
	GC	3 (3.0)	7 (7.1)
	No GC	4 (4.0)	85 (85.9)
		IgA/IgG shift	**No IgA/IgG shift**
	IgA/IgG shift	11 (11.1)	5 (5.1)
	No IgA/IgG shift	11 (11.1)	72 (72.7)
		(Pre-)LEL	**No (pre-)LEL**
	(Pre-)LEL	13 (13.1)	4 (4.0)
	No (Pre-)LEL	3 (3.0)	79 (79.8)

Data is reported as n(%). FS, focus score; GC, germinal centre, LEL, lymphoepithelial lesion.

The higher number of labial gland biopsies with a positive FS was accompanied by a significantly higher FS (p<0.001), relative area of $CD45^+$ infiltrate (p<0.001), number of $CD3^+$ T-lymphocytes/mm^2 (p<0.001) and number of $CD20^+$ B-lymphocytes/mm^2 (p=0.018) in labial gland biopsies compared to their paired parotid gland biopsies. Labial salivary gland biopsies also more

frequently exhibited CD21$^+$ FDC-networks (p=0.004). While Bcl6$^+$ GCs were identified as often in the two salivary gland types, the number of Bcl6$^+$ GCs/mm^2 was significantly higher in parotid gland biopsies (p=0.016) (table 3).

Table 3. Histopathological data of labial and parotid salivary gland biopsies in SjD and non-SjD sicca patients.

	SjD and non-SjD sicca patients (n=99)		
	Labial SG	**Parotid SG**	**P-value**
Surface area of salivary gland section*	11.1 (8.2-15.2)	9.6 (6.3-12.8)	*0.021*
Salivary gland section <4mm^2 (%)	1 (1)	7 (7)	0.07
Focus score*	0.5 (0.0-12.0)	0.0 (0.0-12.0)	*<0.001*
Infiltrated area (CD45$^+$cells) (%)	11.0 (7.0-19.2)	0.8 (0.3-6.2)	*<0.001*
FDC-networks/mm^2*	1.0 (0.0-0.6)	1.0 (0.0-1.2)	0.65
Presence of FDC-networks, n(%)	31 (31.3)	17 (17.2)	*0.004*
GCs/mm^2*	1.0 (0.0-0.2)	0.0 (0.0-0.5)	*0.016*
Presence of GCs, n(%)	7 (7.1)	10 (10.1)	0.72
CD3/CD20 segregation, n(%)*	27 (27.3)	22 (22.2)	0.30
IgA/IgG plasma cell shift, n(%)*	28 (28.3)	20 (20.2)	0.08
LELs/mm^2*	1.0 (0.0-0.4)	1.0 (0.0-1.0)	0.08
Presence of (pre-)LELs, n(%)	16 (16.2)	19 (19.2)	1.00
CD3$^+$cells/mm^2	332 (200-564)	144 (74-330)	*<0.001*
CD20$^+$cells/mm^2	90 (44-236)	24 (11-179)	*0.018*

Data is reported as median (IQR), *median (range) or n(%). LEL, lymphoepithelial lesion; FDC, follicular dendritic cell; GC, germinal centre.

Subgroup diagnosed as SjD

Second, we compared the paired salivary gland biopsies of patients that were diagnosed as SjD based on the decision of the expert panel. Absolute agreement between labial and parotid glands was moderate to good, being 61% for the FS, 69% for GCs, 58% for the IgA/IgG plasma cell shift and 81% for (pre-)LELs (see table 4). Higher FSs (p=0.06) and relative area of CD45$^+$ infiltrates (p<0.001) were observed in the labial compared to parotid glands, in line with the total study population (SjD and non-SjD sicca patients together) (figure 1A-B). Remarkedly, and in contrast to the total population, the number of CD20$^+$ B-lymphocytes/mm^2 (p=0.046) was lower in paired labial compared to parotid gland biopsies of SjD patients (figure 1C). Number of CD3$^+$ T-cells/

mm^2 (p=0.29) (figure 1D) and the maximum organization grade of infiltrates per section (p=0.65) were comparable between paired labial and parotid gland biopsies of SjD patients. Although the number of biopsies which harboured GCs or (pre-)LELs did not differ between the two types of glands, the number of GCs/mm^2 (p=0.016) and severity of LELs were significantly lower in labial gland biopsies (p=0.026) (Figure 1E-F). Almost all salivary gland biopsies (5/7 labial gland biopsies, 10/10 parotid gland biopsies) which exhibited GCs as detected by Bcl6, also revealed clusters of ≥5 AID^+ cells and vice versa. Of note, in 14/21 labial and 5/15 parotid salivary gland biopsies with $CD21^+$ FDC networks, no $Bcl6^+$ and/or AID^+ clusters were observed (supplementary figure 3).

Table 4. Histopathological key features in paired salivary gland sections of SjD patients (n=36).

		Labial salivary gland	
Parotid salivary gland		**FS≥1**	**FS<1**
	FS≥1	19 (52.8)	4 (11.1)
	FS<1	10 (27.8)	3 (8.3)
		GC	**No GC**
	GC	3 (8.3)	7 (19.4)
	No GC	4 (11.1)	22 (61.1)
		IgA/IgG shift	**No IgA/IgG shift**
	IgA/IgG shift	11 (30.6)	4 (11.1)
	No IgA/IgG shift	11 (30.6)	10 (27.8)
		(Pre-)LEL	**No (pre-)LEL**
	(Pre-)LEL	13 (36.1)	4 (11.1)
	No (Pre-)LEL	3 (8.3)	16 (44.4)

Data is reported as n(%). FS, focus score; GC, germinal centre, LEL, lymphoepithelial lesion.

Subgroup diagnosed as non-SjD

Third, we compared the paired biopsies non-SjD sicca patients. A significantly higher FS (p<0.001), relative area of $CD45^+$ infiltrates (p<0.001), number of $CD3^+$ T-lymphocytes (p<0.001) and $CD20^+$ B-lymphocytes (p<0.001) was seen in labial salivary gland biopsies compared to parotid gland biopsies (figure 1).

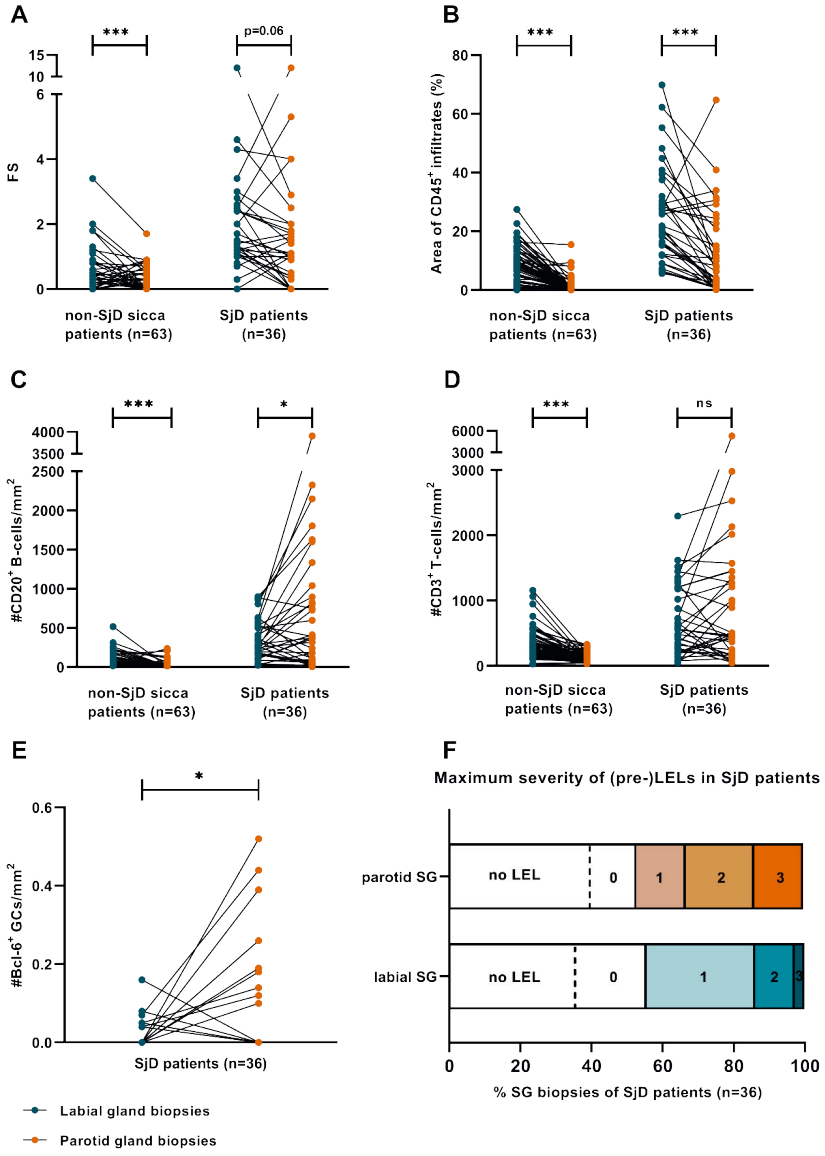


Figure 1. Histopathological comparison of labial and parotid salivary gland biopsies. Focus score (A), relative area of CD45$^+$ infiltrates (B), CD20$^+$ B-lymphocytes (C) and CD3$^+$ T-cells (D) in SjD and non-SjD sicca patients and the number of Bcl6$^+$ GCs per mm^2 (E) and the maximum severity of LELs (F) in salivary gland sections of only SjD patients. LEL stage 0= pre-LEL, lymphocytic infiltration without ductal hyperplasia, LEL stage 1= lymphocytic infiltration with ductal hyperplasia affecting <50% of the epithelium, LEL stage 2= lymphocytic infiltration with ductal hyperplasia affecting 50-100%, LEL stage 3= lymphocytic ductal infiltration and fully circumferentially hyperplastic epithelium without lumen.*p<0.05, ***p=<0.001.

The grade of organisation of infiltrates was comparable between the two paired salivary gland types in non-SjD sicca patients. GCs, IgA/IgG plasma cell shift or (pre-)LELs were virtually absent in non-SjD sicca patients, except for two parotid gland biopsies in which an IgA/IgG shift (n=1) or a pre-LEL (n=1) were observed.

Correlation analysis between paired labial and parotid gland biopsies

In order to assess to what extent the various histopathological features are present in the two types of glands, associations of the features between paired labial and parotid gland biopsies were determined. Correlation of all analysed parameters varied from fair to good in the total study population. When taking only SjD patients into account, correlations were moderate to good for most features, except for fair correlations for the number of FDC-networks/mm^2 and GCs/mm^2. Interestingly, for the SjD patients, the number of CD20$^+$ B-lymphocytes showed a good correlation between the gland types, while for the non-SjD sicca patients a poor correlation was observed (ρ=0.73 vs. ρ=0.11). This was mainly due to a discrepancy in B-lymphocyte numbers between gland types in non-SjD sicca patients (figure 2).

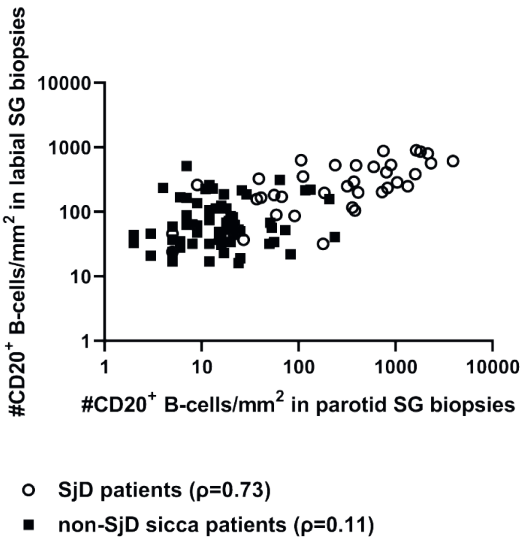


Figure 2. Association between the number of CD20$^+$ B-lymphocytes in paired labial and parotid salivary gland biopsies of non-SjD sicca patients and SjD patients.

Similar correlation coefficients were found for the total study population and SjD patients for FS and area of $CD45^+$ infiltrate (supplementary table 3).

DISCUSSION

In a diagnostic cohort of sicca patients from daily clinical practice, paired labial and parotid salivary gland histopathology appeared comparable in SjD patients. Importantly, absolute agreement of labial glands and parotid glands in terms of the SjD-related features, FS, presence of GCs, plasma cell isotype switch and LELs was high and correlation of most features between the two salivary gland types was generally moderate to good. However, important histopathological differences were also observed.

More and larger lymphocytic infiltrates were seen in labial glands compared to parotid glands, not only in SjD patients, but also in non-SjD sicca patients. This was reflected by a higher FS, amount of infiltrate as well as numbers of T- and B-lymphocytes in the labial glands. The higher number of infiltrating lymphoid cells in labial glands of non-SjD sicca patients argues that lymphocytic infiltrates are frequently present in these glands irrespective of the presence of SjD. The lymphocytic infiltrates in labial glands may develop in SjD patients on top of non-autoimmune related infiltrates, resulting in a higher amount of infiltrate in labial glands also in SjD patients. Reasons for the presence of non-autoimmune related infiltrates in labial glands most clearly seen in non-SjD sicca patients are unknown, but possibilities are gland dysfunction, worse oral health, infections or habitual lip biting.[21] Also dysbiosis in the buccal mucosa microbiome observed in non-SjD sicca patients may contribute to inflammation in the labial salivary glands.[22] Furthermore, labial salivary glands are more easily accessible to microbes in comparison to the parotid gland, primarily due to anatomical differences such as size and length of the excretory ducts. As a consequence of the presence of non-autoimmune related infiltrates in labial glands, patients are potentially misclassified as SjD patients when solely the labial gland FS is used as a histopathological diagnostic criterion. Specificity of the labial gland biopsy for SjD is increased when not only the FS is taken into account, but also other characteristic histo-

pathological features of SjD, i.e., presence of GCs, plasma cell isotype switch, and (pre-) LELs.[18]

In this study, we unequivocally showed the presence of bona fide GCs in both type of glands by staining for Bcl6 and AID. The small discrepancy seen in the number of biopsies with GCs in labial gland biopsies based on either Bcl6 or AID staining can be explained by the fact that the sections stained for these two markers were not adjacent sections.

In this study, we observed that while the number of biopsies with a GC did not significantly differ between paired salivary gland biopsies, more GCs/mm^2 were found in parotid gland biopsies. The higher number of GCs in parotid gland biopsies may indicate that there is a more active humoral immune response in these glands, compared to the labial glands. In addition, a higher absolute B-lymphocyte count was observed in parotid gland biopsies compared to paired labial gland biopsies in SjD patients.

A higher number of B-lymphocytes may have implications for other histopathological features. B-lymphocytes can invade the epithelium of the striated ducts, which probably drives the formation of LELs.[16] Haacke et al. showed that the majority of the intraepithelial B-lymphocytes in SjD patients express the inhibitory Fc-receptor like 4 (FcRL4) protein, which is also abundantly expressed by MALT lymphoma B-lymphocytes in SjD.[23] There are more FcRL4$^+$ B-lymphocytes in parotid glands, compared to labial glands, and these cells actively clonally expand within the epithelium[24,25]. We hypothesized that during this expansion derailment of intraepithelial FcRL4$^+$ B-lymphocytes may result in MALT lymphoma formation, in particular in the parotid gland environment.[25] Taken together, these findings indicate that within the parotid salivary glands there seems to be more pronounced B-lymphocyte activity which is at least partly responsible for the higher number of GCs, more severe LELs and MALT lymphoma development.

The reason for differences in B-lymphocyte numbers and activity between the two types of glands in SjD remains to be elucidated. It is possible that higher levels of certain pro-inflammatory cytokines (e.g., IFNγ, IL-27, BAFF and APRIL) result in more attraction and/or activation of B-lymphocytes in parotid glands.[25] However, transcriptomic analysis of paired parotid and labial salivary gland biopsies of SjD patients showed a high degree of overlap in immune pathway activity between the two salivary gland types.[26]

In conclusion, both labial and parotid gland biopsies have similar histopathological key features and both types of salivary glands can be used for diagnosis and classification of SjD. However, systematic analysis of paired salivary gland biopsies also revealed important differences between these two glands. Labial salivary glands seem to exhibit more non-SjD related inflammation which can obscure diagnosis and classification. In SjD, parotid salivary glands reveal more evident histopathological signs of B-lymphocyte hyperactivity. The results of this study offer novel insights into the pathophysiology of pSS and can be incorporated into guidelines for the histopathological analysis of salivary gland biopsies.

REFERENCES

1. Mariette X, Criswell LA. Primary Sjögren's Syndrome. N Engl J Med. 2018 Mar;378(10):931–9.
2. Pijpe J, Kalk WWI, van der Wal JE, Vissink A, Kluin PhM, Roodenburg JLN, et al. Parotid gland biopsy compared with labial biopsy in the diagnosis of patients with primary Sjogren's syndrome. Rheumatology. 2006 Jul 28;46(2):335–41.
3. Delli K, Dagal EF, Bootsma H, Vissink A, Spijkervet FKL. Patient-reported change of sensibility and pain after parotid and labial gland biopsy applied for primary Sjögren's syndrome diagnostics: one-year follow-up study. Clin Exp Rheumatol. 2018;36 Suppl 112(3):173–6.
4. Deroo L, Genbrugge E, Dochy F, Creytens D, Achten H, De Boeck K, et al. Ultrasound-guided core needle biopsy and incisional biopsy of the parotid gland are comparable in diagnosis of primary Sjögren's syndrome. Rheumatology. 2023 Aug 1;62(8):2765–72.
5. Shiboski CH, Shiboski SC, Seror R, Criswell LA, Labetoulle M, Lietman TM, et al. 2016 American College of Rheumatology/European League Against Rheumatism Classification Criteria for Primary Sjögren's Syndrome: A Consensus and Data-Driven Methodology Involving Three International Patient Cohorts. Arthritis & Rheumatology. 2017 Jan 26;69(1):35–45.
6. Greenspan JS, Daniels TE, Talal N, Sylvester RA. The histopathology of Sjögren's syndrome in labial salivary gland biopsies. Oral Surgery, Oral Medicine, Oral Pathology. 1974 Feb;37(2):217–29.
7. Chisholm DM, Mason DK. Labial salivary gland biopsy in Sjögren's disease. J Clin Pathol. 1968 Sep;21(5):656–60.
8. Fisher BA, Jonsson R, Daniels T, Bombardieri M, Brown RM, Morgan P, et al. Standardisation of labial salivary gland histopathology in clinical trials in primary Sjögren's syndrome. Ann Rheum Dis. 2017;76(7):1161–8.
9. Delli K, Haacke EA, Kroese FGM, Pollard RP, Ihrler S, van der Vegt B, et al. Towards personalised treatment in primary Sjögren's syndrome: baseline parotid histopathology predicts responsiveness to rituximab treatment. Ann Rheum Dis. 2016 Nov;75(11):1933–8.
10. Kroese FG, Abdulahad WH, Haacke E, Bos NA, Vissink A, Bootsma H. B-cell hyperactivity in primary Sjögren's syndrome. Expert Rev Clin Immunol. 2014 Apr 1;10(4):483–99.
11. Nakshbandi U, Haacke EA, Bootsma H, Vissink A, Spijkervet FKL, van der Vegt B, et al. Bcl6 for identification of germinal centres in salivary gland biopsies in primary Sjögren's syndrome. Oral Dis. 2020 Apr 28;26(3):707–10.
12. Cattoretti G, Büttner M, Shaknovich R, Kremmer E, Alobeid B, Niedobitek G. Nuclear and cytoplasmic AID in extrafollicular and germinal center B cells. Blood. 2006 May;107(10):3967–75.
13. Bodeutsch C, De Wilde PCM, Kater L, Van Houwelingen JC, Van Den Hoogen FHJ, Kruize AA, et al. Quantitative immunohistologic criteria are superior to the lymphocytic focus score criterion for the diagnosis of Sjögren's syndrome. Arthritis Rheum. 1992 Sep 16;35(9):1075–87.
14. Daridon C, Pers J, Devauchelle V, Martins-Carvalho C, Hutin P, Pennec Y, et al. Identification of transitional type II B cells in the salivary glands of patients with Sjögren's syndrome. Arthritis Rheum. 2006 Jul 27;54(7):2280–8.
15. Ihrler S, Zietz C, Sendelhofert A, Riederer A, Löhrs U. Lymphoepithelial duct lesions in Sjögren-type sialadenitis. Virchows Archiv. 1999 Apr 22;434(4):315–23.
16. van Ginkel MS, Haacke EA, Bootsma H, Arends S, van Nimwegen JF, Verstappen GM, et al. Presence of intraepithelial B-lymphocytes is associated with the formation of lymphoepithelial lesions in salivary glands of primary Sjögren's syndrome patients. Clin Exp Rheumatol. 2019;37 Suppl 118(3):42–8.

17. Nocturne G, Mariette X. B cells in the pathogenesis of primary Sjögren syndrome. Nat Rev Rheumatol. 2018 Mar;14(3):133–45.

18. van Ginkel MS, Nakshbandi U, Arends S, Haacke EA, Liefers SC, Verstappen GM, et al. Increased Diagnostic Accuracy of the Labial Gland Biopsy in Primary Sjögren Syndrome When Multiple Histopathological Features Are Included. Arthritis & Rheumatology. 2024 Mar 9;76(3):421–8.

19. Delli K, Vissink A, Spijkervet FKL. Salivary gland biopsy for Sjögren's syndrome. Oral Maxillofac Surg Clin North Am. 2014 Feb;26(1):23–33.

20. Bankhead P, Loughrey MB, Fernández JA, Dombrowski Y, McArt DG, Dunne PD, et al. QuPath: Open source software for digital pathology image analysis. Sci Rep. 2017 Dec;7(1):16878.

21. Costa S, Quintin-Roué I, Lesourd A, Jousse-Joulin S, Berthelot JM, Hachulla E, et al. Reliability of histopathological salivary gland biopsy assessment in sjögren's syndrome: A multicentre cohort study. Rheumatology (United Kingdom). 2015;54(6):1056–64.

22. van der Meulen TA, Harmsen HJM, Bootsma H, Liefers SC, Vich Vila A, Zhernakova A, et al. Dysbiosis of the buccal mucosa microbiome in primary Sjögren's syndrome patients. Rheumatology (Oxford). 2018 Dec;57(12):2225–34.

23. Haacke EA, Bootsma H, Spijkervet FKL, Visser A, Vissink A, Kluin PM, et al. FcRL4(+) B-cells in salivary glands of primary Sjögren's syndrome patients. J Autoimmun. 2017 Jul;81:90–8.

24. Visser A, Verstappen GM, van der Vegt B, Vissink A, Bende RJ, Bootsma H, et al. Repertoire Analysis of B-Cells Located in Striated Ducts of Salivary Glands of Patients With Sjögren's Syndrome. Front Immunol. 2020;11:1486.

25. Verstappen GM, Pringle S, Bootsma H, Kroese FGM. Epithelial-immune cell interplay in primary Sjögren syndrome salivary gland pathogenesis. Nat Rev Rheumatol. 2021 Jun;17(6):333–48.

26. Verstappen GM, Gao L, Pringle S, Haacke EA, van der Vegt B, Liefers SC, et al. The Transcriptome of Paired Major and Minor Salivary Gland Tissue in Patients With Primary Sjögren's Syndrome. Front Immunol. 2021 Jul 6;12.

SUPPLEMENTARY MATERIAL

Supplementary table 1. Primary antibodies and staining method used for immunohistochemistry.

Antigen	Clone	Host	Source	Staining method
AID-2	mAID-2	Rat	Thermo Fisher Scientific	Manually
CD3	2GV6	Rabbit	Ventana Roche	Manually
CD20	L-26	Mouse	Ventana Roche	Manually
CD21	2G9	Mouse	Cell Marque Corporation	Automated
CD45	2B11+PD7/26	Mouse	Ventana Roche	Automated
Bcl6	GI19E/A8	Mouse	Ventana Roche	Automated
IgA/IgG	Polyclonal	Rabbit	Ventana Roche	Automated
hmwCK	34βE12	Mouse	Ventana Roche	Manually

Supplementary table 2. Detailed characteristics of sicca patients with discrepancy between expert opinion and ACR-EULAR classification criteria.

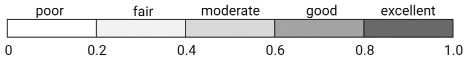
Patient number	Expert opinion	Fulfilment ACR-EULAR classification criteria	Total ACR-EULAR points	FS Labial SG	FS Parotid SG	Anti-SSA/Ro titer	Anti-SSB/La titer	IgG g/L	RF titer IU/ml	OSS	Schirmer's test mm/min (lowest)	UWS ml/min	SWS ml/min	Total ESSDAI score	Total ESSPRI score
1	SjD	No	3	0,7	**1,5**	0	0	8.6	1.2	0	6	0.4	1.1	18	8
2	SjD	No	3	**1,4**	**1,7**	0	0	10.7	0	0	29	0.2	0.9	2	4
3	Non-SjD Sicca	Yes	4	0	0	**164**	0	9.9	1.3	**8**	18	0.2	1.2	5	7
4	Non-SjD Sicca	Yes	4	**1,2**	0,9	0	0	14.4	1.3	1	**2**	0.2	0.6	3	8.3
5	Non-SjD Sicca	Yes	4	0,4	0,6	**240**	0	12.3	0	2	**5**	0.2	0.2	7	6
6	Non-SjD Sicca	Yes	4	**1,3**	0	0	0	9.6	2.1	1	**3**	0.3	1.0	1	7
7	Non-SjD Sicca	Yes	4	**1,8**	0	0	0	18.2	6	0	6	**0.0**	0.0	5	7
8	Non-SjD Sicca	Yes	4	**1,8**	0,5	0	0	11.5	0	3	**4**	0.4	0.5	0	8
9	Non-SjD Sicca	Yes	5	**2,0**	0,6	0	0	10.6	0.9	**7**	**5**	0.3	0.9	0	7
10	Non-SjD Sicca	Yes	5	0	0	**233**	0	11.5	1.4	0	**4**	**0.01**	0.3	2	9
11	Non-SjD Sicca	Yes	6	**3,4**	**1,7**	**17**	0	8.4	0.6	0	35	0.3	1.7	6	9

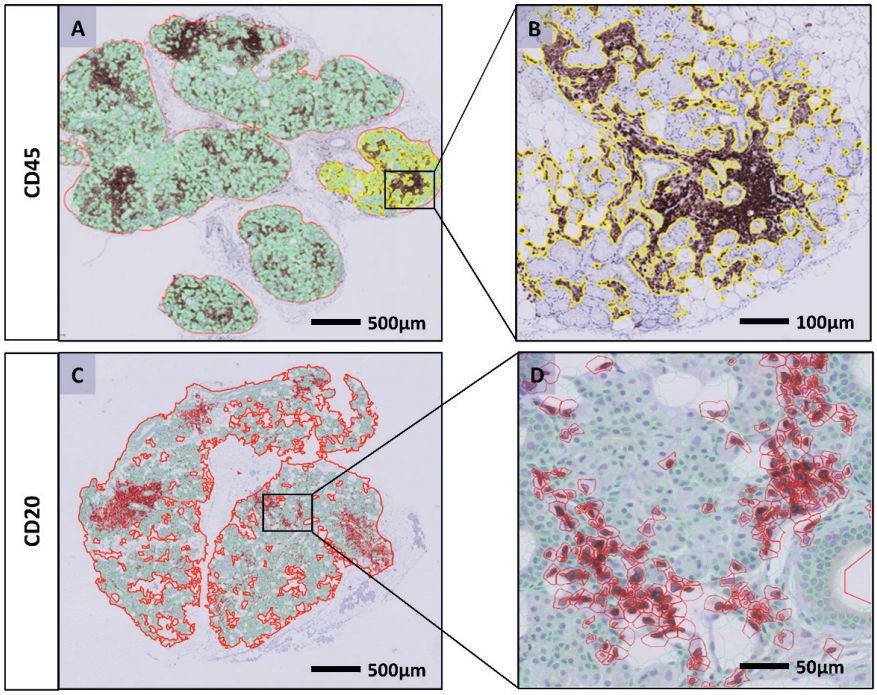
FS, focus score; SSA, Sjögren's syndrome antigen A; OSS, Oscular staining score; UWS, unstimulated whole saliva; SWS, stimulated whole saliva L, Labial salivary gland. Scores that are positive according to the ACR-EULAR criteria are shown in bold.

Supplementary table 3. Correlation coefficients of histopathological features in labial and parotid gland biopsies of the total study population and stratified for SjD patients and non-SjD sicca patients.

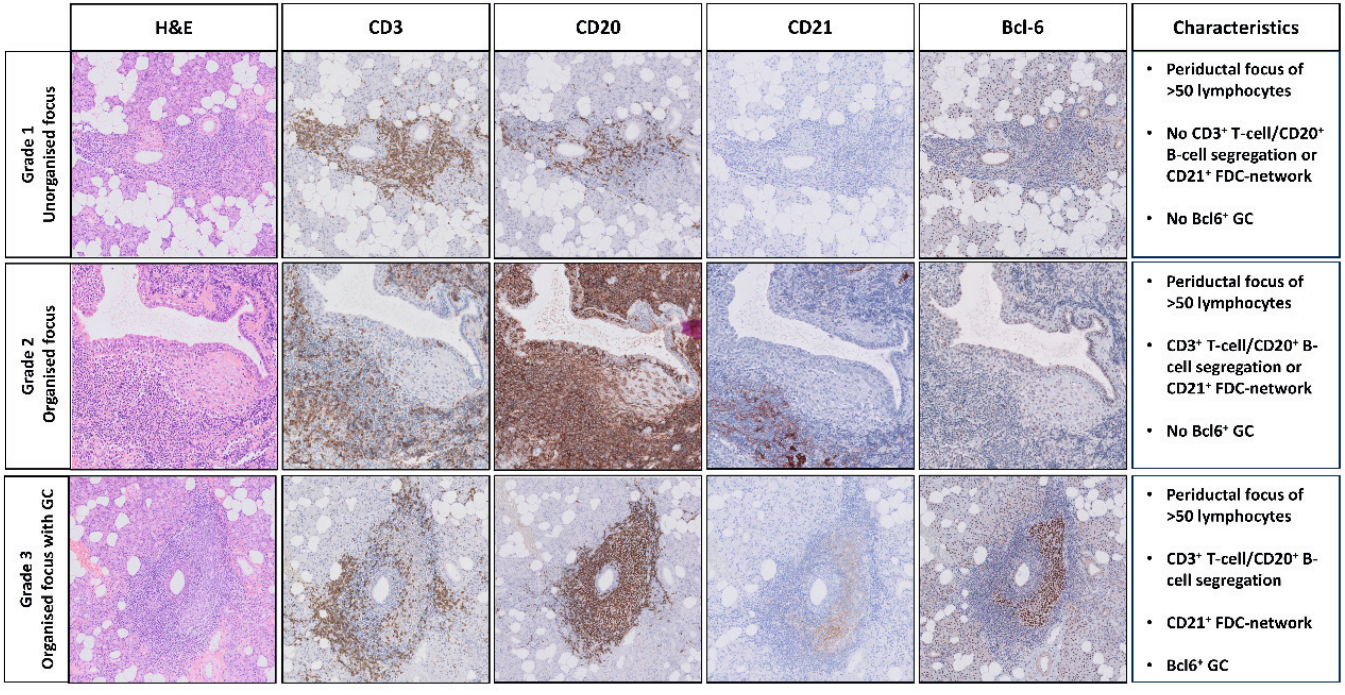
	Total study population (n=99)	SjD patients (n=36)	Non-SjD sicca patients (n=63)
FS	0.60*	0.62*	0.31*
%CD45	0.64*	0.65*	0.37*
LELs/mm²	0.73*	0.56*	n/a
CD3⁺ T-cell/mm²	0.34*	0.45*	0.24
CD20⁺ B-lymphocytes/mm²	0.52*	0.73*	0.11
Bcl6⁺ GCs/mm²	0.39*	0.27	n/a
CD21⁺ FDCs/mm²	0.47*	0.25	0.44*

*P<0.05

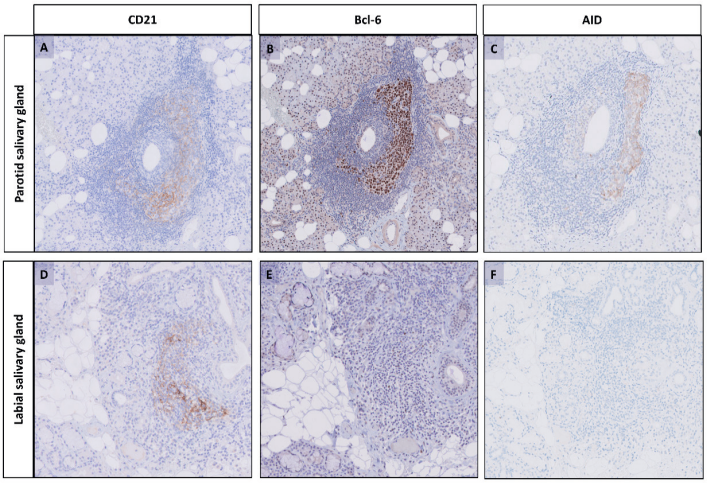




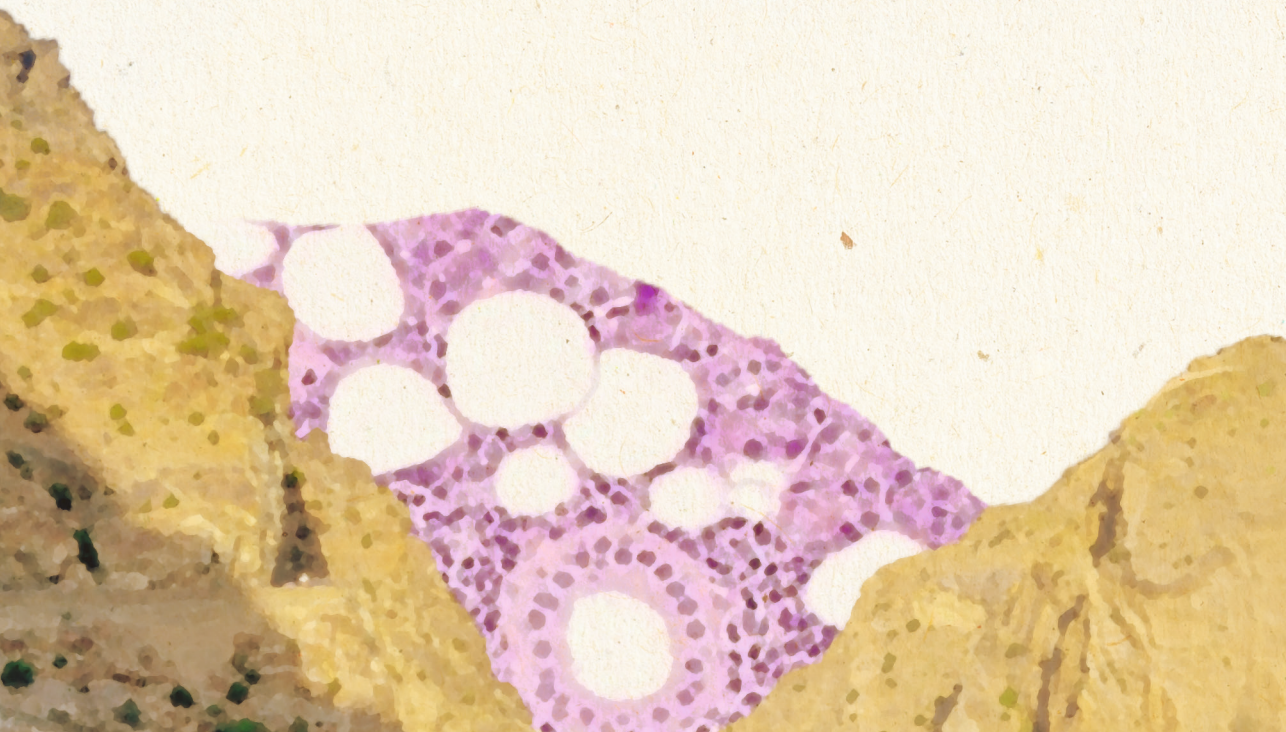
Supplementary figure 1. Quantitative digital image analyses (DIA) of salivary gland sections stained for CD45⁺ lymphocytic infiltrates and CD20⁺ B-lymphocytes using QuPath v0.1.2. For each section, the total area of parenchyma was evaluated by defining regions of interest using the Simple Tissue Detection application, excluding extra- and intra-parenchymal areas with adipose tissue. Hereafter, extra-parenchymal fibrotic and atrophic areas were manually excluded resulting in red encircled areas. Relative area of infiltrates within a labial salivary gland section was assessed by staining for CD45. The total area of 'positive staining' (yellow encircled areas) was calculated and expressed as a percentage per mm^2 glandular tissue (A-B). Absolute and relative B-cell counts within a parotid salivary gland section were analysed by staining for CD20. DAB⁺ cells (red encircled in D) were selected, quantified and expressed as number per mm^2. The method illustrated in C-D was used for sections stained for CD3 and CD20.



Supplementary figure 2. Grading of lymphoid organisation in salivary glands of SjD patients. Grade 1: lymphocytic foci were present, but without expressing a clear T/B-cell segregation in the CD3 and CD20 stainings, without presence of a FDC-network ($CD21^+$) and without presence of a GC ($Bcl6^+$). Grade 2: either a T/B cell segregation within a focus and/or an FDC-network was present, but without presence of a GC. Grade 3: grade 2 features accompanied by the presence of a GC. Sections derived from parotid salivary gland biopsy.



Supplementary figure 3. Salivary gland biopsies of two SjD patients stained for CD21, Bcl-6 and AID. In the first row (A-C) a parotid gland biopsy with a periductal focus with presence of $CD21^+$ FDC-network with $Bcl6^+$ GC and expression of AID. Whereas in the second row (D-F) a labial gland biopsy with a $CD21^+$ FDC-network is present without Bcl6 or AID express.



CHAPTER 4

More severe parotid gland histopathology in pediatric-onset Sjögren's disease compared to adult-onset disease

G.E. Legger[1], U. Nakshbandi[2], M.S. van Ginkel[2], S.C. Liefers[2], L. de Wolff[2], A.J. Stel[2], W. Armbrust[1], F.K.L.Spijkervet[3], A. Vissink[3], S. Arends[2], H. Bootsma[2], B. van der Vegt[4], G.M. Verstappen[2], F.G.M. Kroese[2]

Departments of [1]Paediatric Rheumatology, [2]Rheumatology and Clinical Immunology, [3]Oral and Maxillofacial Surgery and [4]Pathology and Medical Biology, University of Groningen and University Medical Center Groningen, Groningen, the Netherlands

RMD Open 2024;10(3):e004201.

ABSTRACT

Objective

The aim of this study was to assess histopathological features of the parotid glands in paediatric-onset SjD (pedSjD) in comparison to adult-onset SjD (adSjD) patients.

Methods

This study was performed in Groningen, the Netherlands. PedSjD patients from a diagnostic paediatric cohort (n=19), adSjD patients from a diagnostic adult cohort (n=32) and adSjD patients who participated in a clinical trial (n=42) with a baseline parotid gland biopsy were included. Parotid gland biopsies were analysed after (immuno)histological staining for SjD-related histopathological markers and compared between groups.

Results

All characteristic histopathological features of adSjD were also observed in pedSjD. There were no significant differences in lymphoepithelial lesions or immunoglobulin A (IgA)/IgG plasma cell shift between the pedSjD and the adSjD cohorts. However, compared with the diagnostic adSjD cohort (with comparable total EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) scores), pedSjD showed more severe lymphocytic infiltration as reflected by a higher focus score (p=0.003), a higher relative surface area of $CD45^+$ infiltrate (p=0.041), higher numbers of B and T lymphocytes/mm^2 (p=0.004 and p=0.029, respectively), a higher B/T lymphocyte ratio (p=0.013), higher numbers of $CD21^+$ follicular dendritic cell networks/mm^2 (p=0.029) and germinal centres (GC)/mm^2 (p=0.002). Compared with the trial adSjD cohort, with significant higher total ESSDAI scores (p=0.001), only the B/T lymphocyte ratio and numbers of GC/mm^2 were significantly higher in the pedSjD cohort (p=0.023 and p=0.018, respectively).

Conclusion

Patients with pedSjD exhibit more pronounced histopathological features compared with patients with adSjD at diagnosis. Notably, the histopathology

of patients with pedSjD aligns more closely with that observed in an adSjD clinical trial cohort, with even stronger B lymphocyte involvement

INTRODUCTION

Sjögren's disease (SjD) is a chronic, systemic autoimmune disease characterised by dysfunction of exocrine glands. In adults, mainly the lacrimal and salivary glands are involved, resulting in typical ocular and oral sicca symptoms.[1] Predominantly, females are affected with a female-to-male ratio of 10:1. The disease is typically diagnosed in the fourth or fifth decade of life.[2–4] While it is relatively uncommon, SjD can also emerge in children.[5] The prevalence and prognosis of paediatric-onset SjD (pedSjD) still remain unclear. According to a recent study, 1.3% of adult patients with SjD have a paediatric-onset disease.[5] The presenting symptoms in pedSjD differ from those in adults with SjD. Paediatric patients present more often with non-specific extraglandular manifestations like fever and arthralgias.[6] Furthermore, paediatric patients present less often with sicca complaints and more often with symptoms of major salivary gland involvement, reflected by recurrent salivary gland swelling (in particular of the parotid glands), and higher salivary gland ultrasound (SGUS) scores compared with adults.[5,7] For adults, the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria were developed in 2016.[8] Due to a lack of childspecific criteria, the ACR/EULAR classification criteria are also used for classifying pedSjD.

In the current ACR/EULAR classification criteria for SjD, the presence of periductal lymphocytic infiltrates (foci) (focus score (FS) ≥1) is a leading histopathological parameter for classification.[8] These infiltrates are dominated by T and B lymphocytes and also harbour a variety of non-lymphoid cells, including myeloid and plasmacytoid dendritic cells, macrophages and follicular dendritic cells (FDCs). Initially, these infiltrates seem to be unorganised, but they can develop towards ectopic lymphoid tissue with T and B lymphocyte compartmentalisation, presence of $CD21^+$ FDC networks and high endothelial venules[9–11]. In the B lymphocyte areas of ectopic lymphoid tissue with $CD21^+$ FDC networks, germinal centres (GCs) can develop. Identification of GCs can be challenging in H&E stained sections.[9,12] GCs characteristically express the

transcription factor Bcl6 and detection of this protein by immunohistochemistry can assist in detecting (ectopic) GCs unequivocally.[13] Another specific histopathological feature of SjD is infiltration of B lymphocytes into the ductal epithelium, resulting in the development of lymphoepithelial lesion (LELs).[14] These LELs are composed of hyperplastic epithelial cells with high numbers of intraepithelial lymphocytes. In addition to GCs and LELs, another typical histopathological feature of SjD is an increase in the presence of immunoglobulin G (IgG) plasma cells, resulting is the so-called IgA/IgG plasma cell shift.[15] Presence of these features assists in the diagnosis of SjD.[16]

Since patients with pedSjD present more often with major salivary gland involvement, in particular swelling of the parotid glands, compared with patients with adult-onset SjD (adSjD), we hypothesised that histopathological findings of the parotid glands differ between patients with pedSjD and adSjD. To our knowledge, detailed quantitative histopathological studies of (parotid) salivary glands in pedSjD are lacking. Therefore, the aim of this study was to assess and quantify the histopathological features of the parotid glands in patients with pedSjD in comparison to different cohorts of patients with adSjD using both H&E staining and immunohistochemical stainings. Obtaining more insight into the histopathological changes in the salivary glands of patients with pedSjD may increase our knowledge about the underlying pathophysiological mechanism in children and this might lead to a better recognition and treatment of pedSjD.

MATERIALS AND METHODS

Patients and inclusion

This study was performed at the University Medical Centre Groningen (UMCG), a tertiary referral hospital and an expertise centre for SjD in the Netherland. For the paediatric cohort, baseline data was collected from consecutive patients who participated in the Registry of Paediatric Sjögren's Disease LongiTudinal (REpSULT) study.[7] The REpSULT cohort study started in 2020 and includes patients who are referred to the UMCG with a suspicion of having SjD, when the age of symptom onset is ≤16 years. Patients who visited the UMCG before 2020 were entered retrospectively into the database. Patients with a

confirmed diagnosis according to the (paediatric) rheumatologist and who underwent a diagnostic parotid gland biopsy at the UMCG between January 2009 and December 2021 were included in this study.

For adult patients with SjD (age of symptom onset >16 years), data from two previously published adult cohorts were used. The first cohort was a prospective diagnostic adSjD cohort, with comparable inclusion criteria to the paediatric cohort. This cohort consists of consecutive patients, who were referred to the UMCG with suspected SjD between December 2013 and August 2016 and underwent a multidisciplinary evaluation including a parotid gland biopsy.[17] From this cohort, patients who retrospectively fulfilled the ACR/EULAR classification criteria for SjD were included in the current study. This cohort is further referred to as 'diagnostic adSjD cohort'.

The second adult cohort consists of patients who participated in a prospective clinical trial: an open-label, proof-of-concept, phase II study with abatacept (ASAP-II) or a randomised, double-blind, placebo-controlled, phase III trial with abatacept (ASAP-III).[18–20] In general, these patients had active disease according to a moderate to high score on the EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI).[21] From these two clinical trials, patients who underwent a parotid gland biopsy within 1 year before start of the treatment were included in one cohort in the current study. This cohort is further referred to as 'trial adSjD cohort'. From all the cohorts, patients with insufficient parotid biopsy material (≤4 mm^2) and mucosa-associated lymphoid tissue (MALT) lymphoma were excluded

Data collection

Clinical characteristics collected from medical records included gender, age at symptom onset, age at the time of the salivary gland biopsy, and medication use in the 6 months before (history) and at the moment of the biopsy (current). Systemic disease activity was measured using the ESSDAI at the time of the biopsy (or within 1 year from the biopsy for the clinical trial cohort). B-mode salivary gland ultrasound (SGUS) was performed at the time of the biopsy using the MyLabTwice scanner (Esaote). All US images were scored real-time by trained readers. The scoring system by Hocevar was applied (range 0–48).[22]

The international Outcome Measures in Rheumatology (OMERACT) working party have proposed a novel semi-quantitative and simpler scoring

system that evaluates homogeneity and presence of hypoechoic areas in the parotid and submandibular glands (range 0-3). To evaluate the OMERACT scores, we have converted the Hocevar scores to OMERACT scores using a formula published by Rebel et al. [23] Salivary gland function tests included unstimulated whole saliva (UWS) and stimulated saliva (SWS) (by paraffin-chewing) flow rates.[24] In 60% of all the patients, the sum of gland specific (parotid, submandibular and sublingual glands) saliva production (by applying 2% citric acid solution) was used for SWS flow rate. An UWS flow rate of <0.1 ml/min was considered abnormal.

Salivary gland tissue was collected by a biopsy of the parotid gland.[25] Formalin-fixed (4%), paraffin-embedded tissue samples were serially sectioned at 3 µm thickness and sections were subsequently deparaffinised. Tissue sections were stained with H&E to determine the FS (the number of periductal foci (clusters of ≥ 50 lymphocytes) per 4 mm^2) and the presence and stage of LEL (stage 1-3). H&E slides were scored by two trained observers (UN and SL) together with a senior head and neck pathologist (BvdV). A consensus meeting was performed in case of discrepancies between both observers.[26–29] Tissue sections were stained immunohistochemically for CD45 (clone 2B11+PD7/26), for CD21 (clone 2G9) to detect FDC networks, for Bcl-6 (clone GI19E/A8) to detect GCs, for CD3 (clone 2GV6) to detect T-lymphocytes and for CD20 (clone L-26) to detect B-lymphocytes. All stained slides were digitised using a Philips UFS slide scanner (Philips, Best, The Netherlands) and assessed using Philips IntelliSite Pathology Solution software. Quantitative digital image analyses (DIA) of salivary gland sections stained for $CD3^+$ T-lymphocytes, $CD20^+$ B-lymphocytes and $CD45^+$ lymphocytic infiltrates were performed using QuPath v0.1.2.[30] Identification of FDC-networks was done by manually counting the number of CD21-positive stained areas. GCs were identified after staining for Bcl6. GCs were defined as a cluster of ≥5 Bcl6-positive cells. In order to estimate the IgA/IgG plasma cell shift, biopsies were dually stained for IgA and IgG and manually evaluated. A percentage of >30% IgG^+ plasma cells of all IgA and IgG plasma cells in the total parenchyma was considered as a threshold for an IgA/IgG shift. Details on the assessment of FS, LELs, GCs and plasma cell shift have been previously described.[16] An example of H&E and immunological staining of parotid gland sections of a representative pedSjD patient is shown in figure 1A-H.

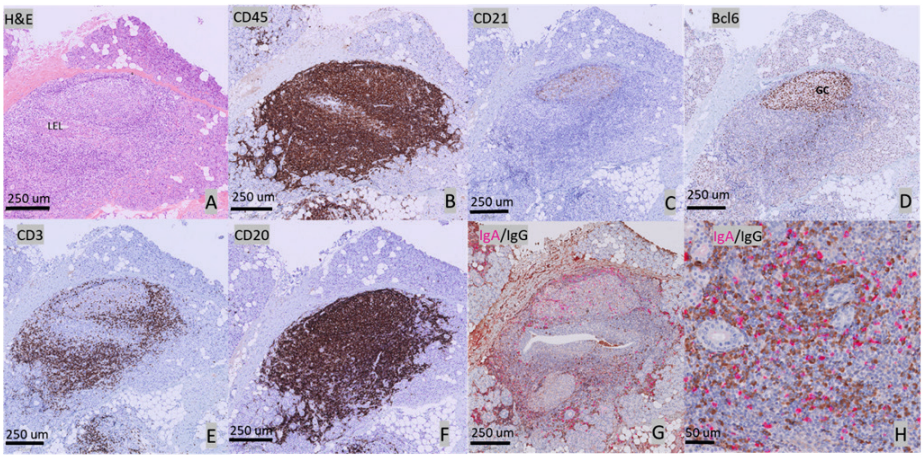


Figure 1. Histopathological features of a parotid gland of a 14-year-old patient with Sjögren's disease (SjD). (A) H&E staining. Presence of a periductal infiltrate of ≥50 lymphocytes (focus) surrounding a striated duct with ductal hyperplasia (lymphoepithelial lesion, LEL). (B) CD45 staining showing lymphocytic infiltration. (C) CD21 staining showing follicular dendritic cell (FDC) networks. (D) Presence of a germinal centre, defined as a cluster of ≥5 adjacent Bcl6+ cells. (E) CD3 staining showing T-cell infiltration. (F) CD20 staining showing B-cell infiltration. (G) Presence of a plasma cell shift, as shown by a relative increase in the number of immunoglobulin G (IgG) (brown) plasma cells compared with the IgA (pink) plasma cells. (H) High magnification image of the IgA/IgG staining.

Laboratory results closest to the date of the biopsy were extracted from the medical records.

Serological parameters collected were antinuclear antibodies (ANA), anti-Sjögren's syndrome-related antigen A (anti-SSA)/anti-SSB antibodies, rheumatoid factor (RF), IgM level and total IgG level. Other laboratory test results included C reactive protein (CRP) level and erythrocyte sedimentation rate (ESR). IgG levels above the normal range for the age, RF-IgM >5 IU/ml, CRP >5 mg/L, ESR >15 mm/h, C3 <0.9 g/L and C4 <0.1 g/L were considered as abnormal.

Statistical analysis

Statistical analyses were performed using IBM Statistical Packages for Social Sciences (SPSS) V.28. Results were expressed as number of patients (%) for categorical parameters and mean (SD) or median (IQR) for normally or

non-normally distributed continuous parameters, respectively. To compare histopathological parameters, symptoms and characteristics between the pedSjD cohort and the diagnostic adSjD cohort and between the pedSjD cohort and the trial adSjD cohort, Fisher's exact or $\chi 2$ tests, or Mann-Whitney U tests were used when appropriate. P-values <0.05 were considered statistically significant. Spearman's correlation coefficient was used to explore the association between histopathological and laboratory parameters in the diagnostic cohorts. A correlation coefficient of <0.2 was interpreted as poor, 0.2-0.4 as fair, 0.4-0.6 as moderate, 0.6-0.8 as good, and >0.8 as excellent.[31]

RESULTS

Patient inclusion

From the REpSULT cohort, 27 out of 28 patients had a confirmed diagnosis of pedSjD. From these 27 patients, eight patients were excluded. Reasons for exclusion were: no parotid gland biopsy (n=4), parotid MALT lymphoma (n=2) and insufficient biopsy material (n=2). Of the 19 pediatric patients included in the current study, 16 (84%) fulfilled the 2016 ACR/EULAR classification criteria and 11 (58%) fulfilled the American-European Consensus Group (AECG) classification criteria.[32] From the previously described diagnostic adSjD cohort[33], 32 out of 37 patients who fulfilled the 2016 ACR/EULAR criteria were included in the diagnostic adSjD cohort in the current study. Reasons for exclusion were insufficient biopsy material (n=2) or MALT lymphoma (n=3).

From the clinical trial adSjD cohorts, 46 out of 95 patients underwent a parotid gland biopsy within one year before the baseline visit. One patient was excluded from the trial adSjD cohort and included in the pedSjD cohort, because this patient had SjD symptoms before the age of 16 years. Three patients were excluded, because of insufficient biopsy material. Thus, in total 42 patients were included in the trial adSjD cohort.

Of the included adult patients, eight patients were part of both the diagnostic adSjD and the trial adSjD cohort.

Differences in clinical characteristics between pedSjD and adSjD

The general patient characteristics of the three cohorts are shown in table 1. Systemic disease activity was comparable between the pedSjD cohort and the diagnostic adSjD cohort (median ESSDAI 5 vs 4, p=0.54). As expected, patients in the trial adSjD cohort had significantly higher systemic disease activity compared with the pedSjD cohort (median ESSDAI 11 vs 5, p=0.001). The score on the glandular domain of the ESSDAI was significantly higher in the pedSjD cohort compared with both the diagnostic adSjD cohort and the trial adSjD cohort (p=0.001 and p=0.001, respectively). The UWS flow rate was significantly higher in the pedSjD cohort compared with both adult cohorts (diagnostic adSjD, p=0.041 and trial adSjD, p=0.046). There were no patients who were treated with biological disease-modifying antirheumatic drugs (eg, rituximab, abatacept) or cyclophosphamide within 6 months prior to the biopsy. The laboratory results for all cohorts are shown in table 2. The pedSjD cohort comprised a relatively lower percentage of anti-SSA^{+} patients (diagnostic adSjD, p=0.004 and trial adSjD, p=0.12) and a lower percentage of patients with an ESR >15 mm/hour compared with the adSjD cohorts (diagnostic adSjD, p=0.010 and trial adSjD, p=0.011).

Table 1. General characteristics.

	pedSjD (n=19)	diagnostic adSjD (n=32)	pedSjD vs. diagnostic adSjD p-value	trial adSjD (n=42)	pedSjD vs. trial adSjD p-value
Gender, female, n (%)	15 (78.9)	31 (96.9)	0.58	38(90.5)	0.24
Age at start symptoms, years*	9 (9-12)	45 (30-58)	*0.001*	44 (30-56)	*0.001*
Age at biopsy, years*	14 (10-17)	52 (46-63)	*0.001*	51 (39-61)	*0.001*
Duration of symptoms at moment of biopsy, years*	3 (2-5)	5 (3-13)	*0.024*	4 (2-8)	0.07
Fulfilment of ACR/EULAR criteria, n(%)	16 (84)	32(100)	*0.022*	40(95)	0.15
ESSDAI total score, range*	5 (3-11)	4 (2-12)	0.54	11(8-16)	*0.001*
ESSDAI score glandular, range domain*	2 (2-4)	0 (0-2)	*0.001*	1 (1-2)	*0.001*
ESSDAI glandular domain pos, n(%)	18 (94.7)	12 (37.5)	*<0.001*	33 (78.6)	0.117
Ultrasound					
Hocevar score total, range*	23 (13-29)$	16 (9-24)$	0.28	16 (11-29)*	0.33
Hocevar score parotis, range*	10 (8-16)$	8 (4-12)$	0.24	8 (4-14)*	0.38
Hocevar score percentage parotis/total score*	52 (48-55)$	52 (36-57)$	0.82	50 (38-58)*	0.78
Omeract score total*	8 (5-10)$	6(1.5-8)$	0.74	6 (3-11)*	0.35
Salivary flow rates					
UWS value, ml/min*	0.18 (0.05-0.55)^	0.09 (0.03-0.19)	*0.041*	0.10 (0.02-0.18)	*0.046*
UWS <0.1 ml/min, n (%)	7 (38.9)^	19 (59.4)	0.16	23 (54.8)	0.26
SWS value, ml/min*	0.4 (0.22-0.59)^	0.65 (0.34-0.94)	0.98	0.26 (0.11-0.45)	*0.033*

Table 1. General characteristics. *(Continued)*

	pedSjD (n=19)		diagnostic adSjD (n=32)		pedSjD vs. diagnostic adSjD p-value	trial adSjD (n=42)		pedSjD vs. trial adSjD p-value
Medication use, n (%)	**Current**	**History**	**Current**	**History**		**Current**	**History**	
Hydroxychloroquine	5 (26)	7 (37)	3 (9)	12 (38)		1 (2)	11 (26)	
tDMARD	1 (5)	0 (0)	3 (9)	8 (25)		0 (0)	7 (17)	
Corticosteroids	1 (5)	2 (11)	3 (9)	1 (3)		0 (0)	6 (14)	

Data is reported as median (IQR)* or n(%). Abbreviations: IQR: interquartile range, ACR-EULAR: American College of Rheumatology/ European League Against Rheumatism, ESSDAI: European alliance of associations for rheumatology Sjogren's syndrome disease activity score, UWSFR: unstimulated whole saliva flow rate, SWSFR: stimulated whole saliva flow rate, NSAID: non-steroid anti-inflammatory drug, tDMARD traditional disease modifying anti-rheumatic drug, Significant p-values are italicized. Missing data: ^0-5, $6-10, *11-15, #16-20

Table 2. Laboratory results.

	pedSjD (n=19)	diagnostic adSjD (n=32)	pedSjD vs. diagnostic adSjD p-value	trial adSjD (n=42)	pedSjD vs.trial adSjD p-value
IgG value, median (IQR) (g/L)	16.1 (13.7-20.0)^	16.6 (12.1-19.7)	0.82	17.9 (13.2-23.5)	0.39
IgG elevated*, n (%)	11 (64.7)^	17 (53.1)	0.44	27 (64.3)	0.98
RF IgM value, median (IQR) (IU/ml)	18 (1.7-73)	23 (4-65)^	0.67	23 (11.8-58.3)	0.52
RF+, (>5 IU/ml), n (%)	12 (63.2)	23 (74.2)^	0.41	35 (83.3)	0.08
Anti-SSA+, n (%)	13 (68.4)	31 (96.9)	*0.004*	36 (85.7)	0.12
Anti-SSB+, n (%)	9 (47.4)	15 (46.9)	0.97	22 (52.4)	0.72
CRP>5, n (%) (mg/L)	2 (10.5)	9 (28.1)	0.18	7 (16.7)	0.71
ESR>15, n (%) (mm/h)	6 (31.6)	22 (68.8)	*0.010*	28 (66.7)	*0.011*
C3<0.9, n (%) (g/L)	0 (0)	2 (6.5)^	0.52	6 (14.3)	0.16
C4<0.1, n (%) (g/L)	1 (5.3)^	0 (0)^	0.38	2 (4.8)	1.00
ANA+, n (%)	16 (88.9)	29 (90.6)	1.00	29 (85.3)	1.00

Abbreviations: IgG: Immunoglobulin G, RF: rheumatoid factor, SSA: Sjögren's syndrome related antigen A, SSB: Sjögren's syndrome related antigen, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, C3: complement 3, C4: complement 4, ANA: anti-nuclear antibodies, IQR: interquartile range *IgG value elevated for age-related normal range. Significant p-values are italicized and bold. Missing data: ^0-5

More severe histopathology in parotid glands of pedSjD patients compared to a diagnostic adSjD cohort

First, we compared the diagnostic pedSjD cohort to the diagnostic adSjD cohort to study the differences in histopathology between pedSjD and adSjD at the time of diagnosis. The results of the histopathological analysis of the biopsies are shown in table 3. Patients with pedSjD showed more lymphocytic infiltration/4 mm^2 reflected by a higher FS (2.6 vs 1.1, p=0.003) and a higher relative surface area of $CD45^+$ lymphocytic infiltrate compared with the diagnostic adSjD cohort (p=0.041). Both T and B lymphocyte subpopulations contributed to the higher level of infiltrate seen in the glandular tissue of patients with pedSjD. The number of B lymphocytes was, however, relatively higher than the number of T lymphocytes as mirrored by the increased B/T lymphocyte ratio in biopsies from patients with pedSjD (p=0.013). When looking at the presence of ectopic lymphoid tissue and the formation of GCs within these structures, we found that both the median numbers of $CD21^+$ FDC networks and $Bcl6^+$ GCs per mm^2 were significantly higher in the pedSjD cohort (p=0.029 and p=0.002 respectively).

Since patients in our pedSjD cohort present more often with major salivary gland swelling compared with the diagnostic adSjD cohort, supported by a significant difference in the ESSDAI glandular domain score (table 1), we performed a subanalysis comparing patients with pedSjD to patients with adSjD who scored positive on the ESSDAI glandular domain. Although only 12 patients (38%) of the diagnostic adSjD cohort had a positive score and could be included in this subanalysis, we still found a significant difference in the FS, amount of $Bcl6^+$ ectopic GCs/mm^2 , $CD20^+$ cells/mm^2 and B/T cell ratio between the pedSjD cohort and the diagnostic adSjD cohort (online supplemental table 1). These results suggest that more frequent parotid gland swelling cannot fully explain the histopathological differences between patients with pedSjD and adSjD.

To exclude potential bias in our results due to a lower frequency of anti-SSA positivity in the pedSjD cohort compared with the adSjD cohorts, we have also compared the biopsy results between anti-SSA+ patients with pedSjD and anti-SSA^+ patients with adSjD (supplementary table 2).

Table 3. Parotid gland biopsy results.

	pedSjD n=19	diagnostic adSjD n=32	pedSjD vs diagnostic adSjD p-value	trial adSjD n=42	pedSjD vs. trial adSjD p-value
Focus score*	2.6 (1.3-3.4)	1.1 (0.3-1.8)^	*0.003*	1.82 (1.2-4.67)	0.79
Focus score ≥1 n(%)	16 (84.2)	21 (65.6)	0.20	36 (85.7)	1.00
CD45+ cells* (% of parenchyma)	20.4 (9.3-35)	10.35 (2.26-24.01)	*0.041*	16.3 (4.9 -28.9)	0.46
LELs/mm2*	0.00 (0.00-0.29)	0.14 (0.00-0.26)	0.94	0.08 (0.00-0.35)	0.79
Presence of LELs, n(%)	9 (48)	18 (56)	0.54	23 (56)	0.53
LEL severity (1-3)*	2 (1-3)	2 (1-3)	0.87	1 (1-2)	0.27
CD21+ FDC-networks/mm2 *	0.21 (0.06-0.79)^	0.00 (0.00-0.35)	*0.029*	0.21 (0.00-0.46)	0.28
Bcl6+ Ectopic GC/mm2*	0.14 (0.04-0.33)	0.00 (0.00-0.12)	*0.002*	0.00 (0.00-0.24)	*0.018*
CD3+ cells/mm2*	1384 (841-1883)	470 (177-1406)	*0.029*	715 (256-1917)	0.15
CD20+ cells/mm2*	1679 (428-2811)	376 (61-1003)	*0.004*	624 (108-1300)	*0.023*
CD20/(CD20+CD3)ratio*	0.51 (0.32-0.58)	0.39 (0.25-0.48)	*0.013*	0.40 (0.23-0.50)	*0.023*
CD3/CD20 segregation, n(%)	14 (74)	18 (56)	0.21	24 (57)	0.22
IgA/IgG plasma cell shift, n(%)	9 (47)	18 (56)	0.54	24 (57)	0.48
IgM+ plasma cells/mm2*	68 (25-156)	42 (10-93)	0.09	6 (29-93)	*0.007*

Data is reported as median (IQR)* or n(%). Abbreviations: adSjD: adult-onset Sjögren's disease, FDC: follicular dendritic cell, GC: germinal centre, IgA: immunoglobulin A, LEL: lymphoepithelial lesion, PedSjD: paediatric-onset Sjögren's disease. Significant p values are italicized. Missing data: ^0-5.

In the subanalysis of anti-SSA-positive patients, we observed the same significant differences between the paediatric and adult patients with SjD as we observed in the total cohort.

To evaluate whether the amount of inflammation in the parotid gland of patients with pedSjD was related to clinical/serological parameters, we tested the correlations between the percentage of $CD45^+$ cells and FS and the following key parameters: levels of ESR, IgG, RF, C3 and C4 in the blood, salivary flow rates and total Hocevar scores. Only a moderate correlation between RF and the percentage of $CD45^+$ cells ($r=0.54$, $p=0.017$) and a good correlation between RF and total Hocevar score were seen ($r=0.78$, $p=0.015$).

Parotid gland biopsies of pedSjD patients harbor higher numbers of B-lymphocytes and germinal centers compared to adult SjD patients with high systemic disease activity

Second, we compared the diagnostic pedSjD cohort with the trial adSjD cohort characterised by a higher systemic disease activity. The results of the histopathological analysis of the biopsies are shown in table 3. The histopathology of the parotid glands of patients with pedSjD was more comparable to the trial adSjD cohort than to the diagnostic adSjD cohort. Remarkably, the number of $CD20^+$ B lymphocytes was higher in the pedSjD cohort compared with the trial adSjD cohort ($p=0.023$), resulting in a higher B/T lymphocyte ratio ($p=0.023$). The number of GCs was also significantly higher in the pedSjD cohort ($p=0.018$).

DISCUSSION

In this study, we assessed in detail key histopathological features in parotid gland biopsies from patients with pedSjD and adSjD. We found that the glandular tissue of patients with pedSjD exhibited all the characteristic histopathological features of SjD that are also seen in adult patients with SjD, including: periductal infiltrates, development of ectopic lymphoid tissue, presence of LELs, GCs and the IgA/IgG plasma cell shift. However, when comparing parotid gland biopsies from the pedSjD cohort to adult-onset patients, the his-

topathological findings appeared to be more severe and were characterised by stronger B lymphocyte involvement in patients with pedSjD.

There were several observations supporting our notion that in patients with pedSjD the histopathological findings in the parotid glands are more severe than in adults.

First, patients with pedSjD present with a higher FS and a higher relative surface area of $CD45^+$ infiltrate, when compared with a diagnostic cohort of adult patients. In our pedSjD cohort, 84.2% of the patients had an FS ≥1 in the parotid gland. This percentage is somewhat lower than in published data from other paediatric SjD cohorts, where percentages between 94 and 100 were reported.[7,34,35] In contrast, only 65.6% of patients in our diagnostic adSjD cohort had an FS ≥1, in line with previous observations from labial and parotid gland biopsies in adults.[26,36] In fact, the amount of infiltrate in patients with pedSjD, as reflected by both FS and area of CD45+ infiltrate, was similar to the amount seen in adult patients of the trial adSjD cohort, which is characterised by a relatively high systemic disease activity.

Second, B lymphocytes seem to contribute more to the periductal infiltrates than T lymphocytes in paediatric patients as mirrored by the increased B/T lymphocyte ratio compared with both cohorts of adult patients with SjD. In adults, the relative number of B lymphocytes in minor (labial) glands appears to increase with the severity of the lesions, namely the FS and amount of infiltrate.[37] Thus, the higher B/T lymphocyte ratio seen in glandular tissues of paediatric patients with SjD underpins the more severe histopathology in patients with pedSjD.

Third, our study showed a more pronounced formation of ectopic lymphoid tissue in parotid glands from patients with pedSjD, compared with the adult diagnostic cohort, indicated by elevated numbers of $CD21^+$ FDC networks in the periductal infiltrates and an increased number of GCs. Intriguingly, the number of GCs in the pedSjD cohort was also higher compared with the trial adSjD cohort. Development of ectopic lymphoid tissue from organised lymphoid infiltrates occurs at sites of chronic inflammation under the influence of chemokines and cytokines.[38] Thus, they can be considered as a later, more mature step in the development of the chronic infiltrate.[39] Continued antigenic stimulation may finally result in GC development within the ectopic

lymphoid tissue. In adult patients with SjD, presence of GCs in salivary glands is associated with increased FS and more severe disease.[40]

The higher level of inflammation in the salivary glands of paediatric patients is reflected in the histopathological observations and in the clinical disease activity scores. The median score on the glandular domain of the ESSDAI was significantly higher in the pedSjD cohort compared with the diagnostic adSjD cohort. The median score on the glandular domain of the ESSDAI in the pedSjD cohort was even significantly higher compared with the trial adSjD cohort, in which the total ESSDAI score was higher compared with the pedSjD cohort. These findings are in line with previous reports, showing that patients with pedSjD more often present with signs of parotid gland swelling and (recurrent) parotitis compared with patients with adSjD.[7,35] Remarkably, the higher degree of inflammation in patients with pedSjD seems restricted to the salivary glands and is not reflected by signs of more active systemic disease. When looking at the total ESSDAI scores, we found no difference between patients with pedSjD and the diagnostic adSjD cohort. Total ESSDAI scores were lower in patients with pedSjD compared with the trial adSjD cohort. Furthermore, the percentage of patients with an ESR >15 mm/hour was significantly lower in the pedSjD cohort compared with both adSjD cohorts. Serological parameters, such as IgG, RF and complement levels, were comparable among all groups. Interestingly, the more severe histopathological findings in the parotid glands in paediatric patients were not reflected by a lower salivary gland function. The UWS flow rate was even significantly higher in the pedSjD cohort compared with both adult cohorts, and no significant differences were found in the percentages of patients with a UWS flow rate <0.1ml/min. Saliva production declines with age[41], which may lead to a lower reserve capacity in older patients, explaining at least part of these differences. A higher saliva production rate in children in general could also explain the lower percentage of pedSjD patients presenting with sicca complaints compared to adult-onset SjD patients. No correlation between salivary flow rates and the severity of the lesions (FS, area of CD45+ infiltrate) was seen in pediatric patients, in line with previous studies in adult SjD.[42,43] Thus, also in children, more severe histopathology is not directly linked to salivary gland dysfunction.

Why children present with more severe histopathology in salivary gland tissue at diagnosis is not clear. An explanation might be that the immune system in humans is in continuous development with age-related patterns of T- and B-lymphocytes subsets in peripheral blood and tissue, which may impact immunoregulation at different life stages.[44,45] Cellular immune responses at local sites of infection also seem to change throughout life. For example, during respiratory tract infections in infants, a more preferential generation of terminally differentiated effector T-lymphocytes over tissue resident memory cells is observed which changes during the early years of childhood.[46] These differences in the immune system in combination with the fact that children are exposed to more diverse external triggers, like recurrent (salivary gland) infections, than adults may result in a different cellular and humoral immune response in children. Other factors that may contribute to the more severe histopathological findings are genetic factors in the form of underlying monogenic immunodeficiencies, like c1q deficiency, but also polymorphisms in genes involved in B-lymphocyte homeostasis or other important immunological pathways.[47–49]

Limitations

This study has some limitations. First, pediatric onset SjD is a rare disease and as a result the number of patients is small. Despite the limited power of this study, we did find significant differences between the cohorts. Second, there is limited data about the (immuno)histopathology of the parotid gland of children without SjD. A part of the differences between the histopathological findings might be caused by age-related differences. Third, the pediatric cohort is composed based on expert diagnosis, since there are currently no child-specific classification criteria for SjD. The lack of international classification criteria may affect generalisability of our findings.

Conclusion

To our knowledge, this is the first study to compare quantitatively all key histopathological features of salivary gland biopsies between patients with pedSjD and adSjD. We have found significant differences between the diagnostic pedSjD and adSjD cohorts and even between the pedSjD cohort and the adSjD cohort with higher systemic disease activity. Our findings suggest that

high disease activity in patients with pedSjD is mainly reflected by signs of glandular inflammation, whereas high disease activity in patients with adSjD is mostly reflected by systemic inflammation. Our findings highlight the need for a different approach in the care of patients with pedSjD with more specific attention to salivary gland inflammation. Our advice is to take the histopathological findings of the salivary gland biopsies into account when evaluating the disease activity in patients with pedSjD and to consider adjusting treatment strategies according to these findings.

REFERENCES

1. Mariette X, Criswell LA. Primary Sjögren's Syndrome. N Engl J Med. 2018 Mar;378(10):931–9.
2. Parisis D, Chivasso C, Perret J, Soyfoo MS, Delporte C. Current State of Knowledge on Primary Sjögren's Syndrome, an Autoimmune Exocrinopathy. J Clin Med. 2020 Jul 20;9(7):2299.
3. Fox RI. Sjögren's syndrome. The Lancet. 2005;366(9482):321–31.
4. Brito-Zerón P, Baldini C, Bootsma H, Bowman SJ, Jonsson R, Mariette X, et al. Sjögren syndrome. Nat Rev Dis Primers. 2016 Jul 7;2(1):16047.
5. Ramos-Casals M, Acar-Denizli N, Vissink A, Brito-Zerón P, Li X, Carubbi F, et al. Childhood-onset of primary Sjögren's syndrome: phenotypic characterization at diagnosis of 158 children. Rheumatology (Oxford). 2021 Oct;60(10):4558–67.
6. Ciurtin C, Cho Y, Al-Obaidi M, Jury EC, Price EJ. Barriers to translational research in Sjögren's syndrome with childhood onset: challenges of recognising and diagnosing an orphan rheumatic disease. Lancet Rheumatol. 2021 Feb;3(2):e138–48.
7. Legger GE, Erdtsieck MB, de Wolff L, Stel AJ, Los LI, Verstappen GM, et al. Differences in presentation between paediatric- and adult-onset primary Sjögren's syndrome patients. Clin Exp Rheumatol. 2021;39 Suppl 133(6):85–92.
8. Shiboski CH, Shiboski SC, Seror R, Criswell LA, Labetoulle M, Lietman TM, et al. 2016 American College of Rheumatology/European League Against Rheumatism classification criteria for primary Sjögren's syndrome. Ann Rheum Dis. 2017 Jan;76(1):9–16.
9. Fisher BA, Jonsson R, Daniels T, Bombardieri M, Brown RM, Morgan P, et al. Standardisation of labial salivary gland histopathology in clinical trials in primary Sjögren's syndrome. Ann Rheum Dis. 2017;76(7):1161–8.
10. Kroese FG, Abdulahad WH, Haacke E, Bos NA, Vissink A, Bootsma H. B-cell hyperactivity in primary Sjögren's syndrome. Expert Rev Clin Immunol. 2014 Apr 1;10(4):483–99.
11. Salomonsson S, Jonsson M V., Skarstein K, Brokstad KA, Hjelmström P, Wahren-Herlenius M, et al. Cellular basis of ectopic germinal center formation and autoantibody production in the target organ of patients with Sjögren's syndrome. Arthritis Rheum. 2003 Nov 4;48(11):3187–201.
12. Haacke EA, van der Vegt B, Vissink A, Spijkervet FKL, Bootsma H, Kroese FGM. Germinal centres in diagnostic labial gland biopsies of patients with primary Sjögren's syndrome are not predictive for parotid MALT lymphoma development. Ann Rheum Dis. 2017 Oct;76(10):1781–4.
13. Haacke EA, van der Vegt B, Vissink A, Spijkervet FKL, Bootsma H, Kroese FGM. Standardisation of the detection of germinal centres in salivary gland biopsies of patients with primary Sjögren's syndrome is needed to assess their clinical relevance. Ann Rheum Dis. 2018 Jun 22;77(6):e32.
14. Ihrler S, Zietz C, Sendelhofert A, Riederer A, Löhrs U. Lymphoepithelial duct lesions in Sjögren-type sialadenitis. Virchows Archiv. 1999 Apr 22;434(4):315–23.
15. Zandbelt MM. The synergistic value of focus score and IgA% score of sublabial salivary gland biopsy for the accuracy of the diagnosis of Sjogren's syndrome: a 10-year comparison. Rheumatology. 2002 Jul 1;41(7):819–23.
16. van Ginkel MS, Nakshbandi U, Arends S, Haacke EA, Liefers SC, Verstappen GM, et al. Increased Diagnostic Accuracy of the Labial Gland Biopsy in Primary Sjögren Syndrome When Multiple Histopathological Features Are Included. Arthritis & Rheumatology. 2024 Mar 9;76(3):421–8.

17. van Ginkel MS, van der Sluis T, Bulthuis MLC, Buikema HJ, Haacke EA, Arends S, et al. Digital image analysis of intraepithelial B-lymphocytes to assess lymphoepithelial lesions in salivary glands of Sjögren's syndrome patients. Rheumatology. 2022 Dec 23;62(1):428–38.

18. Meiners PM, Vissink A, Kroese FGM, Spijkervet FKL, Smitt-Kamminga NS, Abdulahad WH, et al. Abatacept treatment reduces disease activity in early primary Sjögren's syndrome (open-label proof of concept ASAP study). Ann Rheum Dis. 2014 Jul;73(7):1393–6.

19. van Nimwegen JF, Mossel E, van Zuiden GS, Wijnsma RF, Delli K, Stel AJ, et al. Abatacept treatment for patients with early active primary Sjögren's syndrome: a single-centre, randomised, double-blind, placebo-controlled, phase 3 trial (ASAP-III study). Lancet Rheumatol. 2020 Mar;2(3):e153–63.

20. Haacke EA, van der Vegt B, Meiners PM, Vissink A, Spijkervet FKL, Bootsma H, et al. Abatacept treatment of patients with primary Sjögren's syndrome results in a decrease of germinal centres in salivary gland tissue. Clin Exp Rheumatol. 2017;35(2):317–20.

21. Seror R, Ravaud P, Bowman SJ, Baron G, Tzioufas A, Theander E, et al. EULAR Sjögren's syndrome disease activity index: development of a consensus systemic disease activity index for primary Sjögren's syndrome. Ann Rheum Dis. 2010 Jun;69(6):1103–9.

22. Hočevar A, Ambrožič A, Rozman B, Kveder T, Tomšič M. Ultrasonographic changes of major salivary glands in primary Sjögren's syndrome. Diagnostic value of a novel scoring system. Rheumatology. 2005 Jun 1;44(6):768–72.

23. Rebel D, de Wolff L, Delli K, Stel AJ, Kokol HC, Vissink A, et al. Added value of the salivary gland ultrasonography OMERACT score in the ACR/EULAR classification criteria for Sjögren's disease. Semin Arthritis Rheum. 2024 Aug;67:152473.

24. Jensen SB, Vissink A. Salivary Gland Dysfunction and Xerostomia in Sjögren's Syndrome. Oral Maxillofac Surg Clin North Am. 2014 Feb;26(1):35–53.

25. Spijkervet FKL, Haacke E, Kroese FGM, Bootsma H, Vissink A. Parotid Gland Biopsy, the Alternative Way to Diagnose Sjögren Syndrome. Rheumatic Disease Clinics of North America. 2016;42(3):485–99.

26. Pijpe J, Kalk WWI, van der Wal JE, Vissink A, Kluin PhM, Roodenburg JLN, et al. Parotid gland biopsy compared with labial biopsy in the diagnosis of patients with primary Sjogren's syndrome. Rheumatology. 2006 Jul 28;46(2):335–41.

27. Chisholm DM, Mason DK. Labial salivary gland biopsy in Sjögren's disease. J Clin Pathol. 1968 Sep;21(5):656–60.

28. Greenspan JS, Daniels TE, Talal N, Sylvester RA. The histopathology of Sjögren's syndrome in labial salivary gland biopsies. Oral Surgery, Oral Medicine, Oral Pathology. 1974 Feb;37(2):217–29.

29. Kroese FGM, Haacke EA, Bombardieri M. The role of salivary gland histopathology in primary Sjögren's syndrome: promises and pitfalls. Clin Exp Rheumatol. 2018;36 Suppl 1(3):222–33.

30. Bankhead P, Loughrey MB, Fernández JA, Dombrowski Y, McArt DG, Dunne PD, et al. QuPath: Open source software for digital pathology image analysis. Sci Rep. 2017 Dec;7(1):16878.

31. Landis JR, Koch GG. The Measurement of Observer Agreement for Categorical Data. Biometrics. 1977 Mar;33(1):159.

32. Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE, et al. Classification criteria for Sjögren's syndrome: A revised version of the European criteria proposed by the American-European Consensus Group. Vol. 61, Annals of the Rheumatic Diseases. 2002. p. 554–8.

33. van Nimwegen JF, van Ginkel MS, Arends S, Haacke EA, van der Vegt B, Sillevis Smitt-Kamminga N, et al. Validation of the ACR-EULAR criteria for primary Sjögren's syndrome in a Dutch prospective diagnostic cohort. Rheumatology. 2018 May 1;57(5):818–25.

34. McGuirt WF, Whang C, Moreland W. The role of parotid biopsy in the diagnosis of pediatric Sjögren syndrome. Arch Otolaryngol Head Neck Surg. 2002 Nov;128(11):1279–81.

35. Basiaga ML, Stern SM, Mehta JJ, Edens C, Randell RL, Pomorska A, et al. Childhood Sjögren syndrome: features of an international cohort and application of the 2016 ACR/EULAR classification criteria. Rheumatology (Oxford). 2021 Jul 1;60(7):3144–55.

36. Vitali C, Moutsopoulos HM, Bombardieri S. The European Community Study Group on diagnostic criteria for Sjögren's syndrome. Sensitivity and specificity of tests for ocular and oral involvement in Sjögren's syndrome. Ann Rheum Dis. 1994 Oct;53(10):637–47.

37. Christodoulou MI, Kapsogeorgou EK, Moutsopoulos HM. Characteristics of the minor salivary gland infiltrates in Sjögren's syndrome. J Autoimmun. 2010 Jun;34(4):400–7.

38. Bombardieri M, Lewis M, Pitzalis C. Ectopic lymphoid neogenesis in rheumatic autoimmune diseases. Vol. 13, Nature Reviews Rheumatology. 2017. p. 141–54.

39. Sato Y, Silina K, van den Broek M, Hirahara K, Yanagita M. The roles of tertiary lymphoid structures in chronic diseases. Nat Rev Nephrol. 2023 Aug 12;19(8):525–37.

40. Risselada AP, Looije MF, Kruize AA, Bijlsma JWJ, Van Roon JAG. The Role of Ectopic Germinal Centers in the Immunopathology of Primary Sjögren's Syndrome: A Systematic Review. Semin Arthritis Rheum. 2013;42(4):368–76.

41. Ben-Aryeh H, Miron D, Szargel R, Gutman D. Clinical Science Whole-saliva Secretion Rates in Old and Young Healthy Subjects. J Dent Res. 1984 Sep 1;63(9):1147–8.

42. Bookman AAM, Shen H, Cook RJ, Bailey D, McComb RJ, Rutka JA, et al. Whole stimulated salivary flow: Correlation with the pathology of inflammation and damage in minor salivary gland biopsy specimens from patients with primary Sjögren's syndrome but not patients with sicca. Arthritis Rheum. 2011 Jul;63(7):2014–20.

43. Mossel E, van Ginkel MS, Haacke EA, Arends S, Liefers SC, Delli K, et al. Histopathology, salivary flow and ultrasonography of the parotid gland: three complementary measurements in primary Sjögren's syndrome. Rheumatology (Oxford). 2022 May 30;61(6):2472–82.

44. Blanco E, Pérez-Andrés M, Arriba-Méndez S, Contreras-Sanfeliciano T, Criado I, Pelak O, et al. Age-associated distribution of normal B-cell and plasma cell subsets in peripheral blood. Journal of Allergy and Clinical Immunology. 2018 Jun;141(6):2208-2219.e16.

45. Kumar B V., Connors TJ, Farber DL. Human T Cell Development, Localization, and Function throughout Life. Immunity. 2018 Feb;48(2):202–13.

46. Connors TJ, Baird JS, Yopes MC, Zens KD, Pethe K, Ravindranath TM, et al. Developmental Regulation of Effector and Resident Memory T Cell Generation during Pediatric Viral Respiratory Tract Infection. The Journal of Immunology. 2018 Jul 15;201(2):432–9.

47. Hoppenreijs EPAH. Hereditary C1q deficiency and secondary Sjogren's syndrome. Ann Rheum Dis. 2004 Nov 1;63(11):1524–5.

48. Teruel M, Alarcón-Riquelme ME. Genetics of systemic lupus erythematosus and Sjögren's syndrome: an update. Curr Opin Rheumatol. 2016 Sep;28(5):506–14.

49. Lessard CJ, Li H, Adrianto I, Ice JA, Rasmussen A, Grundahl KM, et al. Variants at multiple loci implicated in both innate and adaptive immune responses are associated with Sjögren's syndrome. Nat Genet. 2013 Nov 6;45(11):1284–92.

ACKNOWLEDGEMENTS

Several authors of this publication are members of the European Reference Network ERN ReCONNET.

FUNDING STATEMENT

This work was supported by unrestricted grants from Bristol-Myers Squibb for the ASAP-II and ASAP-III studies to HB and is part of the Veni research programme of GV (project number: 09150162010166), which is financed by the Dutch Research Council (NWO).

CONFLICTS OF INTEREST

HB has received unrestricted research grants, outside the submitted work, from AstraZeneca and consultancy fees from Bristol-Myers Squibb, Roche, Novartis, Union Chimique Belge and Argenx. FK and GV received consultancy fees from Argenx. SA received consultancy fees from Argenx and Novartis. BvdV reports honoraria received by UMCG for expertise or scientific advisory board/consultancy (on request): Visiopharm, Philips, MSD/Merck, DaiichiSankyo/AstraZeneca; speaker's fee from Visiopharm, Diaceutics, MSD/Merck.

STATEMENT OF ETHICS AND CONSENT

All data were obtained as part of the REpSULT cohort, diagnostic sicca cohort or ASAP studies, which were approved by the local ethics committee of the University Medical Center Groningen (UMCG) (REpSULT: METc2019.541, ASAP-II: METc2009.371, ASAP-III: METc2014.118, diagnostic sicca cohort: METc2013.066). All patients provided written informed consent according to the Declaration of Helsinki.

SUPPLEMENTARY MATERIAL

Supplementary Table 1. Parotid gland biopsy results in a subpopulation of patients with major salivary gland enlargement

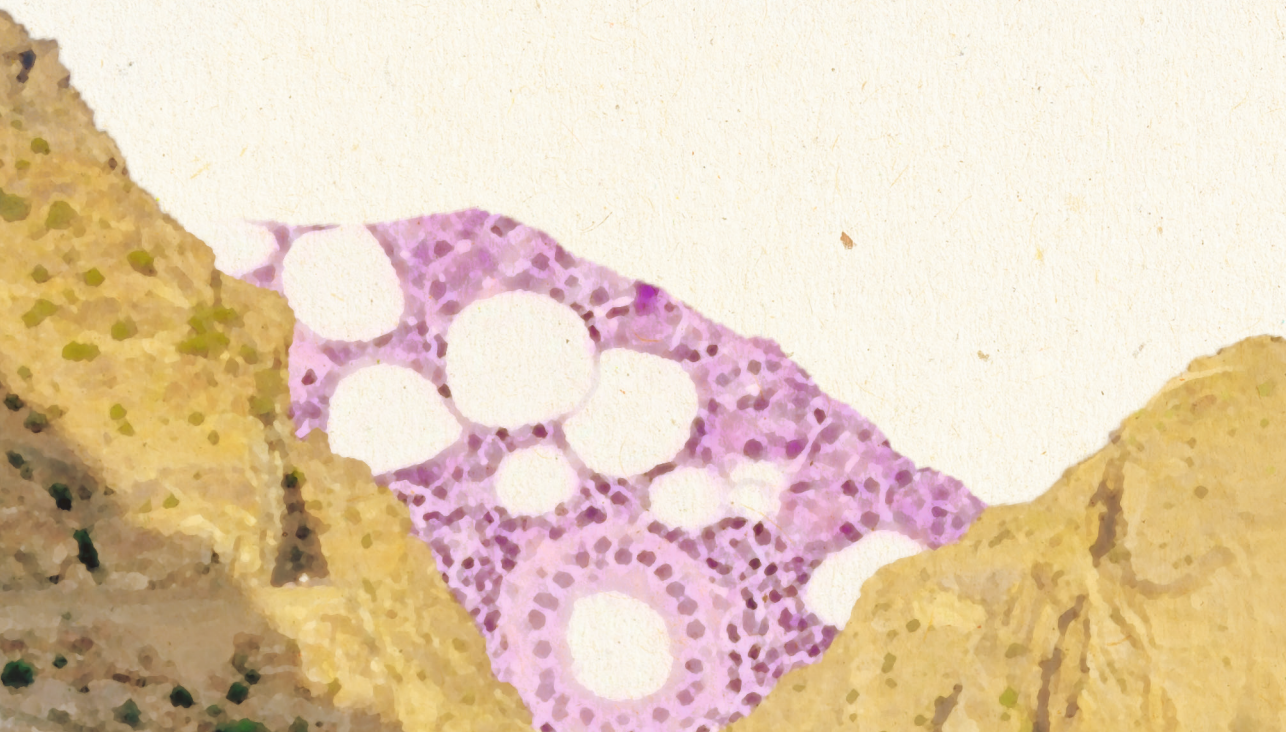
	pedSjD n=18	diagnostic adSjD n=12	pedSjD vs diagnostic adSjD p-value	trial adSjD n=33	pedSjD vs. trial adSjD p-value
Focus score*	2.7 (1.75-3.55)	1.1 (1.0-1.78)^	*0.010*	1.84 (1.32-4.72)	0.72
CD45+ cells * (% of parenchyma)	20.65 (9.82-36.33)	15.22 (3.76-25.41)	0.19	19.84 (6.97-32.94)	0.622
LELs/mm2*	0.05 (0.00-0.31)	0.16 (0.0125-0.26)	0.73	0.14 (0.00-0.49)	0.52
Presence of LELs, n(%)	9 (50)	9 (75)	0.26	21 (66)	0.28
LEL severity (1-3)*	2 (1-3)	2 (1-2.5)	0.78	1 (1-2.5)	0.38
CD21+ FDC-networks/mm2 *	0.21 (0.09-0.86)^	0.00 (0.10-0.38)	0.14	0.26 (0.00-0.49)	0.41
Bcl6+ Ectopic GC/mm2*	0.15 (0.08-0.34)	0.00 (0.00-0.13)	*0.024*	0.00 (0.00-0.32)	0.098
CD3+ cells/mm2*	1444 (977-2058)	470 (177-1406)	0.090	718 (258-1960)	0.168
CD20+ cells/mm2*	1735(592-2818)	488 (98-877)	*0.014*	651 (136-1510)	*0.020*
CD20/(CD20+CD3)ratio*	0.52 (0.36-0.59)	0.39 (0.27-0.50)	*0.042*	0.40 (0.24-0.48)	*0.011*
CD3/CD20 segregation, n(%)	14 (79)	9 (75)	0.60	19 (58)	0.22
IgA/IgG plasma cell shift, n(%)	9 (50)	7 (58)	0.47	17 (52)	0.92
IgM+ plasma cells/mm2*	70 (32.5-168)	24.1 (12.5-51.7-93)	*0.018*	18.4(4.3-80.4)	*0.002*

Data is reported as median (IQR)* or n(%). Abbreviations: LEL: lymphoepithelial lesion,. mm2: square millimeter, IQR: interquartile range, FDC: follicular dendritic cell, GC: germinal center. Significant p-values are italicized. Missing data: ^0-5, $6-10, *11-15, #16-20

Supplementary Tabel 2. Parotid gland biopsy results in anti-SSA positive patients

	pedSjD n=13	diagnostic adSjD n=31	pedSjD vs diagnostic adSjD p-value	trial adSjD n=36	pedSjD vs. trial adSjD p-value
Focus score*	2.8 (1.95-3.7)	1.1 (0.3-1.8)^	*0.002*	2.42 (1.29-5.22)	0.89
CD45+ cells * (% of parenchyma)	28.5 (16.4-40.4-)	10.80 (3.36-24.44)	*0.006*	20.05 (8.57-34.82)	0.28
LELs/mm2*	0.25 (0.00-0.47)	0.14 (0.00-0.26)	0.31	0.14 (0.00-0.46)	0.70^
Presence of LELs, n(%)	8 (62)	18 (58)	1.00	23 (64)	0.88
LEL severity (1-3)*	2^ (1-3)	2 (1-3)	0.89	1 * (1-2)	0.32
CD21+ FDC-networks/mm2 *	0.43 (0.09-1.1)^	0.00 (0.00-0.36)	*0.009*	0.27 (0.00-0.49)	0.16
Bcl6+ Ectopic GC/mm2*	0.2 (0.09-0.37)	0.00 (0.00-0.13)	*<0.001*	0.00 (0.00-0.30)	*0.032*
CD3+ cells/mm2*	1738 (1031-2648)	474 (170-1450)	*0.015*	962 (438-1962)	0.21
CD20+ cells/mm2*	2793(1423-3156)	381 (66.5-1039)	*<0.001*	700 (292-1591)	*0.007*
CD20/(CD20+CD3)ratio*	0.58 (0.51-0.60)	0.41 (0.26-0.48)	*0.013*	0.41 (0.237-0.52)	*0.004*
CD3/CD20 segregation, n(%)	10 (77)	17 (55)	0.20	24 (67)	0.73
IgA/IgG plasma cell shift, n(%)	8 (62)	18 (58)	0.83	24 (67)	0.75
IgM+ plasma cells/mm2*	71(30-180)	44 (10-94)	0.083	33^ (3.7-94)	*0.021*

Data is reported as median (IQR)* or n(%). Abbreviations: LEL: lymphoepithelial lesion,. Mm2: square millimeter, IQR: interquartile range, FDC: follicular dendritic cell, GC: germinal center. Significant p-values are italicized. Missing data: ^0-5, $6-10, *11-15, #16-20



CHAPTER 5

Is the T Follicular Regulatory: Follicular Helper T Cell Ratio in Blood a Biomarker for Ectopic Lymphoid Structure Formation in Sjögren's Syndrome? Comment on the Article by Fonseca et al.

Comment on 'Blood T Follicular Regulatory Cells / T Follicular Helper Cells ratio Marks Ectopic Lymphoid Structure Formation and PD-1+ ICOS+ T Follicular Helper Cells Indicate Disease Activity in Primary Sjögren's Syndrome' by Fonsesca et al (2018)

G.M.P.J. Verstappen[1], U. Nakshbandi[1], E. Mossel[1], E.A. Haacke[2], B. van der Vegt[2], A. Vissink[3], H. Bootsma[1], F.G.M. Kroese[1]

Departments of [1]Rheumatology and Clinical Immunology, [2]Pathology and Medical Biology and [3]Oral and Maxillofacial Surgery, University of Groningen and University Medical Center Groningen, Groningen, the Netherlands

Arthritis & Rheumatology 2018;70(8):1354-1355

We read with great interest the recent article by Fonseca et al in which they elegantly showed that T follicular regulatory (Tfr) cells were enriched in blood as well as in matched minor salivary gland (MSG) biopsy specimens from patients with primary Sjögren's syndrome (SS).[1] They also showed that the Tfr:follicular helper T (Tfh) cell ratio in blood was increased in patients with primary SS compared with patients with non-SS sicca syndrome. Interestingly, this Tfr:Tfh cell ratio in blood correlated with ectopic lymphoid structure formation in MSG tissue.

In our opinion, however, the authors did not show a direct correlation between aberrant Tfr:Tfh cell ratios and ectopic lymphoid structure formation among patients with primary SS. In essence, their study showed that in patients with primary SS the Tfr:Tfh cell ratio in blood was correlated with the numbers of infiltrating lymphocytes, as assessed by flow cytometric analysis of MSG cell suspensions. Additionally, Fonseca and colleagues showed that the Tfr:Tfh cell ratio in blood was increased in patients with focal sialadenitis (defined in their study as a focus score of ≥1) compared with patients without focal sialadenitis. Of note, this comparison was made irrespective of a diagnosis of primary SS, which implied that the majority of patients without focal sialadenitis had non-SS sicca syndrome.

In our study, we assessed the number of circulating Tfr cells and Tfh cells in a larger inception cohort of 98 patients with sicca syndrome and clinically suspected primary SS. MSG biopsy specimens from all patients were assessed in detail using histopathologic analysis. Forty-four patients were classified as having primary SS (43 women, mean age 53 years, mean European League Against Rheumatism Sjögren's Syndrome Disease Activity Index [ESSDAI] score 7), and 54 patients were classified as having non-SS sicca syndrome (46 women, mean age 48 years). Among the 44 patients with primary SS, 80% had never undergone treatment with corticosteroids or disease-modifying antirheumatic drugs.

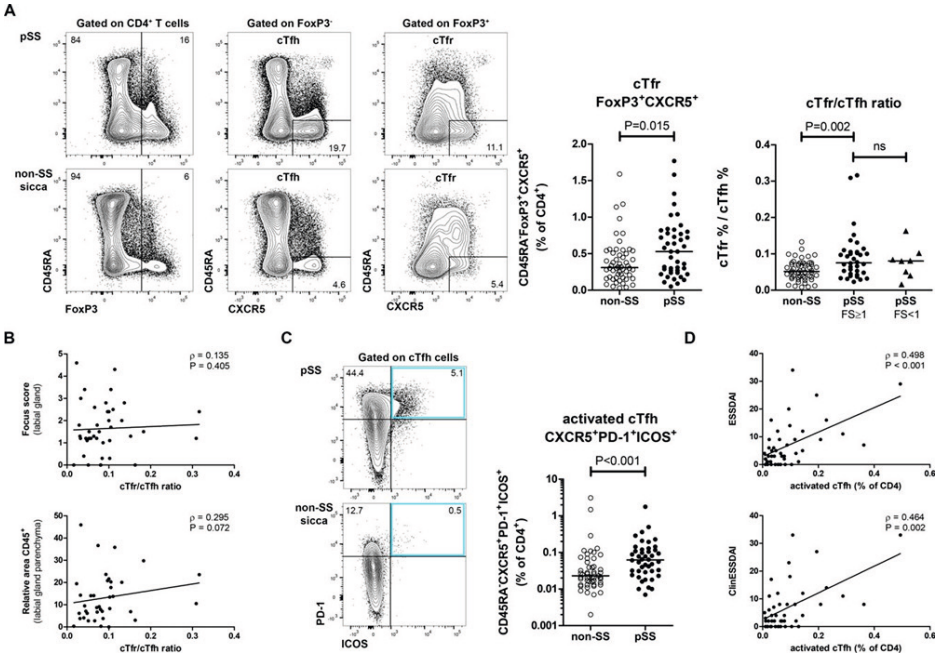


Figure 1. A, The circulating follicular regulatory T (cTfr) cell frequency and the cTfr:circulating follicular helper T (cTfh) cell ratio in blood from patients with non–Sjögren's syndrome (non-SS) sicca syndrome and patients with primary SS (pSS). B, Focus score and area of the CD45+ infiltrate in relation to the Tfr:Tfh cell ratio. C, Frequency of programmed death 1–positive (PD-1+) inducible costimulator–positive (ICOS+) cTfh cells in patients with primary SS and patients with non-SS sicca syndrome. D, Association between activated cTfh cell frequency and with the European League Against Rheumatism Sjögren's Syndrome Disease Activity Index (ESSDAI) and the clinical ESSDAI (ClinESSDAI). In A (right panels) and C (right panel), each symbol represents a single subject; horizontal lines show the median. In B and D, each symbol represents a single subject.

Consistent with the findings by Fonseca et al, the frequencies of Tfr cells and the Tfr:Tfh cell ratio in blood were significantly increased in patients with SS and a focus score of ≥1 compared with patients with non-SS sicca syndrome (Figure 1A). In contrast to what was suggested by Fonseca et al, we could not demonstrate in this larger inception cohort that patients with primary SS who had a focus score of ≥1 in MSG tissue had a higher Tfr:Tfh cell ratio in blood compared with those with a focus score of <1 (Figure 1A). Moreover, neither the focus score nor the area of the CD45+ infiltrate was correlated with the blood Tfr:Tfh cell ratio (Figure 1B). The Tfr:Tfh cell ratio also was not asso-

ciated with the score for ultrasonography of the major salivary glands (sUS) (Spearman's $\rho = 0.04$, $P = 0.831$).

In a previous study we showed that the sUS score was significantly associated with the focus scores in both labial and parotid gland biopsy specimens.[2] Thus, although our data also showed that patients with primary SS have higher Tfr:Tfh cell ratios in blood, we observed no association between this ratio in blood and glandular inflammation.

In addition to our observation of increased levels of Tfr cells and the Tfr:Tfh cell ratio in blood, we also observed a significant increase in the frequency of activated (programmed death 1–positive, inducible costimulator [ICOS]–positive) Tfh cells in patients with primary SS compared with those with non-SS sicca syndrome (Figure 1C), while Fonseca et al observed only a tendency toward higher frequencies of activated Tfh cells. Nonetheless, similar to the observations reported by Fonseca et al, we observed that the frequencies of activated Tfh cells in blood were associated with ESSDAI scores in patients with primary SS (Figure 1D). In addition, we observed that the frequencies of activated Tfh cells correlated with the clinical ESSDAI (ESSDAI without the biologic domain) scores[3], indicating that the correlation is not based only on activity in the biologic domain (e.g., hypergammaglobulinemia).

A previous study by our group[4] also supports an association between activated Tfh cells and disease activity. In that study, circulating Tfh cells in patients with primary SS were studied before and after treatment with abatacept[4]. In that study, we observed a significant decrease in the frequency of activated Tfh cells in blood during treatment. Furthermore, the reduction in ICOS expression by the remaining Tfh cells correlated significantly with the decrease in ESSDAI scores.

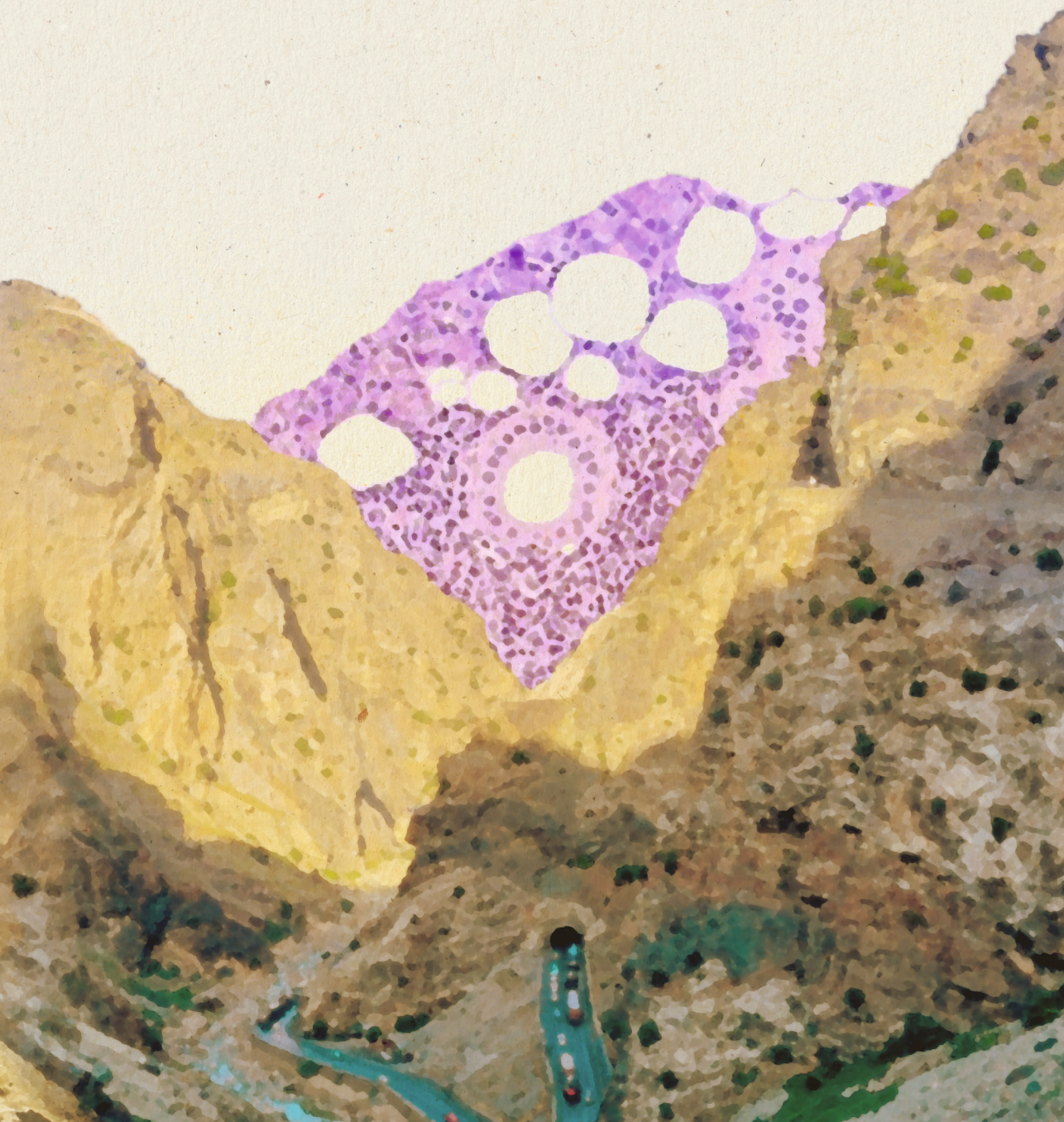
In conclusion, the data presented by Fonseca et al provide evidence that Tfr cells and Tfh cells are important players in the pathogenesis of primary SS. It is likely that these cells are involved in the B cell hyperactivation that characterises this disease, but the levels of these cells in blood may not necessarily reflect the presence of ectopic lymphoid tissue in the salivary glands. Importantly, all available data indicate that Tfh cells contribute significantly to systemic disease activity in primary SS and emphasize that these cells are an important target for treatment.

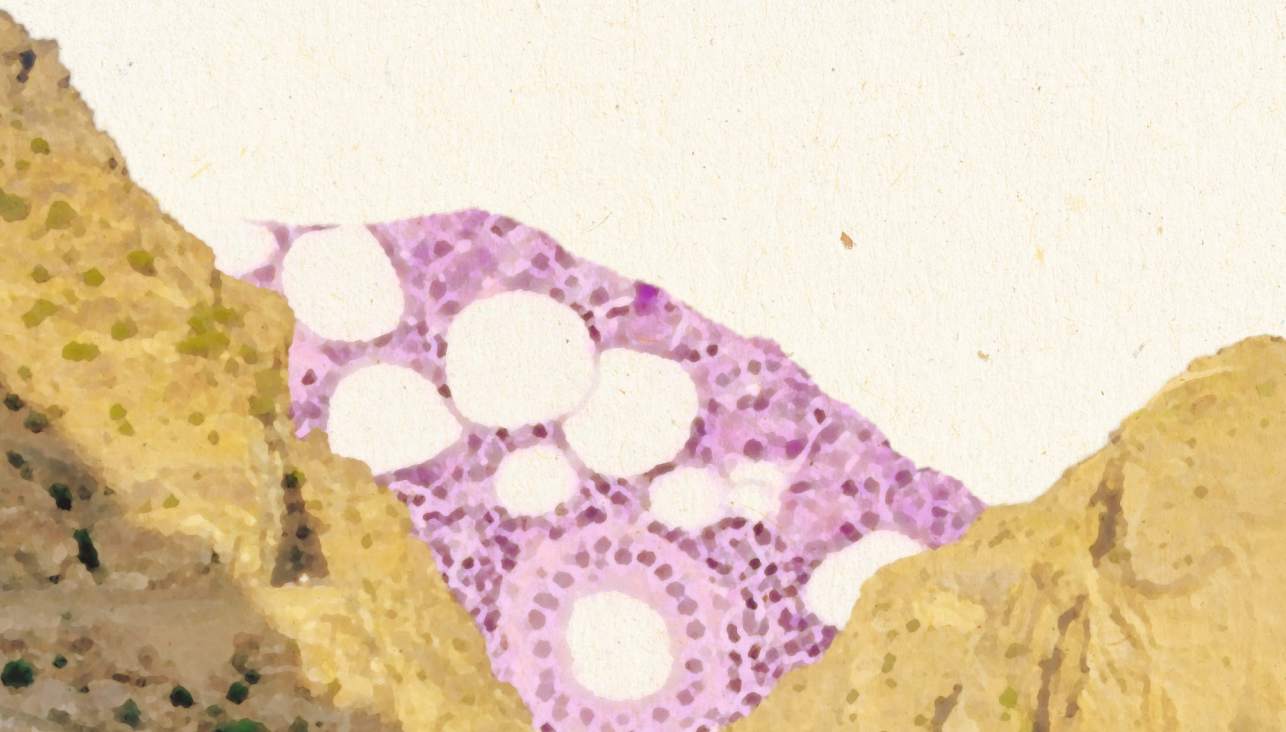
REFERENCES

1. Fonseca VR, Romão VC, Agua-Doce A, Santos M, López-Presa D, Ferreira AC, et al. The Ratio of Blood T Follicular Regulatory Cells to T Follicular Helper Cells Marks Ectopic Lymphoid Structure Formation While Activated Follicular Helper T Cells Indicate Disease Activity in Primary Sjögren's Syndrome. Arthritis & Rheumatology. 2018 May 25;70(5):774–84.
2. Mossel E, Delli K, van Nimwegen JF, Stel AJ, Haacke EA, Kroese FGM, et al. The parotid gland connection: ultrasound and biopsies in primary Sjögren's syndrome. Ann Rheum Dis. 2018 Jul;77(7):e38–e38.
3. Seror R, Meiners P, Baron G, Bootsma H, Bowman SJ, Vitali C, et al. Development of the ClinESSDAI: a clinical score without biological domain. A tool for biological studies. Ann Rheum Dis. 2016 Nov;75(11):1945–50.
4. Verstappen GM, Meiners PM, Corneth OBJ, Visser A, Arends S, Abdulahad WH, et al. Attenuation of Follicular Helper T Cell-Dependent B Cell Hyperactivity by Abatacept Treatment in Primary Sjögren's Syndrome. Arthritis Rheumatol. 2017 Sep;69(9):1850–61.

PART 2

Diagnosis, classification and treatment





CHAPTER 6

Increased diagnostic accuracy of the labial gland biopsy in primary Sjögren's syndrome when multiple histopathological features are included

M.S. van Ginkel[1], U. Nakshbandi[1], S. Arends[1], E.A. Haacke[2], S.C. Liefers[1], G.M.P.J. Verstappen[1], J.F. van Nimwegen[1], E. Brouwer[1], A.J. Stel[1], F.K.L. Spijkervet[3], A. Vissink[3], H. Bootsma[1], B. van der Vegt[2]*, F.G.M. Kroese[1]*

*Shared last authorship

Departments of [1]Rheumatology and Clinical Immunology, [2]Pathology and Medical Biology, [3]Oral and Maxillofacial Surgery, University of Groningen and University Medical Centre Groningen, Groningen, the Netherlands.

Arthritis & Rheumatology 2024 76(3):421-428.

ABSTRACT

Objective

To evaluate the diagnostic accuracy of the labial salivary gland biopsy based on multiple histopathological features in patients with suspected primary Sjögren's syndrome (pSS).

Methods

Patients from a diagnostic sicca cohort with clinically suspected pSS who underwent a labial gland biopsy were included. Patients were categorised as having pSS or non-Sjögren syndrome sicca (non-SS sicca) based on vignettes scored by an expert panel. Labial gland biopsies were analysed for the presence of four histopathological features: focus score (FS) ≥1, prelymphoepithelial and lymphoepithelial lesions, immunoglobulin G plasma cell shift, and germinal centres. Sensitivity and specificity of histologic features were calculated, and the optimal cutoff value for the number of histopathological features needed to diagnose pSS was determined with receiver operating curve analysis.

Results

A total of 38 patients were categorised as having pSS and 65 as having non-SS sicca. In labial gland biopsies of patients with pSS, the prevalence of FS ≥1 was 82%, followed by 68% for pre-lymphoepithelial and lymphoepithelial lesions, 63% for plasma cell shift, and 24% for germinal centres. Although FS ≥1 showed the highest sensitivity for patients with pSS (82%), specificity was higher for the other three features (98%–100%). The presence of two or more (of four) histopathological features had almost comparable sensitivity to FS alone, but specificity increased with 12% to 100%. For fulfillment of American College of Rheumatology/EULAR criteria, specificity increased from84% to 95% when an abnormal biopsy was defined by the presence of two or more histopathological features instead of FS ≥1 only.

Conclusion

The diagnostic accuracy of the labial gland biopsy increases when other histopathological features besides FS are taken into account, by reducing the number of false-positive biopsies.

INTRODUCTION

Primary Sjögren syndrome (pSS) is a chronic systemic autoimmune disease characterized by dry eyes, dry mouth and fatigue, various systemic manifestations, and serological abnormalities.[1] Due to the heterogeneity of the disease, classifying and diagnosing pSS can be challenging. In the past years, multiple classification criteria sets were developed for research purposes to allow selection of well-defined and homogenous populations of patients with pSS for clinical studies. Clinical diagnosis, on the other hand, is still based on expert opinion. Salivary gland biopsies play an important role in both classification and diagnosis of pSS. This is reflected by the prominent place of the salivary gland biopsy in the current American College of Rheumatology (ACR)/EULAR classification criteria for pSS.[2] These criteria consist of two major items (each 3 points), serology and salivary gland histopathology, and three minor items (each 1 point), two ocular function tests and measurement of salivary flow rate. Patients with a total score ≥4 points meet the criteria for pSS. Therefore, either the presence of serum anti-Ro/SSA antibodies or a salivary gland biopsy with ≥1 focus per 4 mm^2 salivary gland tissue is needed to classify a patient as having pSS.

A focus is defined as a periductal infiltrate that consists of ≥50 lymphocytes. The focus score (FS) is calculated by the number of lymphocytic foci per 4 mm^2 of salivary gland tissue. Currently, biopsies with FS ≥1 are considered positive for pSS.[3,4] The sensitivity of the labial gland biopsy (with cutoff FS ≥1) to diagnose pSS varies between 64% and 94% and the specificity between 61% and 100%, dependent on the study.[5] In previous studies, the diagnosis of pSS often relied on the American–European Consensus Group classification criteria, in which the labial gland biopsy (FS ≥1) is also a major item.[5] As a consequence, the outcome of the biopsy has a major influence on positive or negative classification of pSS, leading to potential overestimation of sensitivity and specificity rates. Only a few studies (partly) eliminated circular reasoning, either applying the Japanese criteria without inclusion of the labial gland biopsy or by using the opinion of experienced clinicians as gold standard instead of criteria sets.[6,7] Lymphocytic foci in salivary glands are not restricted to patients with pSS. Lymphocytic infiltrates can also be found in salivary glands of healthy individuals and in patients with human immunodeficiency

virus (HIV) and various autoimmune diseases other than pSS.[8–13] An FS >1 was seen in labial gland biopsies in up to 15% of healthy individuals without sicca symptoms[8]. Older age was associated with higher prevalence of more severe salivary gland lymphocytic infiltrates in a postmortem study of patients without autoimmune diseases and without known head/neck pathology.[14] These observations indicate that salivary gland biopsies may be false positive for pSS, especially in older patients. Vice versa, not all patients with pSS show a labial gland biopsy with FS ≥1; these patients are at risk of being falsely diagnosed with non–Sjögren syndrome (non-SS).[15,16] Previous studies tried to increase the diagnostic accuracy of the labial gland biopsy by analysing FS in more than one section level or by adding immunohistochemical stainings to detect lymphocytic foci more precisely.[17,18] These approaches mainly affected the sensitivity of the labial gland biopsy but did not increase specificity.

Besides periductal infiltrates, other histopathological features that are specific for pSS can be found in salivary gland biopsies of patients with pSS: pre-lymphoepithelial and lymphoepithelial lesions (LELs), a relative increase in the number of immunoglobulin (Ig) G plasma cells (the so-called plasma cell shift), and the presence of germinal centers (GCs; Figure 1). LELs are defined as hyperplastic ductal epithelium with infiltrating lymphocytes.[19,20] As shown recently, they can relatively easy be detected by the presence of intraepithelial $CD20^+$ B lymphocytes.[21] B lymphocytes can also be present in striated ducts with-out hyperplasia of patients with pSS, and these ducts are hypothesized to represent an early stage of LEL formation (pre-LELs).[21].Both the presences of LELs and intraepithelial B lymphocytes are specific for pSS.[21,22] A relative increase in the number of IgG plasma cells, compared to the number of IgA plasma cells, appears to be more specific for pSS than FS alone.[23,24] The formation of GCs is another histopathological feature reflecting B cell hyperactivity in pSS and is present in around one-quarter of the labial glands of patients with pSS.[25,26] Although the diagnostic value of these three histopathological features in pSS was already shown separately, no study explored the added diagnostic value of these features together. Therefore, the aim of this study was to evaluate the diagnostic accuracy of the labial gland biopsy based on multiple histopathological features (FS, pre-LELs and LELs, IgG plasma cell shift, and GCs) in patients suspected of having pSS.

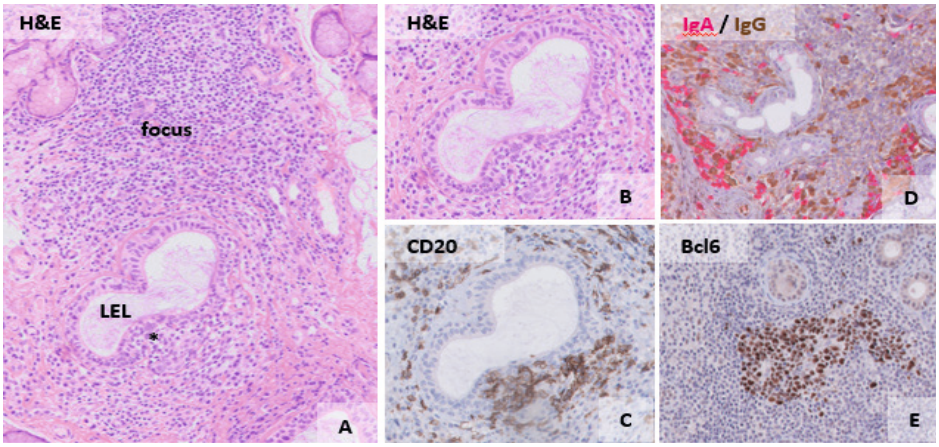


Figure 1. Histopathological features of pSS in labial gland biopsies. (A) Presence of a periductal infiltrate of ≥50 lymphocytes (focus) surrounding a striated duct with ductal hyperplasia (lymphoepithelial lesion – LEL). (B) High resolution image of the same LEL, with ductal hyperplasia in the area with the asterisk*. (C) Consecutive CD20-stained slide, showing presence of intraepithelial $CD20^+$ B-lymphocytes in the same LEL. (D) Presence of a plasma cell shift, as shown by a relative increase in the number of IgG (brown) plasma cells, compared to the IgA (pink) plasma cells. (E) Presence of a germinal centre, defined as a cluster of ≥ 5 adjacent $Bcl6^+$ cells.

METHODS

Patients and inclusion

This prospective diagnostic sicca cohort consisted of consecutive patients with sicca complaints who were suspected of having pSS, were aged ≥18 years, and who underwent a labial salivary gland biopsy during the diagnostic workup for pSS in the University Medical Center Groningen (UMCG), a tertiary referral center for patients with pSS.[27] Patients who were diagnosed with an associated autoimmune disease, positive hepatitis C serology, or (nonspecific) sclerotic sialadenitis within the labial gland biopsy were excluded.

Expert consensus

All patients were categorised as having pSS or non-SS sicca based on expert opinion of a panel of experienced rheumatologists (EB, AJS, and HB). The expert panel independently scored anonymised clinical vignettes as having pSS or non-SS sicca based on information about clinical history, physical ex-

amination, EULAR SS Disease Activity Index score, serological parameters (complete blood count, erythrocyte sedimentation rate, C-reactive protein, antinuclear antibodies, anti-SSA, anti–SSB, rheumatoid factor, IgG, C3 and C4, and cryoglobulinemia), results of the evaluation by an oral and maxillo-facial surgeon (physical examination of the orofacial and neck area, analysis of unstimulated whole saliva [UWS] and stimulated whole saliva), results of ophthalmological evaluation (Schirmer's test, tear break-up time, and ocular staining score [OSS]), and FS of the labial and parotid gland biopsy (if present). In the UMCG, the parotid gland biopsy is part of the standard diagnostic workup of pSS, and most patients in this cohort underwent paired biopsies of the labial and parotid gland. Therefore, the parotid gland FS, if available, was also included in the vignettes for expert diagnosis. The vignettes did not include information about the other histopathological features (pre-LELs and LELs, IgG plasma cell shift, and GCs) or the diagnosis of the treating physician. HB scored all clinical vignettes, and AJS and EB each scored half of the clinical vignettes. In case of a discrepancy in categorisation among the experts, the anonymized clinical vignette was dis-cussed in a consensus meeting with all three experts to reach expert consensus.

Histochemical- and immunohistochemical staining

Formalin-fixed and paraffin-embedded labial gland biopsies of 3-µm sections were stained with haematoxylin and eosin (H&E) and immunohistochemically for high MW cytokeratin (hmwCK;clone 34βE12) to detect epithelial cells, for CD20 (clone L-26) to detect B lymphocytes, and for Bcl-6 (clone GI19E/A8) to detect GCs. These immunohistochemical stainings were manually performed using a standardized procedure as previously described.[21,25] Immunohistochemical dual staining for IgA/IgG with polyclonal antibodies was performed on an automated staining platform (BenchMark XT, Ventana Medical Systems) following the manufacturer's protocols.

Histopathological analyses

All slides were digitized using a whole slide image scanner (Philips), and images were stored on a central image server. The total area of the biopsy was measured digitally on H&E-stained sections. The total number of foci was counted by an experienced pathologist, and FS was calculated. Because pre-

LELs and LELs can be detected by assessing the presence of intraepithelial B lymphocytes within striated ducts, the presence of $CD20^+$ intraepithelial B lymphocytes was analysed by using digital image analysis (DIA). Images from the CD20- and hmwCK-stained sections were loaded into the DIA platform Visiopharm Integrator System. After alignment of the consecutive hmwCK- and CD20-stainedimages, intraepithelial $CD20^+$ B lymphocytes were detected by a DIA algorithm as described before.[21] In case of difficulties with aligning the CD20 and hmwCK images, the presence of intraepithelial $CD20^+$ B lymphocytes was evaluated manually on the digitized slides. For the presence of a plasma cell shift, percent-ages of IgA^+ and IgG^+ plasma cells were manually evaluated on the digital slides. A relative decrease of IgA^+ plasma cells (≤70% of all IgA^+ and IgG^+ plasma cells) in the total parenchyma was considered as a plasma cell shift.[23] GCs, defined as a cluster of five or more adjacent Bcl-6^+ cells,[25] were manually detected and counted on digitised slides.

Statistical analyses

Patient characteristics were described as mean ± SD or number (%) as appropriate and were compared between patients with pSS and patients with non-SS sicca using the independent samples t-test and chi-square or Fisher's exact test. P values of less than 0.05 were considered statistically significant. The prevalence of histopathological features was calculated in patients with pSS and patients with non-SS sicca, and sensitivity and specificity analyses were performed for all histopathological features separately. Receiver operating curve (ROC) analysis was performed to determine the optimal cutoff value for the number of histopathological features according to the highest Youden's index. The performance of the ACR/EULAR criteria after adjusting the biopsy item (two or more histopathological features instead of FS ≥1 only) to predict expert categorisation was evaluated with the area under the ROC curve (AUC), which was interpreted as no discrimination (0–0.5), poor (0.5–0.7), fair (0.7–0.8), good (0.8–0.9), or excellent (0.9–1.0) accuracy. All analyses were performed in IBM SPSS Statistics, version 28.

RESULTS

Inclusion and patient characteristics

In total, 113 consecutive patients from a prospective diagnostic cohort suspected of having pSS, were evaluated. Ten patients were excluded from this study due to the presence of an associated autoimmune disease (n = 7), positive serology for hepatitis C (n = 2), or the presence of sclerotic sialadenitis in the labial gland biopsy (n = 1). Of the remaining 103 included patients, 38 patients (37%) were categorised as having pSS according to the expert panel, and 65 patients (63%) were categorised as having non-SS sicca. Patient characteristics of both patient groups are shown in Table 1.

Table 1. Patient characteristics of included patients in the prospective diagnostic sicca cohort.

	Primary Sjogren's patients (expert opinion) N=38	Non-SS sicca patients (expert opinion) N=65	P-value
Female, n (%)	37 (94%)	56 (86%)	0.087
Age at time of biopsy	52.6 ± 14.8	49.4 ± 12.7	0.243
Presence of anti-SSA	31 (82%)	7 (11%)	*<0.001*
Labial gland biopsy FS≥1	31 (82%)	8 (12%)	*<0.001*
Parotid gland biopsy FS≥1*	22 (63%)	1 (2%)	*<0.001*
Schirmer ≤5 mm/5min	31 (82%)	38 (59%)	*0.016*
OSS ≥ 5*	17 (47%)	8 (12%)	*<0.001*
UWS ≤ 0.1 ml/min	22 (58%)	26 (40%)	0.079

OSS: Ocular Staining Score; UWS: Unstimulated Whole Saliva. *97 patients underwent paired labial and parotid gland biopsies in this cohort, OSS was available in 101 patients.

High prevalence of histopathological features other than positive focus score in pSS patients

In the group categorised as having pSS, 31 of 38 patients (82%) had a positive FS (≥1). Furthermore, pre-LELs and LELs, assessed by the presence of intraepithelial B lymphocytes, were found in 68%, followed by the presence of a plasma cell shift in 63% and the presence of GCs in 24% of patients with pSS (Figure 2A; Table 2). In the subgroup of patients categorised as having pSS with an FS <1 (7 of 38), two patients showed presence of histopathological features

associated with pSS other than a positive FS: One patient showed a presence of pre-LELs and LELs/intraepithelial B lymphocytes and a plasma cell shift, and the other patient showed a presence of pre-LELs and LELs/intraepithelial B lymphocytes only. The other five patients with pSS with an FS of <1 did not reveal any of the other three histopathological features (Figure 2C).

In the group categorised as having non-SS sicca by the experts, 8 of 65 patients (12%) had a positive FS. Importantly, the biopsies of these eight patients with non-SS did not exhibit other histopathological features besides the positive FS (Figure 2B and 2D). In only one additional patient with non-SS sicca with an FS <1.0 (FS = 0.6), pre-LELs and LELs/intraepithelial B lymphocytes were found. Plasma cell shifts and GCs were completely absent in patients with non-SS sicca, leading to a specificity of 100% for these two features (Figure 2D, Table 2).

Combination of histopathological features improves diagnostic specificity

ROC analysis for the number of positive histopathological features in the labial gland biopsy (calculated as the sum [score 0–4]) of all four features (ie, FS ≥ 1, presence of pre-LELs and LELs assessed by the presence of intraepithelial B lymphocytes, presence of a plasma cell shift, and presence of GCs) showed excellent accuracy for predicting expert categorisation, with an AUC of 0.919 (95% confidence interval0.850–0.998) and an optimal cutoff value of two or more histopathological features, including any combination of the four features. The presence of two or more features showed almost similar sensitivity compared to FS alone (79% for two or more features vs 82% for FS alone), whereas the specificity increased with 12% to 100% because none of the patients with non-SS sicca showed more than one histopathological feature (Figure 2D; Table 2). Also, the positive predictive value increased (100% for two or more features vs 80% for FS alone), and the negative predictive value remained 89% for both FS and two or more histologic features (Table 2). The majority of patients with pSS (29 of 38) showed a positive FS together with one or two of the other features, whereas one patient with pSS had a biopsy that showed two histopathological features without a positive FS (presence of pre-LELs and LELs/intraepithelial B lymphocytes and plasma cell shift; Figure 2C).

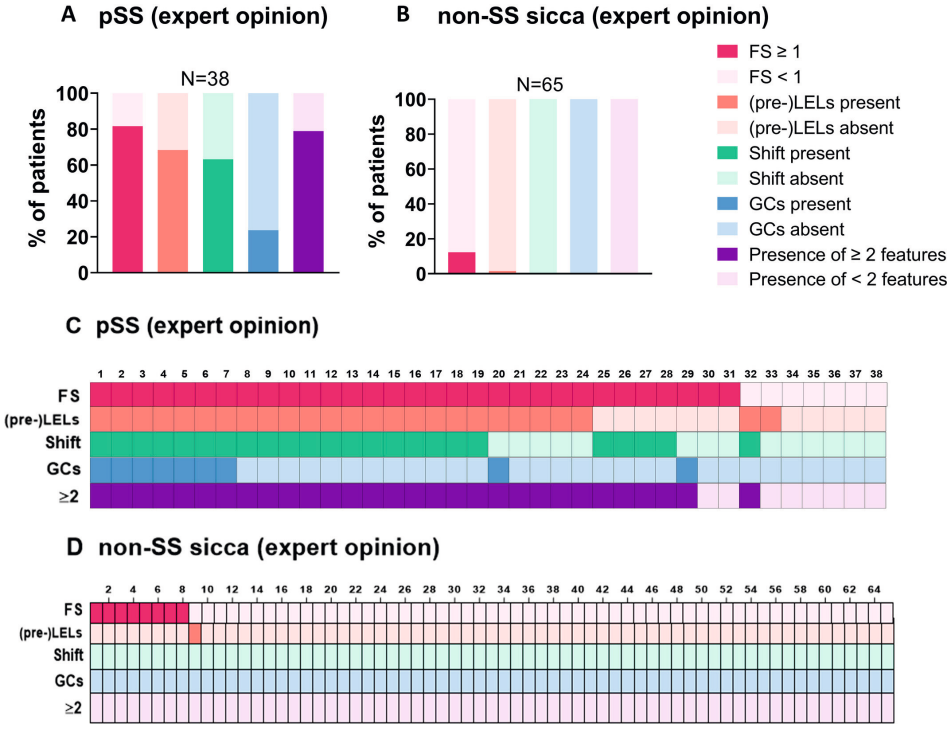


Figure 2. Presence of histopathological features in labial gland biopsies of pSS and non-SS sicca patients. (A, B) Percentages of pSS patients (A) and non-SS sicca patients (B) with presence of the following histopathological features within labial gland biopsies: a positive focus score (FS≥1), presence of (pre-)LELs/intraepithelial B-lymphocytes, presence of a plasma cell shift (shift), presence of germinal centres (GCs), and presence of ≥2 of these 4 histopathological features. (C, D) Presence of the four histopathological features presented for each individual pSS patient (C) and non-SS sicca patient (D) separately.

Table 2. Sensitivity, specificity, positive and negative predictive value of histopathological features for the diagnosis of pSS.

		Sensitivity	Specificity	PPV	NPV
Focus score≥1	N=103	82% (31/38)	88% (57/65)	80% (31/39)	89% (57/64)
(pre-)LELs/Intraepithelial B-lymphocytes	N=101	68% (26/38)	98% (62/63)	96% (26/27)	84% (62/74)
Plasma cell shift	N=103	63% (24/38)	100% (65/65)	100% (24/24)	82% (65/79)
Germinal centres	N=102	24% (9/38)	100% (64/64)	100% (9/9)	69% (64/93)
≥2 histopathological features (any combination)	N=102	79% (30/38)	100% (64/64)	100% (30/30)	89% (64/72)

PPV: positive predictive value; NPV: negative predictive value; LELs: lymphoepithelial lesions

Combination of histopathological features improves performance of ACR-EULAR classification

Of the patients categorised as having non-SS sicca by the experts with an FS ≥1.0 (n = 8), seven were classified as having pSS according to the ACR/EULAR criteria (Figure 3A). These seven patients had relatively low ACR/EULAR scores (4–6), indicating that these patients would not fulfill the ACR/EULAR criteria without a positive labial gland biopsy (Figure 3B). Furthermore, these patients had lower OSS and higher Schirmer's test results and UWS rates compared to patients categorised as having pSS by the experts (Figure 3C–G). Also, most of these patients were anti-SSA negative (seven of eight), except for one patient with a low anti-SSA titre of <25 (Figure 3C). The patients categorised as having non-SS sicca by the experts with a positive FS were not older compared to the other groups, and no differences were found in smoking behaviour among the groups.

The sensitivity and the specificity of the original ACR/EULAR criteria (FS ≥1) were 92% and 84% in this cohort, respectively. When the original biopsy item was replaced for the biopsy item presence of two or more histopathological features, sensitivity decreased with 2% to 90%, but, importantly, the specificity increased by 11%, resulting in a specificity of 95%. The optimal cutoff value of the ACR/EULAR criteria remained 4 points (Table 3). The slight decrease in sensitivity is explained by one patient who changed from being diagnosed with pSS (original ACR/EULAR criteria) to non-SS (adjusted ACR/EULAR criteria). This patient had an FS of 2.6 without other histopathological features, was SSA negative, and was categorised as having pSS by the experts (Table 3). Importantly, the increase in specificity is explained by seven patients changing from being diagnosed with pSS (original ACR/EULAR criteria) to non-SS (adjusted ACR/EULAR criteria). All these seven patients had a positive FS in their labial gland biopsy only without any of the other three histopathological features, and these patients were all categorised as having non-SS sicca by the experts.

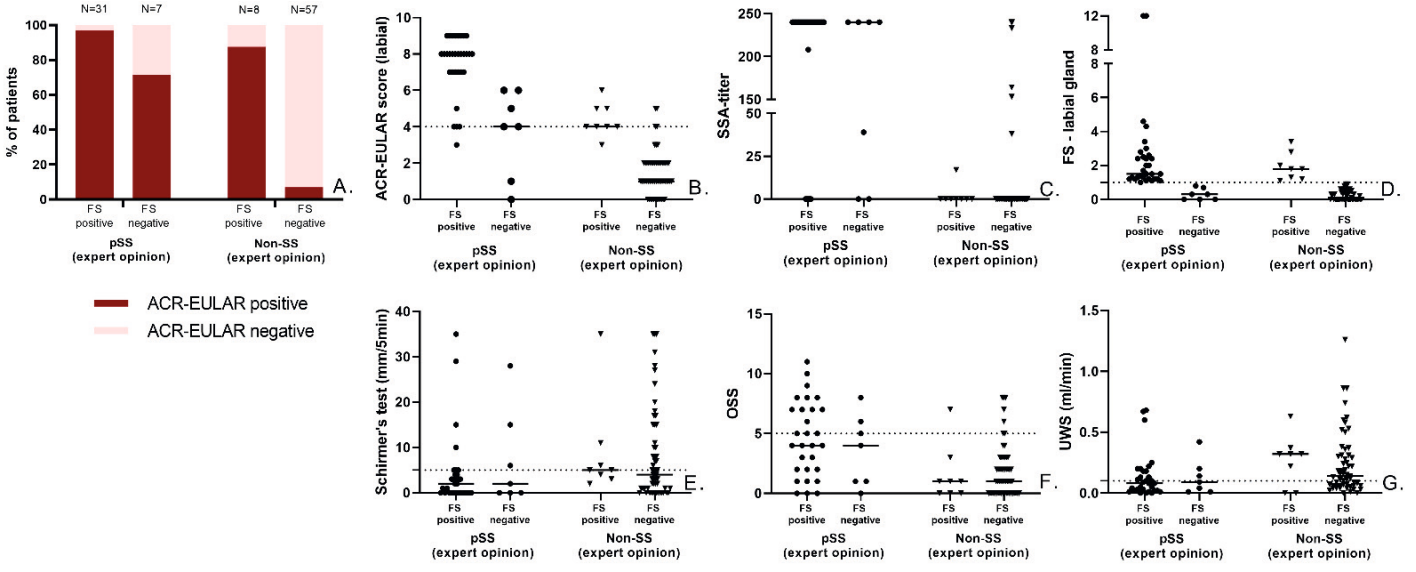


Figure 3. Fulfilment of ACR-EULAR criteria and clinical characteristics of patients divided in four groups based on expert classification and focus score. (A) Fulfilment of the ACR-EUALR criteria and (B) the ACR-EULAR scores (range 0-9) presented in four groups based on expert classification (pSS versus non-SS sicca) and the focus score (FS; positive versus negative). (C) Focus scores (FS) in the labial biopsy, and (D) SSA-titers, (E) ocular staining scores (OSS), (F) Schirmer's test scores and (G) unstimulated whole saliva (UWS), all presented for the same four groups based on expert classification (pSS versus non-SS sicca) and positivity of the focus score.

Table 3. Analysis of cut-off value of the adjusted ACR-EULAR score when FS≥1 is replaced by presence of ≥2 histological features.

	Cut-off value	Sensitivity	Specificity
Original ACR-EULAR score (FS≥1)	3	94.7%	78.1%
	4	**92.1%**	**84.4%**
	5	78.9%	93.8%
Adjusted ACR-EULAR score (≥2 histopathological features)*	3	92.1%	89.1%
	4	**89.5%**	**95.3%**
	5	81.6%	98.4%

ACR-EULAR: American College of Rheumatology – European League Against Rheumatism
*≥2 histopathological features include the four pSS specific features: focus score ≥1, presence of (pre-)LELs/intraepithelial B-lymphocytes, presence of a plasma cell shift, presence of germinal centres.

DISCUSSION

In a diagnostic cohort of patients with sicca clinically suspected of having pSS, we showed here that the specificity and the positive predictive value of the labial gland biopsy both increase to 100% when, besides FS ≥1, the following histopathological features are taken into account: presence of pre-LELs and LELs (assessed by the presence of intraepithelial B lymphocytes), a plasma cell shift, and GCs. The presence of two or more histopathological features in the labial gland biopsy performed was best to diagnose pSS according to expert opinion. When we replaced the biopsy item (FS ≥1) in the current ACR/EULAR criteria with the biopsy item "presence of two or more histopathological features", the specificity of the ACR/EULAR criteria increased by >10%. Thus, the diagnostic accuracy of the labial gland biopsy as well as the performance of the ACR/EULAR criteria increases when other histopathological features besides FS are taken into account. This leads to a lower number of false-positive labial gland biopsies and thereby a lower number of patients with misclassified pSS.

In this cohort, patients who solely had a positive FS in the labial gland biopsy without one of the other three histopathological features were mostly categorised as having non-SS sicca by the experts. This indicates that patients are at risk of having a false-positive biopsy for pSS when the histopathological criteria for pSS solely rely on the FS. Seven of the eight patients with a

positive biopsy based on FS ≥1 who were categorised as having non-SS by the experts, however, did fulfill the ACR/EULAR criteria for having pSS (including the labial gland biopsy result). For these patients, expert categorisation as having non-SS sicca was based on a combination of clinical characteristics, serology, and results of ocular and oral tests. These patients with non-SS sicca were mostly SSA negative and had higher Schirmer scores, higher UWS, and lower OSS (Figure 3). Also, in most (six of seven) of these patients, the FS of the parotid gland was <1. Together, this indicates that these patients may represent a subgroup of patients with sicca complaints but without specific clinical characteristics of pSS except for a (false) positive labial gland biopsy. It is known that salivary gland biopsies can be positive for pSS in healthy individuals without sicca symptoms (≤15%).[8] Lymphocytic infiltrates in the salivary glands are found in patients with diseases other than pSS, such as patients with myasthenia gravis, bone marrow transplant recipients, patients with HIV, and patients with hepatitis C.[11–13,28] The reason for the presence of lymphocytic infiltrates in the glandular tissue of patients with non-SS sicca potentially causing false-positive biopsies for pSS is not completely understood. These infiltrates might be caused by local injury, such as chewing or biting or subclinical infections.[8]

Classification criteria for pSS are developed and widely used for the selection of patients for clinical studies and trials.[2,29] The pathophysiology of sicca symptoms in the subgroup of misclassified patients with pSS with a false-positive biopsy could be different from patients with pSS. Therefore, this could influence studies that investigate pSS pathogenesis or treatment efficacy. Preferably, the misclassified patients should not be included in these studies. In contrast to the FS, the presence of pre-LELs and LELs/intraepithelial B lymphocytes, a plasma cell shift, and GCs were highly specific for pSS in the presented cohort of patients with sicca. These three features are all signs of hyperactivity of B lymphocytes, which is a hallmark of pSS, and seem to better reflect the severity of the lymphocytic infiltrate than FS alone. Adjusting the biopsy item to two or more histopathological features in the ACR/EULAR criteria resulted in a higher specificity and a lower number of patients with misclassified (false-positive) pSS. Sensitivity, however, slightly decreased in the adjusted ACR/EULAR criteria. This decrease in sensitivity was caused by only one patient with pSS who was reclassified from having

pSS (original criteria with FS only) to non-SS (adjusted criteria with two or more histopathological features). In comparison, previous studies showed that adding salivary gland ultrasonography to the ACR/EULAR criteria only slightly changed the performance but increased feasibility of the criteria.[30,31] By taking the four histopathological criteria into account instead of FS alone, the performance of the ACR/EULAR criteria clearly improved. Regarding feasibility, the immunohistochemistry to detect pre-LELs and LELs/intraepithelial B lymphocytes, a plasma cell shift, and GCs can be performed in most diagnostic pathology laboratories. Detection of intraepithelial B lymphocytes can also be performed manually in case of an absence of DIA software.[19] Before adjusting the biopsy item in the ACR/EULAR criteria, our findings should be validated in another diagnostic cohort. However, the current results already show that for diagnosis, clinicians (rheumatologists and pathologists) should be cautious when interpreting the results of biopsies with only a positive FS (and no other pSS-related histopathological features) because this may lead to a false-positive diagnosis of pSS in the absence of serum anti-SSA antibodies.

A strength of this study is the use of expert opinion as the gold standard. Although the FS was included in the clinical vignettes, the experts were blinded for the other three histopathological features and the diagnosis of the treating physician. Blinding the experts for the additional histopathological features is essential to evaluate its diagnostic accuracy. Blinding for FS would have resulted in categorisation difficulties and misclassified patients because FS plays an important role in diagnosing pSS. Furthermore, the use of an expert panel and vignettes with extensive clinical information minimised the impact of circular reasoning compared to using the classification criteria as the gold standard.[5] Because parotid gland biopsies are part of the standard diagnostic workup in our centre, the experts also had access to the parotid gland FS in most patients (Table 1), which could have influenced expert opinion. However, by including both the labial and parotid FSs in the vignettes, they stayed as close as possible to the normal diagnostic workup. Besides labial and parotid FSs, the experts took the combination of all clinical characteristics, serological results, and oral and ocular examination into account, thereby limiting the impact of the parotid FS on expert categorisation. This is also illustrated by the fact that some patients in this cohort had a positive labial gland biopsy FS and a corresponding negative parotid gland biopsy FS but

were categorised as having SS by the experts (10 of 97). Of these 10 patients, 4 were SSA negative, and these patients were categorised as having pSS on the combination of clinical features included in the vignettes (even without SSA antibodies and with a negative parotid FS). Importantly, our analysis showed that three of these four patients did have a presence of other histopathological features besides FS in their labial gland biopsy, further underlining the increase in specificity that these features give.

In conclusion, these data show that the diagnostic accuracy of the labial gland biopsy to diagnose pSS increases when the histopathological features pre-LELs and LELs, assessed by the presence of intraepithelial B lymphocytes, a plasma cell shift, and GCs are taken into account besides the FS, leading to a lower number of false-positive labial gland biopsies in patients clinically suspected of having pSS.

REFERENCES

1. Brito-Zerón P, Baldini C, Bootsma H, Bowman SJ, Jonsson R, Mariette X, et al. Sjögren syndrome. Nat Rev Dis Primers. 2016 Jul 7;2(1):16047.
2. Shiboski CH, Shiboski SC, Seror R, Criswell LA, Labetoulle M, Lietman TM, et al. 2016 American College of Rheumatology/European League Against Rheumatism Classification Criteria for Primary Sjögren's Syndrome: A Consensus and Data-Driven Methodology Involving Three International Patient Cohorts. Arthritis & Rheumatology. 2017 Jan 26;69(1):35–45.
3. Greenspan JS, Daniels TE, Talal N, Sylvester RA. The histopathology of Sjögren's syndrome in labial salivary gland biopsies. Oral Surgery, Oral Medicine, Oral Pathology. 1974 Feb;37(2):217–29.
4. Daniels TE, Cox D, Shiboski CH, Schiødt M, Wu A, Lanfranchi H, et al. Associations between salivary gland histopathologic diagnoses and phenotypic features of Sjögren's syndrome among 1,726 registry participants. Arthritis Rheum. 2011;63(7):2021–30.
5. Guellec D, Cornec D, Jousse-Joulin S, Marhadour T, Marcorelles P, Pers JO, et al. Diagnostic value of labial minor salivary gland biopsy for Sjögren's syndrome: a systematic review. Autoimmun Rev. 2013 Jan;12(3):416–20.
6. Morbini P, Manzo A, Caporali R, Epis O, Villa C, Tinelli C, et al. Multilevel examination of minor salivary gland biopsy for Sjögren's syndrome significantly improves diagnostic performance of AECG classification criteria. Arthritis Res Ther. 2005 Jan 17;7(2):R343.
7. Obinata K, Sato T, Ohmori K, Shindo M, Nakamura M. A comparison of diagnostic tools for Sjögren syndrome, with emphasis on sialography, histopathology, and ultrasonography. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology. 2010 Jan;109(1):129–34.
8. Radfar L, Kleiner DE, Fox PC, Pillemer SR. Prevalence and clinical significance of lymphocytic foci in minor salivary glands of healthy volunteers. Arthritis Care Res (Hoboken). 2002 Oct 15;47(5):520–4.
9. Segerberg-Konttinen M, Konttinen YT, Bergroth V. Focus score in the diagnosis of Sjögren's syndrome. Scand J Rheumatol Suppl. 1986;61:47–51.
10. Drosos AA, Andonopoulos AP, Costopoulos JS, Papadimitrio CS, Moutsopoulos HM. Prevalence of primary Sjögren's Syndrome in an elderly population. Rheumatology. 1988;27(2):123–7.
11. Lindahl G, Hedfors E. Focal lymphocytic infiltrates of salivary glands are not confined to Sjögren's syndrome. Scand J Rheumatol Suppl. 1986;61:52–5.
12. Lindahl G, Lönnquist B, Hedfors E. Lymphocytic infiltration and HLA-DR expression of salivary glands in bone marrow transplant recipients: a prospective study. Clin Exp Immunol. 1988 May;72(2):267–73.
13. Ihrler S, Zietz C, Sendelhofert A, Menauer F, Blasenbreu-Vogt S, Löhrs U. Zur Differenzialdiagnose lymphoepithelialer Speicheldrüenläsionen. Pathologe. 2000 Nov 15;21(6):424–32.
14. Takeda Y, Komori A. Focal lymphocytic infiltration in the human labial salivary glands: a postmortem study. Journal of Oral Pathology & Medicine. 1986 Feb 28;15(2):83–6.
15. Sharma R, Chaudhari KS, Kurien BT, Grundahl K, Radfar L, Lewis DM, et al. Sjögren Syndrome without Focal Lymphocytic Infiltration of the Salivary Glands. J Rheumatol. 2020 Mar;47(3):394–9.
16. MONSALVE DM, ANAYA JM. With Minor Salivary Gland Biopsy in Sjögren Syndrome, Is a Negative Result Possible? J Rheumatol. 2020 Mar 1;47(3):310–2.

17. Trivedi A, Cornejo KM, O'Donnell P, Dresser K, Deng A. Employing immunohistochemical staining to labial minor salivary gland biopsies from patients with Sjogren's syndrome increases diagnostic certainty. Journal of Oral Pathology & Medicine. 2021 Jan 27;50(1):98–102.
18. Bredahl SK, Reibel J, Pedersen AML. Value of multilevel sectioning of labial salivary gland biopsies in the diagnosis of Sjögren's syndrome. Oral Surg Oral Med Oral Pathol Oral Radiol. 2021 Jan;131(1):85–91.
19. van Ginkel MS, Haacke EA, Bootsma H, Arends S, van Nimwegen JF, Verstappen GM, et al. Presence of intraepithelial B-lymphocytes is associated with the formation of lymphoepithelial lesions in salivary glands of primary Sjögren's syndrome patients. Clin Exp Rheumatol. 2019;37 Suppl 118(3):42–8.
20. Ihrler S, Zietz C, Sendelhofert A, Riederer A, Löhrs U. Lymphoepithelial duct lesions in Sjögren-type sialadenitis. Virchows Archiv. 1999 Apr 22;434(4):315–23.
21. van Ginkel MS, van der Sluis T, Bulthuis MLC, Buikema HJ, Haacke EA, Arends S, et al. Digital image analysis of intraepithelial B-lymphocytes to assess lymphoepithelial lesions in salivary glands of Sjögren's syndrome patients. Rheumatology. 2022 Dec 23;62(1):428–38.
22. Leroy JP, Pennec YL, Letoux G, Youinou P. Lymphocytic infiltration of salivary ducts: a histopathologic lesion specific for primary Sjögren's syndrome? Arthritis Rheum. 1992 Apr;35(4):481–2.
23. Zandbelt MM. The synergistic value of focus score and IgA% score of sublabial salivary gland biopsy for the accuracy of the diagnosis of Sjogren's syndrome: a 10-year comparison. Rheumatology. 2002 Jul 1;41(7):819–23.
24. Bodeutsch C, De Wilde PCM, Kater L, Van Houwelingen JC, Van Den Hoogen FHJ, Kruize AA, et al. Quantitative immunohistologic criteria are superior to the lymphocytic focus score criterion for the diagnosis of Sjögren's syndrome. Arthritis Rheum. 1992 Sep 16;35(9):1075–87.
25. Nakshbandi U, Haacke EA, Bootsma H, Vissink A, Spijkervet FKL, van der Vegt B, et al. Bcl6 for identification of germinal centres in salivary gland biopsies in primary Sjögren's syndrome. Oral Dis. 2020 Apr 28;26(3):707–10.
26. Kroese FGM, Haacke EA, Bombardieri M. The role of salivary gland histopathology in primary Sjögren's syndrome: promises and pitfalls. Clin Exp Rheumatol. 2018;36 Suppl 1(3):222–33.
27. van Nimwegen JF, van Ginkel MS, Arends S, Haacke EA, van der Vegt B, Sillevis Smitt-Kamminga N, et al. Validation of the ACR-EULAR criteria for primary Sjögren's syndrome in a Dutch prospective diagnostic cohort. Rheumatology. 2018 May 1;57(5):818–25.
28. Maldonado JO, Beach ME, Wang Y, Perez P, Yin H, Pelayo E, et al. HCV Infection Alters Salivary Gland Histology and Saliva Composition. J Dent Res. 2022 May 20;101(5):534–41.
29. Arends S, de Wolff L, Deroo L, Verstappen GM, Vissink A, Kroese FGM, et al. Selection of study endpoints and patients for clinical trials in primary Sjögren's syndrome. Vol. 40, Clinical and experimental rheumatology. Italy; 2022. p. 2225–32.
30. van Nimwegen JF, Mossel E, Delli K, van Ginkel MS, Stel AJ, Kroese FGM, et al. Incorporation of Salivary Gland Ultrasonography Into the American College of Rheumatology/European League Against Rheumatism Criteria for Primary Sjögren's Syndrome. Arthritis Care Res (Hoboken). 2020 Apr;72(4):583–90.
31. Jousse-Joulin S, Gatineau F, Baldini C, Baer A, Barone F, Bootsma H, et al. Weight of salivary gland ultrasonography compared to other items of the 2016 ACR/EULAR classification criteria for Primary Sjögren's syndrome. J Intern Med. 2020 Feb 12;287(2):180–8.

ACKNOWLEDGEMENTS

Several authors of this publication are members of the European Reference Network ReCONNET.

FUNDING STATEMENT

Supported by the Jan-Kornelis de Cock Foundation (Groningen, The Netherlands) and in part by the Innovative Medicine Initiative-2 (NECESSITY) (grant 806975). This publication is part of project 09150162010166 of the Veni research program (to Dr. Verstappen), which is financed by the Dutch Research Council. There was no involvement of these funding sources in the study design, data collection, analysis, and interpretation of data, or writing of this report.

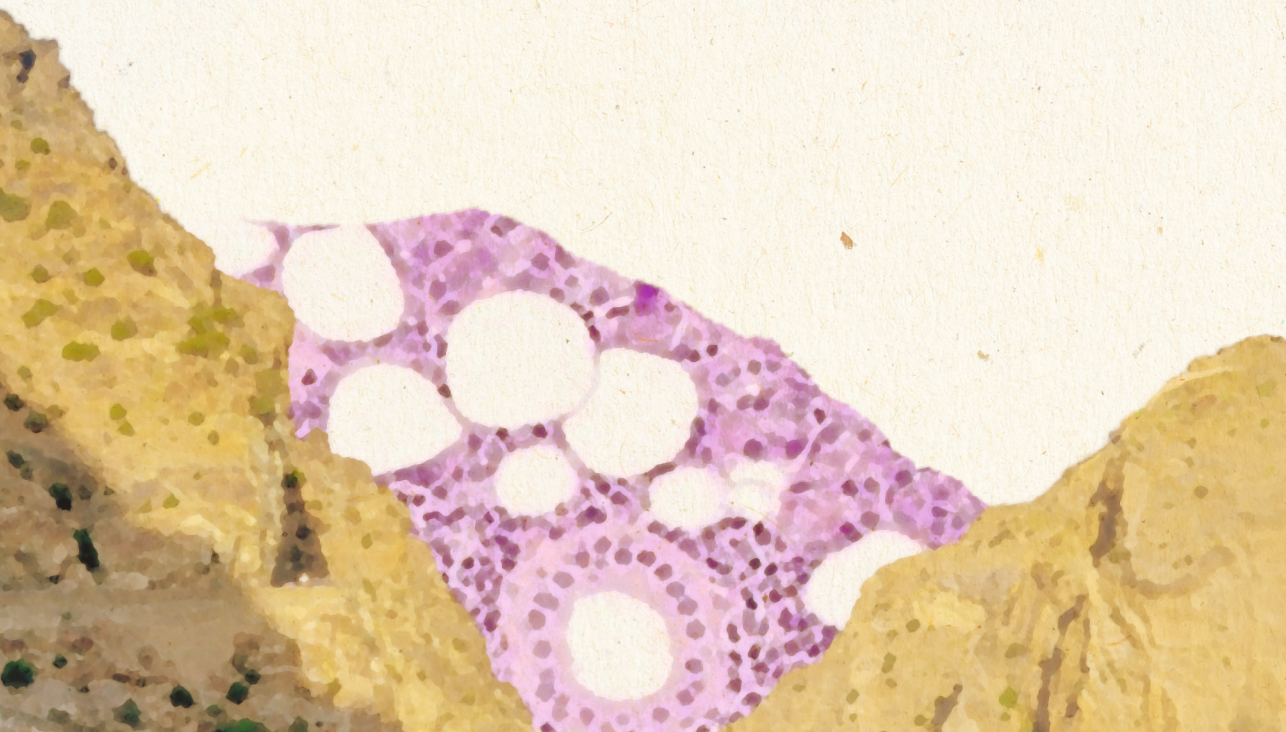
AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. van Ginkel had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design
van Ginkel, Nakshbandi, Arends, Haacke, Verstappen, van Nimwegen, Spijkervet, Vissink, Bootsma, van der Vegt, Kroese.

Acquisition of data
van Ginkel, Nakshbandi, Liefers, Brouwer, Stel, Bootsma, van der Vegt.

Analysis and interpretation of data
van Ginkel, Nakshbandi, Arends, van der Vegt, Kroese.



CHAPTER 7

Addition of other histopathological key features besides focus score increases the diagnostic accuracy of parotid gland biopsies in Sjögren's disease

U. Nakshbandi[1], S. Arends[1], M.S. van Ginkel[1], E.A. Haacke[2], S.C. Liefers[1], G.M.P.J. Verstappen[1], J.F. van Nimwegen[1], E. Brouwer[1], A.J. Stel[1], F.K.L. Spijkervet[3], A. Vissink[3], B. van der Vegt[2], F.G.M. Kroese[1], H. Bootsma[1]

Departments of [1]Rheumatology and Clinical Immunology, [2]Pathology and Medical Biology and [3]Oral and Maxillofacial Surgery, University of Groningen and University Medical Centre Groningen, Groningen, the Netherlands

Submitted

Major or minor salivary gland biopsies play an important role for both clinical diagnosis and classification of Sjögren's disease (SjD).[1,2] This importance is reflected by its prominent place in the ACR-EULAR classification criteria.[3] However, in the current classification criteria a biopsy is considered positive solely based on the presence of focus score ≥1. A focus is defined as a periductal infiltrate consisting of ≥50 lymphocytes per 4 mm^2 salivary gland tissue.[4,5] Other histopathological Sjögren-related features may also be present, namely the presence of intra-epithelial B-lymphocytes with or without ductal hyperplasia (hereafter referred to as (pre-)lymphoepithelial lesions (LELs)), a relative increase in the number of IgG plasma cells (the so-called plasma cell shift) and germinal centres (GCs), but are currently not considered in the classification of SjD.[6–9] In our earlier work, we observed that the diagnostic accuracy of the *labial* gland biopsy increased when these additional features were taken into account.[10] Presence of ≥2 (out of 4) histopathological features resulted in almost comparable sensitivity to FS alone (79% for ≥2 features versus 82% for FS alone), but the specificity improved from 88% to 100%, thereby reducing the number of false positive cases.

In SjD, a parotid gland biopsy is a good and safe alternative for a labial gland biopsy.[11] Also, the applicability of the parotid gland biopsy has increased as ultrasound guided core needle biopsies seem to show comparable results to incisional parotid gland biopsies.[12] While histopathology between labial and parotid gland biopsies is overall comparable, some important differences have been identified: parotid glands show more B-lymphocyte related features (e.g. more $CD20^+$ B-lymphocytes, more GCs/mm^2 and more severe LELs) and labial glands exhibit more inflammation unrelated to SjD.[13] Therefore, in this study, we aimed to assess the impact of these additional histopathological features on the diagnostic accuracy of the parotid gland biopsy in patients suspected for SjD.

Patients from a diagnostic sicca cohort with clinically suspected SjD, who underwent a parotid gland biopsy, were included as earlier described.[13] Patients were categorised as SjD or non-SjD sicca based on anonymised clinical vignettes scored by an expert panel. Experts based their opinion on clinical history, physical examination, serological parameters, FS of labial and parotid gland biopsy, as well as the results of evaluation by an oral- and maxillo-facial surgeon and ophthalmologist.

All parotid gland biopsies were similarly processed, stained and analysed for the presence of FS ≥1, (pre-)LELs, IgG plasma cell shift and GCs. The accuracy and optimal cut-off value with sensitivity and specificity for the FS alone and for the number of histopathological features (FS≥1, (pre-)LELs, IgG plasma cell shift and GCs) (range 0-4) in the parotid gland biopsies to predict SjD or non-SjD sicca based on expert opinion was determined with Receiver Operating Characteristic (ROC) analysis. For detailed patient inclusion and exclusion criteria, staining protocols and statistical analysis see our earlier work.[10,13]

Of the 99 patients, 36 patients were categorised as SjD and 63 as non-SjD sicca patients by the experts. ROC analysis for FS in the parotid gland biopsies showed excellent accuracy to predict expert distinction between SjD and non-SjD sicca with an AUC of 0.828 (95% CI 0.732-0.924). The optimal cut-off value for this distinction in our cohort was a FS of 0.9, with a sensitivity of 67% and specificity of 97%. However, results for the currently applies cut-off value of 1.0 were similar, with a sensitivity of 64% and specificity of 98%. In the group categorised as SjD, 23 of 36 patients (64%) had a FS ≥1, (pre-)LELs were found in 17 patients (47%), followed by plasma cell shift in 15 patients (42%) and GCs in 10 patients (28%)(table 1, figure 1A).

Table 1. Sensitivity and specificity of histopathological features in parotid salivary gland biopsies for the diagnosis of SjD according to expert opinion (n=99).

	Sensitivity	Specificity
Focus score ≥1	64% (23/36)	98% (62/63)
(Pre-) lymphoepithelial lesions	47% (17/36)	100% (63/63)
Plasma cell shift	42% (15/36)	98% (62/63)
Germinal centres	28% (10/36)	100% (63/63)
≥1 histopathological features	72% (26/36)	97% (61/63)
≥2 histopathological features (any combination)	53% (19/36)	100% (63/63)

In the subgroup of patients categorised as SjD with FS<1 (13/36), three patients showed presence of additional histopathological features: all three showed a plasma cell shift, one patient also had (pre-)LELs and a GCs. The remaining 11 patients did not reveal any of the additional histopathological features (figure 1C). In the group categorised as non-SjD sicca by the experts,

only 1 of 63 patients (2%) had a FS≥1. A plasma cell shift was found in one non-SjD sicca patient, leading to a specificity of 98% (figure 1B-D). No (pre-) LEL and GCs were observed in non-SjD patients.

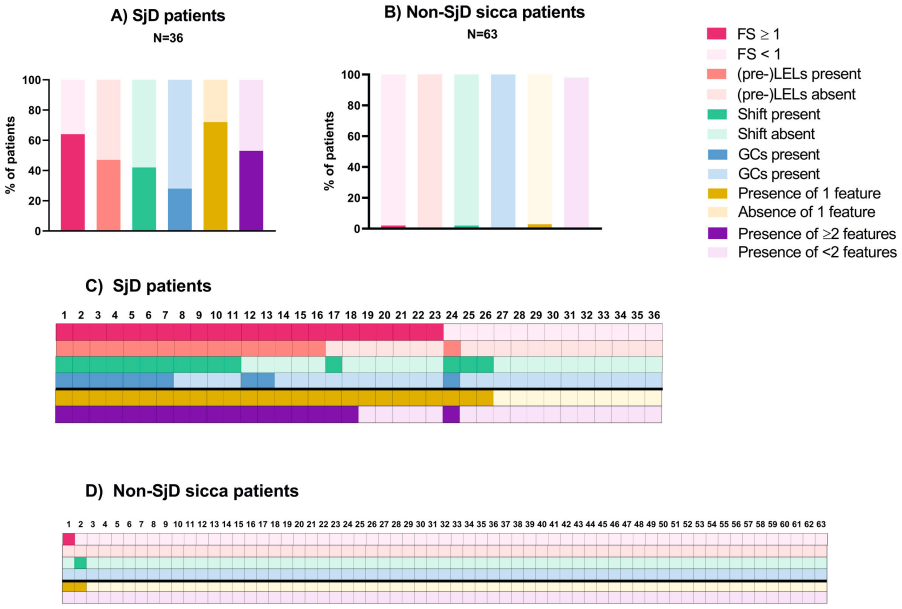


Figure 1. Presence of histopathological features in parotid gland biopsies of SjD and non-SjD sicca patients according to expert opinion. Percentages of SjD patients (A) and non-SjD sicca patients (B) with presence of a positive focus score (FS≥1), (pre-)LELs, plasma cell shift (shift), germinal centres (GCs), 1 feature and of ≥2 of these 4 histopathological features. Presence of histopathological features presented for each individual SjD patient (C) and non-SjD sicca patient (D) separately.

ROC analysis for the number of positive histopathological features in the parotid gland biopsies showed excellent accuracy to predict expert categorisation of SjD with an AUC of 0.851 (95% CI 0.759-0.943), with an optimal cut off value of ≥1 feature. Compared to FS alone, the presence of ≥1 out of any of the 4 features increased the sensitivity from 64% to 72%, while the specificity remained comparable (from 98% to 97%). The presence of ≥2 histopathological features decreased the sensitivity to 53% (compared to 64% for FS alone), while specificity was similar (100% compared to 98%).

Discrepancies between diagnosis based on expert opinion and classification criteria (n=10) are shown in supplementary table 1. From the patients categorised as SjD patients with a FS<1 (n=13), 9 patients would fulfil the ACR-EULAR classification criteria without taking the biopsy into account.

Overall, the diagnostic accuracy of the parotid gland increases when additional histopathological features besides FS are taken into account. While for labial gland biopsies the specificity increased by taking other features into account, for parotid gland biopsies the sensitivity increases. This discrepancy is most likely due to the observation that in contrast to parotid glands, labial glands contain more often non-autoimmune related lymphocytic infiltrates. The specificity of the parotid gland biopsy is already very high since almost all non-SjD sicca patients showed a FS<1 (97%, 61/63).

In conclusion, we show that the parotid gland biopsy has a good sensitivity and excellent specificity to predict the diagnosis of SjD patients. In line with earlier finding for the labial gland biopsy, addition of other histopathological features besides FS also improves the diagnostic accuracy of the parotid biopsy.

REFERENCES

1. Fox RI. Sjögren's syndrome. The Lancet. 2005;366(9482):321–31.
2. Kroese FGM, Haacke EA, Bombardieri M. The role of salivary gland histopathology in primary Sjögren's syndrome: promises and pitfalls. Clin Exp Rheumatol. 2018;36 Suppl 1(3):222–33.
3. Shiboski CH, Shiboski SC, Seror R, Criswell LA, Labetoulle M, Lietman TM, et al. 2016 American College of Rheumatology/European League Against Rheumatism Classification Criteria for Primary Sjögren's Syndrome: A Consensus and Data-Driven Methodology Involving Three International Patient Cohorts. Arthritis & Rheumatology. 2017 Jan 26;69(1):35–45.
4. Chisholm DM, Mason DK. Labial salivary gland biopsy in Sjögren's disease. J Clin Pathol. 1968 Sep;21(5):656–60.
5. Greenspan JS, Daniels TE, Talal N, Sylvester RA. The histopathology of Sjögren's syndrome in labial salivary gland biopsies. Oral Surgery, Oral Medicine, Oral Pathology. 1974 Feb;37(2):217–29.
6. Ihrler S, Zietz C, Sendelhofert A, Riederer A, Löhrs U. Lymphoepithelial duct lesions in Sjögren-type sialadenitis. Virchows Archiv. 1999 Apr 22;434(4):315–23.
7. van Ginkel MS, Haacke EA, Bootsma H, Arends S, van Nimwegen JF, Verstappen GM, et al. Presence of intraepithelial B-lymphocytes is associated with the formation of lymphoepithelial lesions in salivary glands of primary Sjögren's syndrome patients. Clin Exp Rheumatol. 2019;37 Suppl 118(3):42–8.
8. Bodeutsch C, De Wilde PCM, Kater L, Van Houwelingen JC, Van Den Hoogen FHJ, Kruize AA, et al. Quantitative immunohistologic criteria are superior to the lymphocytic focus score criterion for the diagnosis of Sjögren's syndrome. Arthritis Rheum. 1992 Sep 16;35(9):1075–87.
9. Nakshbandi U, Haacke EA, Bootsma H, Vissink A, Spijkervet FKL, van der Vegt B, et al. Bcl6 for identification of germinal centres in salivary gland biopsies in primary Sjögren's syndrome. Oral Dis. 2020 Apr 28;26(3):707–10.
10. van Ginkel MS, Nakshbandi U, Arends S, Haacke EA, Liefers SC, Verstappen GM, et al. Increased Diagnostic Accuracy of the Labial Gland Biopsy in Primary Sjögren Syndrome When Multiple Histopathological Features Are Included. Arthritis & Rheumatology. 2024 Mar 9;76(3):421–8.
11. Pijpe J, Vissink A, Kallenberg CGM, Spijkervet FKL. Parotid gland biopsy compared with labial biopsy in the diagnosis of Sjogren's syndrome: reply. Rheumatology. 2006 Jul 28;46(2):371–371.
12. Deroo L, Genbrugge E, Dochy F, Creytens D, Achten H, De Boeck K, et al. Ultrasound-guided core needle biopsy and incisional biopsy of the parotid gland are comparable in diagnosis of primary Sjögren's syndrome. Rheumatology. 2023 Aug 1;62(8):2765–72.
13. Nakshbandi U, van Ginkel MS, Verstappen GMPJ, Spijkervet FKL, Arends S, Haacke EA, et al. Histopathological comparison of Sjögren-related features between paired labial and parotid salivary gland biopsies of sicca patients. Rheumatology. 2024 Oct 1;63(10):2670–7.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on re-quest from the corresponding author. The data are not publicly available.

FUNDING STATEMENT

This study was supported by the Jan-Kornelis de Cock Foundation (Groningen, the Netherlands) and in part by Innovative Medicine Initiative (IMI)-2 project number 806975 (NECESSITY). This publication is part of project number 09150162010166 of the Veni research programme of GMV, which is financed by the Dutch Research Council (NWO). UMCG is member of the European Reference Network ERN ReCONNET. There was no involvement of these funding sources in the study design, data collection, analysis and interpretation of data, or writing of this report.

CONFLICT OF INTEREST DISCLOSURE

All unrelated to the current manuscript.

AUTHOR CONTRIBUTIONS

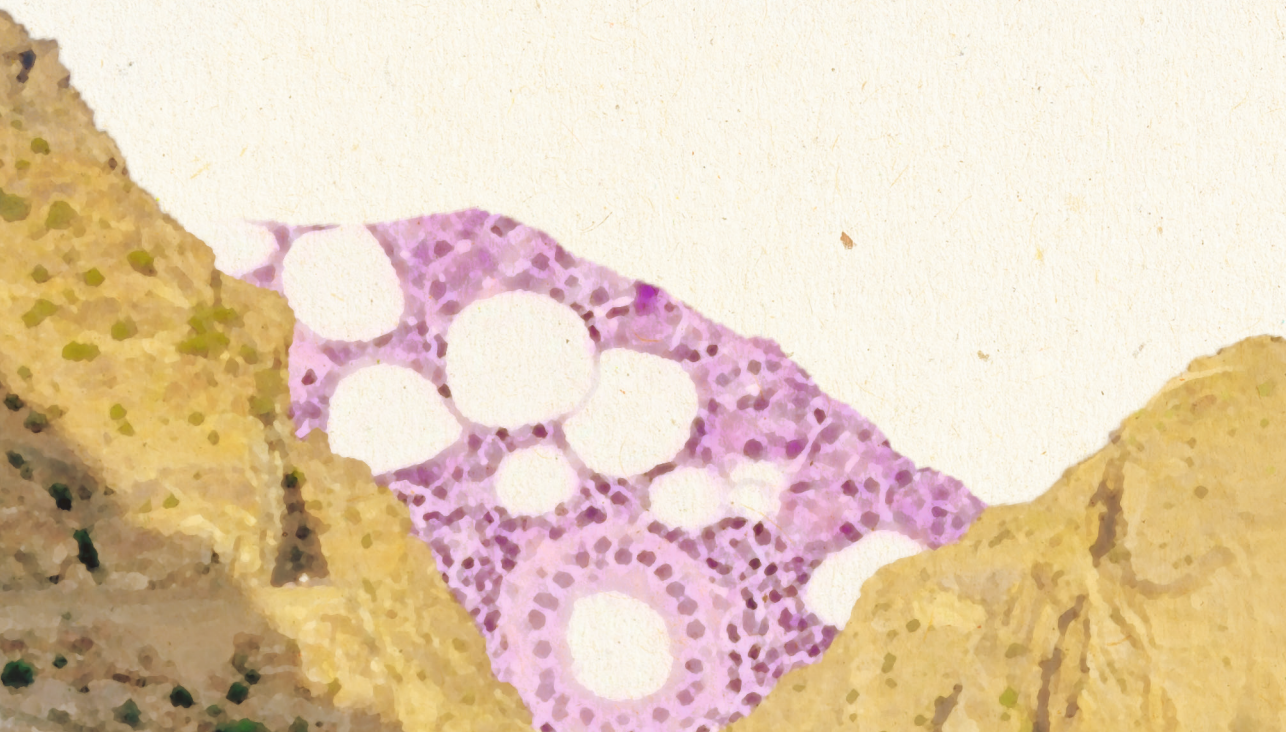
UN, MSvG, SA, FGMK, BvdV, HB and AV were involved in study concept and design. HB and AV recruited patients. FKLS performed all salivary gland biopsies. UN, MSvG, EAH, JFvN, EB, AJS and BvdV collected data. UN, MSvG, SA, EAH, SCL, GMV, AV, HB, BvdV and FGMK, analysed and interpreted the data. All authors critically reviewed the manuscript and approved the final version to be published.

SUPPLEMENTARY MATERIAL

Supplementary table 1. Detailed characteristics of sicca patients with discrepancy between expert opinion and ACR-EULAR classification criteria.

Patient number	Expert opinion	Fulfilment ACR-EULAR classification criteria (based on PSG)	Total ACR-EULAR points (based on PSG)	FS Parotid SG	FS Labial SG	Anti-SSA/Ro titer	Anti-SSB/La titer	IgG g/L	RF titer IU/ml	OSS (highest)	Schirmer's test mm/min (lowest)	UWS ml/min	SWS ml/min	Total ESSDAI score	Total ESSPRI score	Hocevar total score
1	SjD	No	-	0	**1,1**	0	0	13,1	2,8	-	**0**	**0,02**	0,43	2	8	5
2	SjD	No	1	0	**2,6**	0	0	11,9	0	3	**3**	0,60	2,33	3	4	-
3	SjD	No	1	0	**2,0**	0	0	20,2	0	**5**	10	0,18	0,93	9	-	12
4	SjD	No	1	0,4	**1,0**	0	0	18,8	0	2	**4**	0,25	0,25	5	7	20
5	SjD	No	3	**1,5**	0,7	0	0	8,6	1,2	0	6	0,42	1,09	18	8	8
6	SjD	No	3	**1,7**	**1,4**	0	0	10,7	1,2	0	29	0,20	0,86	2	4	14
7	Non-SjD Sicca	Yes	4	0	0	**164**	0	9,9	1,3	**8**	18	0,24	1,18	5	7	-
8	Non-SjD Sicca	Yes	4	0,6	0,4	**240**	0	12,3	1,9	2	**5**	0,19	0,19	7	6	3
9	Non-SjD Sicca	Yes	5	0,0	0,0	**233**	0	11,5	1,4	0	**4**	**0,01**	0,32	2	9	7
10	Non-SjD Sicca	Yes	6	**1,7**	**3,4**	**17**	0	8,4	0,6	0	35	0,32	1,65	6	9	5

FS, focus score; SSA, Sjögren's syndrome antigen A; SSB, Sjögren' syndrome antigen B; RF, Rheumatoid factor; OSS, Oscular staining score; UWS, unstimulated whole saliva; SWS, stimulated whole saliva. Scores that are positive according to the ACR-EULAR criteria are shown in bold.



CHAPTER 8

Histopathological key features in labial salivary gland biopsies and their relation with serological and functional characteristics and with expert diagnosis in patients clinically suspected for Sjögren's disease

U. Nakshbandi[1], G.M.P.J. Verstappen[1], S. Arends[1], S.C. Liefers[1], E.A. Haacke[2], M.S. van Ginkel[1], J. Alberga[3], D. Farris[4], C. Lessard[4], A. Rasmussen[4], F.K.L. Spijkervet[3], Hendrika Bootsma[1], *Frans G.M. Kroese[1], *Arjan Vissink[3], *Bert van der Vegt[2]

*Shared last authorship

Departments of [1]Rheumatology and Clinical Immunology, [2]Pathology and Medical Biology, [3]Oral and Maxillofacial Surgery, University of Groningen and University Medical Center Groningen, Groningen, the Netherlands, [4]Oklahoma Medical Research Foundation, Oklahoma, the United States

Manuscript in preparation

ABSTRACT

Objectives

This study aims to assess to what extent key histopathological features (focus score (FS), IgA/IgG plasma cell shift, (pre-)lymphoepithelial lesions (LELs) and germinal centres (GCs)), correlate with each other, with serological/functional parameters and with expert diagnosis in patients suspected for Sjögren's disease (SjD).

Methods

Consecutive patients with clinically suspected SjD who underwent a labial gland biopsy were included in this international multicentre study. Labial gland biopsies were analysed for the presence of FS≥1, (pre-)LELs, IgA/IgG plasma cell shift and GCs. Major serological (anti-SSA antibodies, anti-SSB antibodies, rheumatoid factor and total IgG levels) and functional (unstimulated whole saliva (UWS), stimulated whole saliva (SWS), Schirmer's test) parameters assessed during diagnostic work-up were collected too. Agreement between the four key histopathological features was evaluated. In addition, differences in the number of histopathological key features between patients with normal or abnormal major serological and functional parameters were assessed. Univariable and multivariable logistic regression analyses were performed to assess the relation of histopathological, serological and functional parameters with expert diagnosis (SjD or non-SjD sicca).

Results

In total, 262 patients were included, 42.4% categorised as SjD and 57.6% as non-SjD sicca by experts. Of the 262 labial salivary gland biopsies, 37.0% had FS ≥1. 27.5% (pre-)LELs, 26.5% IgA/IgG plasma cell shift and 5.0% GCs. Fair to moderate inter-feature agreement was found between FS ≥1, (pre)LELs, and IgA/IgG plasma cell shift, whereas poor agreement was observed for these features with the presence of GCs. The number of histopathological key features present was significantly associated with serological test results, but not with functional oral and ocular test results. The number of features resulted in the highest explained variance to predict diagnosis, which was higher compared

to FS ≥1. In multivariable analysis, presence of histopathological features and anti-SSA were independently associated with expert diagnosis.

Conclusion

The presence of histopathological key features (FS ≥1, LELs, IgA/IgG plasma cell shift and GCs) are generally interrelated and associated with serological parameters in patients suspected for SjDs. The assessment of additional histopathological features, besides FS, is relevant for making the diagnosis of SjD.

INTRODUCTION

Sjögren's disease (SjD) is a systemic autoimmune disease characterized by chronic inflammation of salivary and lacrimal glands. As a result, most SjD patients present with typical sicca symptoms: dry mouth and eyes.[1] The chronic inflammation is reflected by periductal lymphocytic infiltrates, called foci. A focus is defined as a periductal infiltrate consisting of 50 or more mononuclear cells. The focus score (FS) is the number of foci per 4/mm^2 glandular tissue.[2]

The significance of inflamed salivary glands in SjD is underscored by the prominent role of the salivary gland biopsy in the 2016 ACR-EULAR classification criteria for SjD. According to these criteria, a classification as SjD requires a FS ≥1 and/or presence of anti-Sjögren's syndrome A (anti-SSA) antibodies. It is important to note that not all patients with SjD have a positive biopsy, and foci can also be present in non-SjD patients, sometimes even with a FS ≥1.[3,4]

The extent of severity of salivary gland inflammation correlates significantly with serological abnormalities, including the presence of serum anti-SSA and anti-Sjögren's syndrome B (anti-SSB) antibodies, rheumatoid factor (RF), as well as with extra glandular manifestations.[5] Although glandular inflammation is a hallmark of SjD, there is no strong correlation between the severity of salivary gland inflammation, in terms of amount of infiltrate, and a decrease in salivary flow.[6] The relationship between salivary flow and other histopathological features besides a FS ≥1 remains largely unexplored. Beyond FS, salivary gland biopsies of SjD patients frequently exhibit other characteristic histopathological features. These key features are: presence of (pre-)lymphoepithelial lesions (LELs), relative increase in IgG plasma cells,

and presence of germinal centres (GCs).[3,7–9] LELs are characterised by the infiltration of lymphocytes into striated ducts along with concurrent hyperplasia of the ductal epithelium. LELs can be subdivided into several stages, according to the severity of the lesion. The current staging of LELs is based on the degree of hyperplasia: stage 1 (<50% epithelial hyperplasia), stage 2 (>50% epithelial hyperplasia), and stage 3 (circumferential hyperplastic epithelium with occluded lumen)[10]. In addition, a pre-LEL stage can be recognised. In this stage intra-epithelial B-lymphocytes are found in between the epithelial cells, but without concurrent ductal hyperplasia.[10] Another feature characteristic for SjD is the presence of a relative increase in the number of IgG plasma cells at the expense of IgA plasma cells.[8] When more than 30% of plasma cells in the salivary gland biopsy express IgG, this is called an IgA/IgG plasma cell shift.[3] Finally, periductal infiltrates in SjD may become more organised and can harbour GCs[9,11]. Importantly, addition of the presence of (pre-)LELs, IgA/IgG plasma cell shift and GCs to FS ≥1 increases the diagnostic accuracy of labial gland biopsies[4]. Furthermore, changes in histopathological features besides FS are seen after treatment with immunosuppressants. For example, the number and severity of LELs/mm^2 and number of GCs/mm^2 was significantly reduced in patients treated with rituximab, but did not change in placebo-treated patients.[12] Together, these data suggest that assessment of multiple histopathological features, including but not limited to FS, is of value in the diagnostic work-up of patients suspected of having SjD. To investigate the potential added value, this study aims to assess to what extent key histopathological features (FS, IgA/IgG plasma cell shift, (pre-)LELs and GCs), correlate with each other, with serological/functional parameters and with expert diagnosis in patients suspected for SjD.

MATERIALS AND METHODS

Patients and inclusion

In this international multicentre diagnostic cohort study, consecutive sicca patients clinically suspected for SjD were prospectively included at the University Medical Centre Groningen (UMCG) (n=113) and at the Oklahoma Medical Research Foundation (OMRF) (n=169) between 2014 and 2017. Suspected SjD

patients underwent a complete diagnostic workup, including a labial salivary gland biopsy in all patients. Patients with an age <18 years, a diagnosis with an associated auto-immune disease, salivary gland mucosa associated lymphoid tissue (MALT) lymphoma, positive hepatitis C serology, (non-specific) sclerotic sialoadenitis within the labial gland biopsy and insufficient biopsy material (<4 mm^2) were excluded from the current study.

Clinical characteristics

In both the UMCG and OMRF, the various parameters were collected according to a validated standardised operations protocol. All patients included at the UMCG were categorised as SjD or non-SjD sicca patients based on expert diagnosis by a panel of experienced rheumatologists involved in the care of SjD.[4] The expert panel independently scored anonymised clinical vignettes with information about clinical history, physical examination, EULAR Sjögren's syndrome disease activity index (ESSDAI) score, serological parameters, results of the evaluation by expert oral and maxillofacial surgeons and results of ophthalmological evaluation, as previously published.[4] All patients included at the OMRF participated in a half-day clinical evaluation to assess the extent of oral, ocular and rheumatologic manifestations of SjD. Results were interpreted by a team of experts in oral medicine, ophthalmology and rheumatology, providing the diagnosis.

Serological data on presence of autoantibodies (anti-SSA and anti-SSB), RF and elevated serum IgG plasma cells were collected. The following functional tests of salivary glands and tear glands were performed and analysed: unstimulated whole saliva (UWS) flow rate, stimulated whole saliva (SWS) flow rate and Schirmer's test.

Histochemical and immunohistochemical staining

Formalin-fixed and paraffin-embedded labial gland biopsies of 3µm sections were histochemically stained with haematoxylin and eosin (H&E) and immunohistochemically for high molecular weight cytokeratin (hmw-CK) to identify salivary gland epithelial cells, for CD20 to identify B-lymphocytes and aid (pre-)LEL identification, anti-IgA and anti-IgG double staining to identify IgA or IgG plasma cells for assessing the presence of an IgA/IgG plasma cell shift, and for Bcl-6 to identify GCs. Sections were either manually stained or using

an automated staining platform (BenchMark XT, Ventana Medical Systems) following the manufacturer's protocols. For staining protocol and antibodies used see our previous work.[3]

Histopathological analyses

The FS was calculated on an H&E-stained salivary gland section. The surface area was digitally measured by manually outlining the contours of the parenchyma (Philips IntelliSite Pathology Solution, Philips, Netherlands). For the assessment of pre-LELs and LELs, sections consecutively stained for hmwCK and CD20, were digitally aligned as described previously.[10] Briefly, a Digital Image Analysis (DIA) algorithm in Visiopharm Integrator System (Visiopharm, Hørsholm, Denmark) was used to identify hmwCK$^+$ striated ducts and presence of CD20$^+$ B-lymphocytes within the epithelium of the striated ducts. All hmwCK$^+$ducts within the sections with a positive intra-epithelial CD20 staining according to the algorithm were manually screened for the presence of (pre-)LELs. A LEL was defined as the presence of lymphocytic infiltration into striated ducts along with ductal hyperplasia. A pre-LEL was defined as B-lymphocytic infiltration but without concurrent ductal hyperplasia.[10]

GCs were defined as a cluster of 5 or more Bcl6$^+$ cells as previously described[9]. For the IgA/IgG plasma cell ratio, percentages of IgA$^+$ and IgG$^+$ plasma cells were manually estimated on the digital slides. A relative decrease of IgA$^+$ plasma cells (≤70% of all IgA$^+$ and IgG$^+$ plasma cells together) in the total parenchyma was considered as an IgA/IgG plasma cell shift. All sections were analysed by trained researchers (UN, MvG, SCL) under supervision of an experienced head and neck pathologist (BvdV). Disagreements were resolved during a consensus meeting.

Statistical analyses

Data were analysed using SPSS version 28 statistical software (SPSS Inc., Chicago, IL). Results were expressed as number of patients (%), mean ± SD, or median (IQR; p25-p75) for categorical, normally distributed, and non-normally distributed data, respectively. Cohen's Kappa was calculated to assess inter-feature agreement within labial salivary gland biopsies of suspected SjD patients. Inter-feature agreement was defined as the concordance between histopathological key features observed in labial gland biopsies. Kappa

(k) 0.00 to 0.20 'poor, 0.21 to 0.40 'fair', 0.41 to 0.60 'moderate', 0.61 to 0.80 'good, and above 0.81 'excellent agreement.[13] To test associations between the number of histopathological key features and major serological and functional parameters, salivary gland biopsies of suspected SjD patients were categorised according to the number of histopathological key features present (no features, one feature, two features or three to four features). Mann Whitney U test was used to assess the differences in the number of features present between patients with normal or abnormal serological and functional tests. Univariable logistic regression was performed with clinical diagnosis by experts (SjD/non-SjD sicca) as dependent variable to evaluate which parameters are associated with clinical diagnosis. The explained variance of these variables was expressed using the Nagelkerke R^2. Hereafter, parameters were entered into a forward conditional multivariable logistic regression model. Selection of variables for multivariable modelling was based on clinical relevance, the p-value (<0.05) and explained variance according to the univariable logistic regression analysis and multi-collinearity. P-value <0.05 was considered statistically significant.

RESULTS

Patients

In total, 282 suspected SjD patients from the diagnostic OMRF and UMCG cohort were evaluated. Twenty patients were excluded from this study due to presence of an associated auto-immune disease (n=15), positive hepatitis C serology (n=2), insufficient biopsy material (n=2) and sclerotic sialadenitis (n=1) (supplementary figure 1). Of the remaining 262 patients, 42.4% (111/262) were categorised as SjD according to the expert diagnosis and 57.6% (151/262) as non-SjD sicca. Patient characteristics of the total study population are shown in table 1.

Table 1. Clinical and serological characteristics of suspected Sjögren's disease patients.

	Total study population (n=262)
Clinical parameters	
ACR-EULAR+	86 (32.8)
Age, years	51 ± 13
Female, n (%)	243 (92.7)
Schirmer lowest eye, mm	7 [3-16]
SWS ml/min	1.7 [0.9-2.5]
UWS ml/min	0.1 [0.0-0.2]
Serological parameters	
Anti-SSA positive, n (%)	70 (26.7)
Anti-SSB positive, n (%)	39 (14.9)
RF positive, n (%)	44 (16.8)
IgG g/L	10.6 [8.9-13.1]*

Data are represented as mean ± SD, median [IQR] or number (%) * Serum IgG data available from 259 patients. Abbreviations: SWS, stimulated whole saliva; UWS, unstimulated whole saliva; SSA, Sjögren's syndrome antigen A; SSB, Sjögren's syndrome antigen B; RF, rheumatoid factor.

Prevalence of histopathological features and inter-feature agreement in patients clinically suspected for SjD

For this analysis, all 262 patients clinically suspected of having SjD were included, irrespective of final expert diagnosis (SjD or non-SjD sicca). Of all features characteristic for SjD, FS ≥1 was most frequently observed in labial gland biopsies (37.0%). (Pre-)LELs and/or an IgA/IgG plasma cell shift were observed in 27.5% and 26.5% of the labial gland biopsies, respectively. GCs were observed in only 5.0% of labial gland biopsies.

In 13% of biopsies with a FS of zero (n=60) (i.e. a complete absence of foci), one additional key histopathological feature was present: seven biopsies with a pre-LEL and one with an IgA/IgG plasma cell shift. The other 87% of biopsies without a focus did not show other histopathological key features. In contrast, in labial gland biopsies with a FS between 0 and 1 (n=92) 32% of biopsies showed other key features: (pre-)LELs, IgA/IgG plasma cell shift and one biopsy a GC. In labial gland biopsies with a FS of 1 or higher (n=101), 66% showed additional features besides a FS ≥1. In biopsy-positive patients, one

additional feature was present in 28% of patients, and 38% presented with two or three additional features (table 2). Thus, the presence of (pre-)LELs, an IgA/IgG plasma cell shift, or a GC is associated with the presence of foci.

Table 2. Frequencies of histopathological key features for different FS groups (total cohort).

	FS=0	FS>0 and <1	FS≥1
(pre-)LEL (n=67)	7/60 (11.7)	11/101 (10.9)	49/92 (53.3)
IgA/IgG plasma cell shift (n=67)	1/61 (1.6)	24/103 (23.3)	42/94 (44.7)
GC (n=12)	0/61 (0)	1/103 (9.7)	11/94 (11.7)
No features (n=121)	52/60 (86.7)	69/101 (68.3)	0/92 (0)
1 feature (n=68)	8/60 (13.3)	29/101 (28.7)	31/92 (33.7)
2 features (n=28)	0/60 (0)	2/101 (2.0)	26/92 (28.3)
3 to 4 features (n=36)	0/60 (0)	1/101 (1.0)	35/92 (38.0)

Data are represented as number (%). Abbreviations: FS, Focus score; LEL, lympho-epithelial lesion; GC, germinal centre. The number of subjects varies across analyses due to missing data. Specific sample sizes are indicated.

The relation between histopathological key features, as reflected by the inter-feature agreement, varied from fair to moderate for the presence of FS ≥1, (pre-)LELs and IgA/IgG plasma cell shift (table 3). Highest agreement was observed between the presence of FS ≥1 and (pre-)LELs ($k=0.47$, $p<0.001$). Poor to fair agreement was observed between FS ≥1, (pre-)LELs and IgA/IgG plasma cell shift and presence of GCs ($k=0.16$, $k=0.17$, $k=0.20$, respectively). Thus, although the strength of these associations varies, the presence of histopathological key features are generally interrelated in suspected SjD patients.

Table 3. Presence of histopathological key features in labial salivary gland biopsies (total cohort).

	No (pre)LEL	(pre)LEL		
FS<1 (n=161)	143 (77.3)	18 (25.7)	K =0.466	
FS≥1 (n=94)	42 (22.7)	52 (74.3)	P<0.001	
Total (n=255)	185 (100)	70 (100)		
	No IgA/IgG plasma cell shift	**IgA/IgG plasma cell shift**		
FS<1 (n=164)	139 (72.8)	25 (36.2)	K=0.325	**No (pre)LEL (n=187)**
FS≥1 (n=96)	52 (27.2)	44 (63.8)	P<0.001	**(pre-)LEL (n=69)**
Total (n=260)	191 (100)	69 (100)		**Total (n=256)**
	No GC	**GC**		
FS<1 (n=165)	164 (66.1)	1 (7.1)	K=0.155	**No (pre)LEL (n=185)**
FS≥1 (n=97)	84 (33.9)	13 (92.9)	P<0.001	**(pre-)LEL (n=70)**
Total (n=262)	248 (100)	14 (100)		**Total (n=255)**

Data are represented as number (%). Abbreviations: FS, Focus score; LEL, lympho-epithelial lesion; GC, germinal centre. The number of subjects varies across analyses due to missing data. Specific sample sizes are indicated where applicable. Kappa (k) 0.00 to 0.20 'poor, 0.21 to 0.40 'fair', 0.41 to 0.60 'moderate', 0.61 to 0.80 'good, and above 0.81 'excellent agreement.[13]

Number of histopathological key features in relation to serological and functional parameters

For this analysis, again all 262 patients clinically suspected for having SjD were included, irrespective of final expert diagnosis (SjD or non-SjD sicca). Salivary gland biopsies were categorised based on the number of histopathological features (FS ≥1, (pre-)LELs, IgA/IgG plasma cell and GCs) present as outlined in table 4. The number of histopathological key features present was significantly higher in patients with abnormal compared to normal serological parameters (anti-SSA positivity (P<0.001), anti-SSB positivity (P<0.001), RF positivity (P<0.001) and elevated IgG (P<0.001)). However, no significant differences were observed between the number of histopathological features present in suspected SjD patients with and without abnormal function test results (UWS, SWS and Schirmer's test) (table 4).

No IgA/IgG plasma cell shift	**IgA/IgG plasma cell shift**					
152 (81.3)	33 (49.3)	K=0.317				
35 (18.7)	34 (50.7)	P<0.001				
187 (100)	67 (100)					
No GC	**GC**			**No GC**	**GC**	
182 (75.2)	3 (23.1)	K=0.170	**No IgA/IgG plasma cell shift (n=191)**	189 (76.5)	2 (15.4)	K=0.201
60 (24.8)	10 (76.9)	P<0.001	**IgA/IgG plasma cell shift (n=69)**	58 (23.5)	11 (84.6)	P<0.001
242 (100)	13 (100)		**Total (n=260)**	148 (100)	13 (100)	

Histopathological key features in relation to clinical diagnosis of SjD

In univariable logistic regression, all individual histopathological key features were significantly associated with expert diagnosis of SjD. Of the individual features, (pre)LELs showed the highest explained variance based on the R^2 ((pre)LEL: 13.5%, IgA/IgG plasma cell shift: 12.5%, FS≥1: 8.8 %, GCs: 4.8%). Among all variables, the number of features had the highest explained variance for expert diagnosis (21.9%, table 5). The OR for expert diagnosis increased significantly when multiple histopathological features were present. This implies that the presence of multiple features enhances the odds of having SjD, and this effect is amplified with the addition of more features. As expected, all serological parameters (anti-SSA, anti-SSB, RF, elevated total IgG levels) were strongly associated with SjD (p<0.001), while abnormal UWS, SWS and Schirmer results were not.

Table 5. Logistic regression with clinical diagnosis by expert diagnosis (SjD/non-SjD sicca) as dependent variable and histopathological, serological and functional parameters as independent variables.

	Univariable analysis			Multivariable analysis	
	R^2	OR (95% CI)	P-value	OR (95% CI)	P-value
FS ≥1	0.088	3.024 (1.788-5.116)	<0.001		b
(pre)LELs	0.135	4.571 (2.498-8.365)	<0.001		b
IgA/IgG plasma cell shift	0.125	4.380 (2.397-8.002)	<0.001		b
GCs	0.048	7.457 (1.618-34.360)	0.010		b
Number of histopathological features					
-1 feature	0.219	1.204 (0.641-2.262)	0.563	1.167 (0.609-2.234)	0.641
-2 features		3.335 (1.425-7.805)	0.006	2.832 (1.176-6.821)	0.020
-3 to 4 features		17.263 (5.699-52.294)	<0.001	8.591 (2.641-27.947)	<0.001
anti-SSA	0.188	6.552 (3.467-12.382)	<0.001	2.477 (1.177-5.215)	<0.001
anti-SSB	0.160	10.641 (3.989-28.382)	<0.001		c
RF	0.145	7.366 (3.254-16.673)	<0.001		c
Elevated IgG g/L	0.121	8.345 (3.097-22.486)	<0.001		d
UWS<0.1ml/min	0.005	1.290 (0.789-2.110)	0.310		a
SWS<0.7 ml/min	0.001	0.883 (0.477-1.634)	0.692		a
Schirmers lowest eye <5 mm	0.13	1.500 (0.915-2.460)	0.108		a

a: parameters were not tested in multivariable regression analysis because of a p-value ≥0.05 in univariable regression analysis.

b: FS≥1, (pre-)LELs, IgA/IgG plasma cell shift and GCs were not tested in multivariable regression analysis because the number of histopathological features was included.

c. anti-SSB and RF were not tested in multivariable regression analysis because anti-SSA and IgG were included.

d: elevated IgG was not selected during multivariable regression analysis (p≥0.05).

In multivariable logistic regression analyses with conditional forward selection (table 4), the explained variance improved from 21.9% (histopathological features alone) to 26.8% with the addition of anti-SSA antibodies. The final model identified two or more histopathological features (two features OR 2.83 95% CI 1.18-6.82; three to four features OR 8.59 95% CI 2.64-27.95) and presence of anti-SSA antibodies (OR 2.48 CI 1.18-5.22) as independent predictors of expert diagnosis of SjD.
For individual patient information about the presence of histopathological key features, serological and functional characteristics for SjD and non-SjD sicca patients, see supplementary figure 2.

DISCUSSION

This study explored the association between four histopathological key features, their relation with serological/functional parameters and with expert diagnosis in patients clinically suspected for SjD.

Histopathological analysis of labial salivary gland biopsies of suspected SjD patients revealed that while the strength of these associations varies, the presence of histopathological key features (presence of FS≥1, (pre)LELs, IgA/IgG plasma cell shift, GCs) are generally interrelated in suspected SjD patients. Furthermore, the total number of SjD related key features present was associated with the presence of serological (anti-SSA and anti-SSB antibodies, RF and elevated IgG levels) but not functional (UWS, SWS, Schirmer's test) abnormalities. We further demonstrate that the presence of multiple histopathological features, along with anti-SSA antibodies, serve as independent predictors of SjD diagnosis. Additionally, the presence of multiple key features provide a stronger predictive value than FS≥1 alone.

The current study revealed that labial gland biopsies with a FS between 0 and 1 generally lack additional histopathological features. This finding is consistent with evidence suggesting that the development of LELs and GCs requires the presence of (B-)lymphocytic infiltration.[9,10] Interestingly, while (pre-)LELs and a IgA/IgG plasma cell shift may develop independently, GCs are predominantly observed when all other histopathological key features are present. However, the inter-feature agreement between GCs and the other

histopathological features was low, likely due to the relatively low prevalence of GCs in these diagnostic cohorts, limiting their co-occurrence with other features. However, longitudinal studies are needed to confirm whether these histopathological key features develop in a specific order.

The presence of a periductal infiltrates alone, without additional histopathological features, does not necessarily indicate SjD, as even a FS ≥1 has been observed in non-SjD sicca patients.[3] Moreover, in our study we observed that the explained variance for SjD of solely the FS was only 8.8% while this was 21.9% when all key features were taken into account. Consequently, the diagnostic value of labial gland biopsies can be enhanced by incorporating other histopathological markers in the diagnostic evaluation, something we have previously shown.[4]

In this cohort, we also observed labial gland sections from non-SjD patients that exhibited additional histopathological features, in addition to the presence of foci. This raises the possibility that some of these non-SjD patients may actually represent early SjD patients, where glandular inflammation has begun, but other hallmarks of disease—such as systemic, serological or functional characteristics - are not yet fully developed. Longitudinal follow-up studies are needed to determine whether these patients eventually progress to SjD, hereby assessing the potential value of these histopathological features as early indicators of disease development. Besides salivary gland involvement, the presence of anti-SSA/Ro, anti-SSB/La antibodies, RF and elevated serum IgG levels has been associated with SjD.[15] In this study, we observed that as the number of histopathological key features present within the labial gland increases, the frequency of all SjD-related serological abnormalities increases as well. Multiple studies have illustrated the association between the presence of histopathological features and serological abnormalities. For example, Tengner et al. showed a strong positive correlation between serum levels of anti-SSA and anti-SSB antibodies and the number of plasma cells in the minor salivary glands of SjD patients.[16] Indeed, plasma cells within salivary glands are involved in the secretion of SjD related auto-antibodies.[17,18] In addition, the presence of (pre-)LELs has been linked to elevated levels of serum IgG, as well as an increased number of plasma cells and higher RF levels.[10,19] Moreover, Risselada et al. reported that patients with GCs were more likely to test positive for RF, anti-SSA, and anti-SSB antibodies compared to

patients without GCs.[11] These findings emphasize that the histopathological changes in the salivary glands are closely associated with specific serological markers, potentially reflecting autoimmune activity in SjD.

An important aspect of clinical disease is the impaired function of salivary and lacrimal glands.[1] The hypothesis is that progressive glandular inflammation can result in gland destruction and therefore decreased saliva and tear production. However, in this study, approximately half of the sicca patients with a salivary gland biopsy had no key features, but show abnormal UWS and Schirmer's test results. Furthermore, no significant differences were found in the number of key features between patients with normal and abnormal functional test results (UWS, SWS, Schirmer's test). This is consistent with previous studies, which showed that there is no correlation between the extent of labial gland infiltration and saliva production.[6,20] These findings suggest that even without histopathological glandular involvement, impaired salivary gland function can be present, making other causes for hyposalivation more likely.

To evaluate the predictive value of histopathological key features for SjD and non-SjD sicca, expert diagnosis was used in this study and it was not solely based on the ACR-EULAR classification criteria. We decided to take expert diagnosis and not fulfilment of classification criteria as primary outcome, because the latter is strongly influenced by the result of the FS, hampering independent assessment. In our study experts had access to FS but were not informed of the additional histopathological features (the presence of (pre-) LELs, IgA/IgG plasma cell shift, or GCs). This minimises the risk of bias related to these features, underscoring validity of the results of this study.

A methodological limitation was that, at the UMCG, anonymised vignettes were used for the clinical diagnosis[21], whereas at the OMRF, clinical diagnoses were made directly by a team of experts as part of routine diagnostics. This difference in diagnostic approach introduces potential heterogeneity, which may affect the identification of SjD and non-SjD patients.

In conclusion, the presence of histopathological key features (FS ≥1, LELs, IgA/IgG plasma cell shift and GCs) are generally interrelated and associated with serological parameters in patients suspected for SjDs. Our results demonstrate that assessment of additional histopathological features, besides FS, is relevant for making the diagnosis of SjD.

REFERENCES

1. Brito-Zerón P, Baldini C, Bootsma H, Bowman SJ, Jonsson R, Mariette X, et al. Sjögren syndrome. Nat Rev Dis Primers. 2016 Jul 7;2(1):16047.
2. Shiboski CH, Shiboski SC, Seror R, Criswell LA, Labetoulle M, Lietman TM, et al. 2016 American College of Rheumatology/European League Against Rheumatism Classification Criteria for Primary Sjögren's Syndrome: A Consensus and Data-Driven Methodology Involving Three International Patient Cohorts. Arthritis & Rheumatology. 2017 Jan 26;69(1):35–45.
3. Nakshbandi U, van Ginkel MS, Verstappen GMPJ, Spijkervet FKL, Arends S, Haacke EA, et al. Histopathological comparison of Sjögren-related features between paired labial and parotid salivary gland biopsies of sicca patients. Rheumatology. 2024 Oct 1;63(10):2670–7.
4. van Ginkel MS, Nakshbandi U, Arends S, Haacke EA, Liefers SC, Verstappen GM, et al. Increased Diagnostic Accuracy of the Labial Gland Biopsy in Primary Sjögren Syndrome When Multiple Histopathological Features Are Included. Arthritis & Rheumatology. 2024 Mar 9;76(3):421–8.
5. Gerli R, Muscat C, Giansanti M, Danieli MG, Sciuto M, Gabrielli A, et al. Quantitative assessment of salivary gland inflammatory infiltration in primary Sjogren's syndrome: Its relationship to different demographic clinical and serological features of the disorder. Br J Rheumatol. 1997;36(9).
6. Mossel E, van Ginkel MS, Haacke EA, Arends S, Liefers SC, Delli K, et al. Histopathology, salivary flow and ultrasonography of the parotid gland: three complementary measurements in primary Sjögren's syndrome. Rheumatology (Oxford). 2022 May 30;61(6):2472–82.
7. Ihrler S, Zietz C, Sendelhofert A, Riederer A, Löhrs U. Lymphoepithelial duct lesions in Sjögren-type sialadenitis. Virchows Archiv. 1999 Apr 22;434(4):315–23.
8. Bodeutsch C, De Wilde PCM, Kater L, Van Houwelingen JC, Van Den Hoogen FHJ, Kruize AA, et al. Quantitative immunohistologic criteria are superior to the lymphocytic focus score criterion for the diagnosis of Sjögren's syndrome. Arthritis Rheum. 1992 Sep 16;35(9):1075–87.
9. Nakshbandi U, Haacke EA, Bootsma H, Vissink A, Spijkervet FKL, van der Vegt B, et al. Bcl6 for identification of germinal centres in salivary gland biopsies in primary Sjögren's syndrome. Oral Dis. 2020 Apr 28;26(3):707–10.
10. van Ginkel MS, Haacke EA, Bootsma H, Arends S, van Nimwegen JF, Verstappen GM, et al. Presence of intraepithelial B-lymphocytes is associated with the formation of lymphoepithelial lesions in salivary glands of primary Sjögren's syndrome patients. Clin Exp Rheumatol. 2019;37 Suppl 118(3):42–8.
11. Risselada AP, Looije MF, Kruize AA, Bijlsma JWJ, Van Roon JAG. The Role of Ectopic Germinal Centers in the Immunopathology of Primary Sjögren's Syndrome: A Systematic Review. Semin Arthritis Rheum. 2013;42(4):368–76.
12. Delli K, Haacke EA, Kroese FGM, Pollard RP, Ihrler S, van der Vegt B, et al. Towards personalised treatment in primary Sjögren's syndrome: baseline parotid histopathology predicts responsiveness to rituximab treatment. Ann Rheum Dis. 2016 Nov;75(11):1933–8.
13. Landis JR, Koch GG. The measurement of observer agreement for categorical data. Biometrics. 1977 Mar;33(1):159–74.
14. Seror R, Bootsma H, Saraux A, Bowman SJ, Theander E, Brun JG, et al. Defining disease activity states and clinically meaningful improvement in primary Sjögren's syndrome with EULAR primary Sjögren's syndrome disease activity (ESSDAI) and patient-reported indexes (ESSPRI). Ann Rheum Dis. 2016 Feb;75(2):382–9.

15. García-Carrasco M, Mendoza-Pinto C, Jiménez-Hernández C, Jiménez-Hernández M, Nava-Zavala A, Riebeling C. Serologic features of primary Sjögren's syndrome: clinical and prognostic correlation. Int J Clin Rheumtol. 2012 Dec;7(6):651–9.
16. Tengnér P, Halse AK, Haga HJ, Jonsson R, Wahren-Herlenius M. Detection of anti-Ro/SSA and anti-La/SSB autoantibody-producing cells in salivary glands from patients with Sjögren's syndrome. Arthritis Rheum. 1998 Dec;41(12):2238–48.
17. Szyszko EA, Aqrawi LA, Jonsson R, Brokstad KA, Skarstein K. Non-proliferating plasma cells detected in the salivary glands and bone marrow of autoimmune NOD.B10.H2b mice, a model for primary Sjögren's syndrome. Autoimmunity. 2016;49(1):41–9.
18. Steinmetz TD, Verstappen GM, Suurmond J, Kroese FGM. Targeting plasma cells in systemic autoimmune rheumatic diseases – Promises and pitfalls. Immunol Lett. 2023 Aug 1;260:44–57.
19. Steinmetz TD, Verstappen GM, Schulz SR, de Wolff L, Wilbrink R, Visser A, et al. Association of Circulating <scp>Antibody-Secreting</scp> Cell Maturity With Disease Features in Primary Sjögren's Syndrome. Arthritis & Rheumatology. 2023 Jun 22;75(6):973–83.
20. Jonsson R, Kroneld U, Bäckman K, Magnusson B, Tarkowski A. Progression of sialadenitis in Sjögren's syndrome. Br J Rheumatol. 1993 Jul;32(7):578–81.
21. van Nimwegen JF, van Ginkel MS, Arends S, Haacke EA, van der Vegt B, Sillevis Smitt-Kamminga N, et al. Validation of the ACR-EULAR criteria for primary Sjögren's syndrome in a Dutch prospective diagnostic cohort. Rheumatology. 2018 May 1;57(5):818–25.

ACKNOWLEDGEMENTS

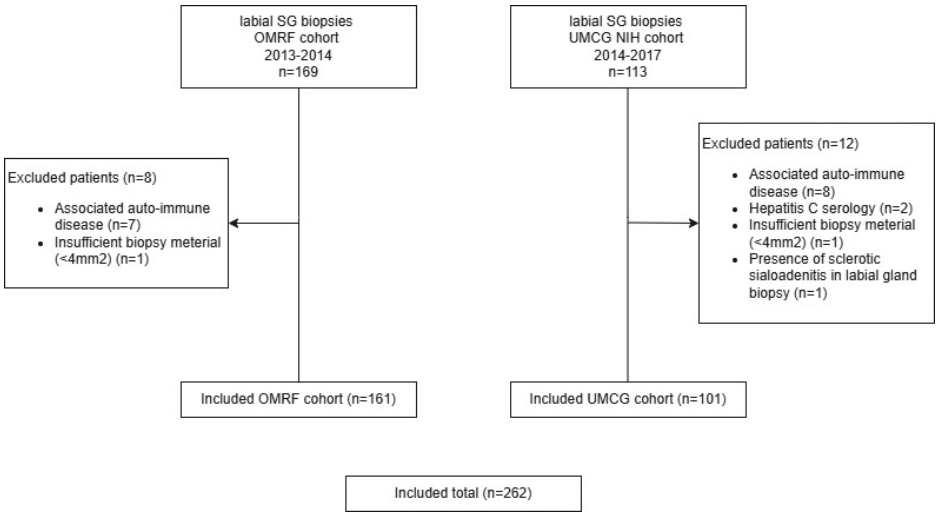
Several authors of this publication are members of the European Reference Network ReCONNET and of NECESSITY.

FUNDING STATEMENT

NIH (RFA-DE-06-004): Salivary Biomarkers for primary Sjögren's Syndrome Detection- Definitive Validation. There was no involvement of these funding sources in the study design, data collection, analysis and interpretation of data, or writing of this report. This project has received funding from the Innovative Medicines Initiative 2 Joint Undertaking (JU) under grant agreement number 806975. JU receives support from the European Union's Horizon 2020 research and innovation program and EFPIA. The present article reflects only the authors' view and JU is not responsible for any use that may be made of the information it contains.

SUPPLEMENTARY MATERIAL

Supplementary figure 1. Flowchart of patient inclusion.

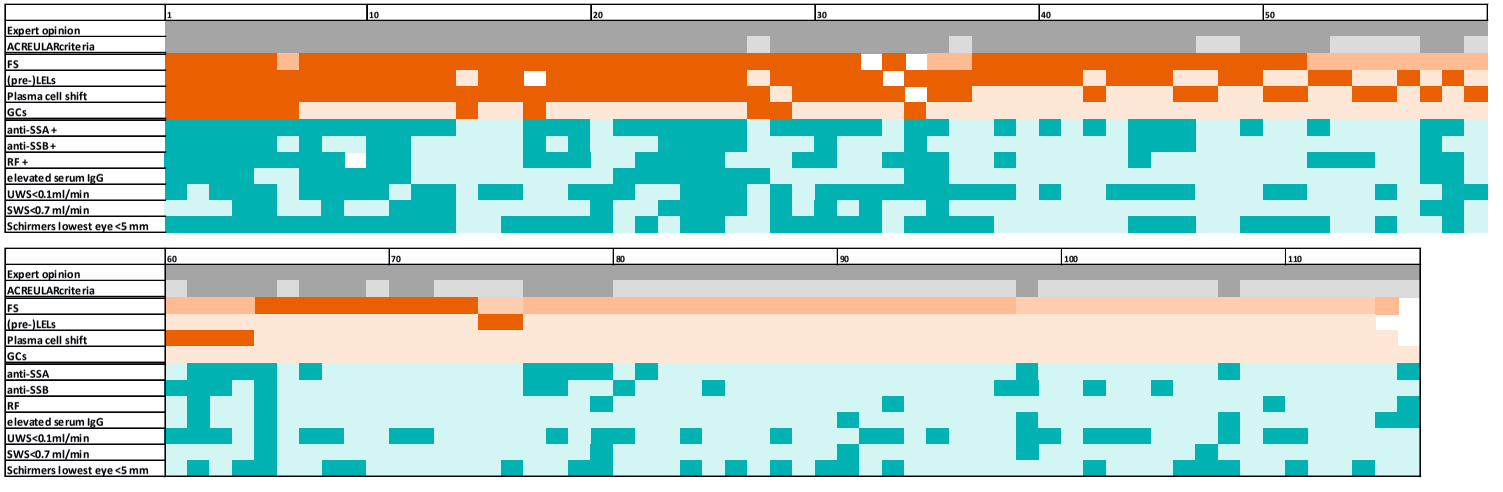


Supplementary table 1. Histopathological key features in labial gland biopsies of suspected SjD

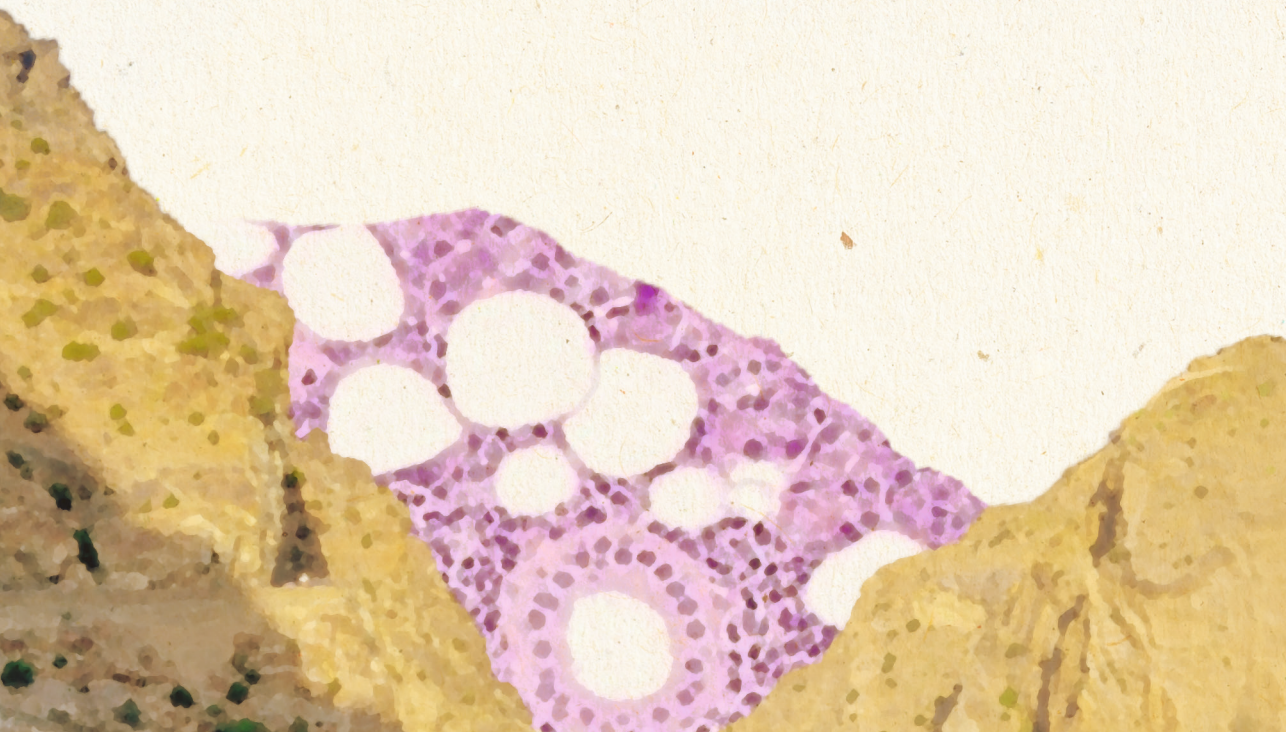
	FS=0	FS>0 and <1	FS≥1
(pre-)LEL (n=67)	7/60 (11.7)	11/101 (10.9)	49/91 (53.8)
IgA/IgG plasma cell shift (n=67)	1/61 (1.6)	24/103 (23.3)	42/94 (44.7)
GC (n=12)	0/61 (0)	1/103 (9.7)	11/94 (11.7)
No features (n=121)	52/60 (86.7)	69/101 (68.3)	0/91 (0)
1 feature (n=68)	8/60 (13.3)	29/101 (28.7)	31/91 (34.1)
2 features (n=28)	0/60 (0)	2/101 (2.0)	26/91 (28.6)
≥3 features (n=36)	0/60 (0)	1/101 (1.0)	35/91 (38.5)

Supplementary figure 2. Histopathological features of labial salivary gland biopsies, serological and clinical characteristics of SjD and non-SjD sicca patients.

A. **SjD patients**



B. **Non-SjD sicca patients**



CHAPTER 9

Abatacept has a limited effect on salivary gland inflammation in Sjögren's disease patients

U. Nakshbandi[1], S.C. Liefers[1], S. Arends[1], F.K.L. Spijkervet[2], G.M.P.J. Verstappen[1], A. Vissink[2], L. de Wolff[1], B. van der Vegt[3], H. Bootsma[1], F.G.M. Kroese[1]

Departments of [1]Rheumatology and Clinical Immunology, [2]Oral and Maxillofacial Surgery and [3]Pathology and Medical Biology, University of Groningen and University Medical Center Groningen, Groningen, the Netherlands

Clin Exp Rheumatol. 2024 Dec;42(12):2362-2368

ABSTRACT

Objectives

This study aimed to assess (1) effects of abatacept on salivary gland histology of Sjögren's disease (SjD) patients, (2) the predictive value of salivary gland histopathological characteristics at baseline for clinical response to abatacept treatment.

Methods

Patients (n=41) who participated in the Dutch ASAP-II and ASAP-III trials and international abatacept trial (IM101603) from whom a labial (n=13) or parotid (n=28) salivary gland biopsy was obtained at baseline and after 24 weeks of treatment with abatacept were included. Biopsies were analysed for SjD related histopathological features before and after abatacept (n=25) or placebo (n=16) treatment. Histopathological data at baseline were compared between clinical responders and non-responders to abatacept treatment.

Results

Comparison between abatacept- and placebo-treated patients revealed virtually no differences in histopathological parameters of parotid and labial salivary gland biopsies of SjD patients at baseline and 24 weeks after therapy. In labial glands, only the number of IgA plasma cells/mm^2 differed between the two groups over time (p=0.034). Correspondingly in parotid glands, the number of IgA plasma cells increased in the abatacept group (p=0.049) after 24 weeks. The number of CD20$^+$ B-cells/mm^2 in parotid glands of the placebo group increased compared to baseline (p=0.021). There were no evident differences in baseline histopathological parameters between CRESS or ClinESSDAI responders and non-responders treated with abatacept.

Conclusions

Abatacept has limited effects on salivary gland histology in SjD patients after 24 weeks of treatment. Besides possibly affecting numbers of IgA plasma cells and preventing increases in B-lymphocyte infiltration, salivary gland histopathology could not predict response to abatacept treatment in SjD patients.

INTRODUCTION

Sjögren's disease (SjD) is a systemic auto-immune disease, characterised by chronic inflammation of exocrine glands. In SjD, salivary and lacrimal glands are frequently affected causing typical sicca complaints such as xerostomia and dry eyes.[1] In addition to exocrine glands, extra-glandular organ systems can be involved, underlining the systemic nature of the disease.[2]

In SjD patients, minor and major salivary glands are typically infiltrated by lymphoid cells. These infiltrates are predominantly located around striated ducts and mainly consist of T- and B-lymphocytes, together with a variety of non-lymphoid cells.[1,3] The infiltrates may even be organised in ectopic lymphoid tissue as witnessed by evidently segregated B- and T-cell rich areas, presence of follicular dendritic cell (FDC) networks and development of germinal centres (GCs).[4] Another histopathological feature of salivary gland tissue of SjD patients is the presence of lymphoepithelial lesions (LELs) composed of lymphocyte-containing striated ducts together with hyperplasia of ductal epithelial cells.[5] Finally, a marked increase in the number of IgG plasma cells can be seen, leading to a decline in the IgA/IgG plasma cell ratio (the so-called plasma cell shift).[6]

While much is still unknown about the pathogenesis of SjD, B-cell hyperactivity has been recognised to play an essential role.[7] Activated $CD4^+$ T-cells enable and contribute to hyperactivity of B-cells. Therefore, inhibition of T-cell dependent B-cell hyperactivity may be a promising target for treatment of SjD. Abatacept, a fusion protein of CTLA-4 and IgG1-Fc, inhibits the interaction between CD80/CD86 on antigen-presenting cells, including B-cells, and CD28 on T-cells. Thereby the delivery of co-stimulatory signals essential for T-cell activation is prevented.[8] Notwithstanding promising effects in an open-label phase II trial, clinical effects were not conclusive in two placebo-controlled randomised trials with a decrease in EULAR Sjogren's syndrome disease activity index (ESSDAI) as clinical endpoint.[9,10] The absence of a clear clinical response could partially be attributed to the large placebo response in ESSDAI. However, post-hoc analysis using the recently developed Composite of Relevant Endpoints in Sjögren's Syndrome (CRESS) showed discrimination between abatacept and placebo[11]. Previous studies demonstrated that abatacept treatment induces multiple biological effects in SjD patients,

including a reduction in the number of circulating follicular helper T-cells (Tfh), normalisation of the elevated levels of Bruton's tyrosine kinase (BtK) in B-cells, and decreased levels of disease-relevant biomarkers in serum, such as rheumatoid factor and CXCL13.[10,12,13] Despite these biological effects witnessed in peripheral blood, there were no significant histopathological changes when comparing parotid salivary gland biopsies prior to and after abatacept treatment in the open-label phase II study.[14] However, major limitations of the study were the small number of patients and lack of a placebo group. Hence the aims of this study were (1) to assess the effect of abatacept on parotid and labial gland tissue of patients with SjD in comparison with baseline conditions and the placebo group and (2) to assess the predictive value of histological characteristics present in labial and parotid salivary glands at baseline with regard to clinical response to abatacept treatment.

MATERIALS AND METHODS

Patients

For this study, patient samples from the open-label phase II Abatacept Sjögren Active Patients (ASAP) trial (ASAP-II) and the randomised placebo-controlled ASAP trial (ASAP-III) carried out at the University Medical Centre Groningen (UMCG; Groningen, the Netherlands), as well as the multi-centre international abatacept trial (IM101603) were combined. Abatacept was either intravenously (ASAP-II) or subcutaneously (ASAP-III and IM101603) applied. Intravenous abatacept infusions (10mg/kg) administrated on days 1,15, and 29 and monthly thereafter for 24 weeks.[9,10,15] For subcutaneous application, patients had received instructions to administer injections at home, once a week, for 24 weeks. Injections contained 125 mg of abatacept or placebo. Informed consent was obtained from all patients according to Declaration of Helsinki principles. For trial information such as randomisation, follow-up time and clinical endpoints see previously published papers.[9,10,15] All patients with a labial or parotid salivary gland biopsy at baseline and after 24 weeks of abatacept or placebo treatment, were included in this study. Exclusion criteria were insufficient biopsy material (<1mm^2), a focus score (FS) <1 at baseline and development of a MALT lymphoma (supplementary figure 1A).

(Immuno-)histological staining and evaluation

Paraffin-embedded salivary gland biopsies (labial or parotid) of all three trials were similarly processed and analysed as previously described.[16] Sections were stained with haematoxylin-eosin (H&E) and for cytokeratin (CK) 8/18, CD3, CD20, CD45, CD21, Bcl6, IgA, IgG and IgM. In addition, an immunohistochemical double staining technique was performed for IgA and IgG. Primary antibodies used are listed in supplementary table 1. Slides were digitized using a Philips UFS slide scanner (Philips, Best, the Netherlands). H&E-stained sections were used to determine the FS, defined as the number of periductal foci (clusters of ≥50 lymphocytes) per 4 mm^2. Staining for CK was used to visualise ductal epithelium in order to support the H&E and CD20 staining in the identification of the number of LELs/mm^2 and assessing the maximum severity of the LELs per section.[5] All sections were evaluated for B/T cell segregation in one or more foci on the basis of staining adjacent sections for CD20 and CD3. Presence and number of $CD21^+$ FDC-networks and $Bcl6^+$ GCs per section were assessed as previously described.[4] Dual staining for IgA and IgG was used to determine the presence of an IgA/IgG plasma cell shift (≥30% IgG plasma cells). Analyses were performed by a trained researcher (UN), an experienced lab-technician (SL) together with a senior head and neck pathologist (BvdV). Discrepancies between observers were resolved in a consensus meeting.

Digital image analysis

Tissue sections stained with H&E and for CD3, CD20, CD45, IgA, IgG and IgM were analysed using Digital Image Analysis (DIA) algorithms in QuPath (version 0.2.3) as previously described.[16] Briefly, the total area of salivary gland parenchyma was digitally measured on the H&E stained section. The number of $CD3^+$ T-cells, $CD20^+$ B-lymphocytes and IgA, IgG and IgM plasma cells per mm^2 was determined. The relative surface area of $CD45^+$ lymphocytic infiltrate was assessed in relation to the total surface area of salivary gland parenchyma.

Baseline histopathological characteristics in relation to clinical response

From all SjD patients included in the current study, a parotid or labial salivary gland biopsy was taken (within two years) before initiation of abatacept treatment. To evaluate the predictive value for response, salivary gland histopathology at baseline was compared between clinical responders and non-re-

sponders at 24 weeks of abatacept treatment. To define clinical treatment response, patients were categorised as responders or non-responders based on the CRESS, minimal clinically important improvement (MCII) in Clinical ESSDAI (ClinESSDAI) and ClinESSDAI low disease activity (LDA) (supplementary figure 1B).[11,17–19] Total CRESS response was defined as response on ≥3 out of the 5 items. MCII was defined as a decrease of ≥3 points. ClinESSDAI LDA was defined as a score <5.

Statistical analysis

Data were analysed using SPSS version 28 statistical software (SPSS Inc, Chicago, IL). Results were expressed as number of patients (%), mean±SD, or median (IQR) for categorical, normally distributed, and non-normally distributed data, respectively. Mann-Whitney U test and Fisher's Exact test were used to compare differences between the abatacept and placebo groups or between responders and non-responders. Wilcoxon signed-rank test and McNemar's test were used to compare differences over time within treatment groups. The difference between placebo and abatacept groups for change in histopathological parameters over time was evaluated using generalised estimating equations (GEE). The GEE model included baseline values of the dependent variable, treatment, time and interaction of treatment and time. In order to reach normal distribution of residuals most histopathological parameters were transformed. The exchangeable correlation structure was used for all variables. Results of the GEE model were not given if it was not possible to obtain a normal distribution of residuals or if the power was too low. P-values <0.05 were considered statistically significant.

RESULTS

For demographic, serological and clinical patient characteristics see table 1. At baseline, no differences in histopathological parameters between the placebo and abatacept groups were observed (see supplementary table 2).

Table 1. Demographic, clinical and serological characteristics of Sjögren's disease patients treated with placebo or abatacept.

	Placebo (n=18)	Abatacept (n=31)	p-value
Clinical parameters			
Age, years	46 [37-60]	46 [34-57]	0.71
Female, n (%)	18 (100)	30 (96.8)	0.63
Disease duration, years	1 [0-2]	1 [1-3]	0.13
Concomitant treatment, n (%)			
- **Oral corticosteroids**	4 (22.2)	4 (12.9)	0.32
- **NSAIDs**	4 (22.2)	15 (48.3)	0.06
- **Hydroxychloroquine**	4 (22.2)	4 (12.9)	0.32
Schirmer, mm/5 min	4 [1-8]	7 [3-18]	0.14
SWS, ml/min	0.30 [0.15-1.05]	0.39 [0.15-0.57]	0.96
UWS, ml/min	0.10 [0.04-0.20]	0.11 [0.06-0.22]	0.75
ESSDAI, total	10 [8-17]	12 [8-16]	0.84
ESSDAI, glandular domain	1 [1-2]	1 [0-2]	0.95
ESSPRI	7 [6-8]	7 [6-8]	0.73
Serological parameters			
RF	22 [6-100]	43 [15-100]	0.22
Anti-SSA positive, n (%)	16 (88.9)	30 (96.8)	0.30
Anti-SSB positive, n (%)	8 (44.4)	21 (67.7)	0.07
IgG, g/L	16 [13-20]	18 [14-27]	0.17

NSAIDs=Non-steroid inflammatory drugs, ESSDAI=EULAR Sjögren's syndrome disease activity index, ESSPRI=EULAR Sjögren's syndrome patient reported index, RF=rheumatoid factor, SSA=Sjögren's syndrome-related antigen A, SSB=Sjögren's syndrome-related antigen B. SWS=stimulated whole saliva, UWS=unstimulated whole saliva. Values are presented as median [IQR] unless otherwise specified.

Effect of abatacept and placebo treatment over time on salivary gland biopsies of SjD patients

Comparison between the abatacept and placebo group by GEE analysis revealed that there were virtually no differences over time (baseline and week 24) in FS, amount of infiltrate, number and severity of LELs, $CD21^+$ FDC-networks, $Bcl6^+$ GCs, number of $CD3^+$ T-cells, number of $CD20^+$ B-cells, presence of B/T-cell segregation, presence of IgA/IgG shift and the number of IgG and IgM plasma cells/mm^2 neither in parotid nor labial salivary gland biopsies (see table 2).

Table 2. Histopathological and immunohistochemical data of parotid and labial salivary gland biopsies of Sjögren's disease patients before and after placebo or abatacept therapy.

	Parotid salivary gland biopsies							Labial salivary gland biopsies						
	Placebo (n=11)			Abatacept (n=17)				Placebo (n=5)			Abatacept (n=8)			
	Baseline	Week 24	P-value	Baseline	Week 24	P-value	GEE P-value	Baseline	Week 24	P-value	Baseline	Week 24	P-value	GEE P-value
Focus score	1.8 (1.6-3.4)	1.5 (1.0-2.9)	0.53	4.3 (1.6-7.6)	3.3 (1.2-7.3)	0.62	0.55^	2.0 (1.4-2.3)	1.8 (1.5-5.5)	0.50	2.0 (1.7-4.6)	5.8 (1.4-9.3)	0.16	0.85^^
LELs/mm²	0 (0-0.13)	0.00 (0-0.18)	0.25	0.27 (0-0.53)	0.12 (0-0.74)	0.65	n/a[a]	0 (0-0.18)	0.12 (0-0.22)	0.29	0 (0-0.28)	0 (0-0.24)	0.72	n/a[a]
LEL⁺ patients, n(%)*	5 (45.5)	5 (45.5)	1.00	10 (58.8)	9 (52.9)	1.00	0.77	2 (40.0)	3 (60.0)	1.00	2 (25.0)	3 (37.5)	1.00	*
FDC-networks/mm²	0.23 (0-0.49)	0.31 (0.0-1.2)	0.09	0.41 (0-0.57)	0.16 (0-0.92)	0.64	*	0.22 (0.07-0.86)	0.23 (0.09-0.52)	0.23	0.38 (0.04-0.67)	0.28 (0-0.63)	0.75	0.84^
FDC-network⁺ patients, n(%)*	8 (72.7)	8 (72.7)	1.00	10 (58.8)	9 (52.9)	1.00	0.78	4 (80.0)	5 (100.0)	*	6 (75.0)	5 (62.5)	1.00	*
GCs/mm²	0.11 (0-0.33)	0 (0-0.20)	0.40	0 (0-0.29)	0 (0-0)	0.23	n/a[a]	0 (0-0.10)	0 (0-0.09)	0.32	0 (0-0.09)	0 (0-0)	1.00	n/a[a]
GC⁺ patients, n(%)*	6 (54.5)	4 (36.4)	0.69	5 (29.4)	3 (17.6)	0.50	*	1 (20.0)	1 (20.0)	1.00	2 (25.0)	1 (12.5)	1.00	*
CD3⁺cells/mm²	1212 (464-1904)	1547 (350-3053)	0.16	666 (277-1211)	711 (480-2144)	0.15	0.87^	528 (396-1054)	666 (485-1430)	0.35	1317 (404-2088)	1092 (495-1517)	0.33	0.16
CD20⁺cells/mm²	596 (232-1285)	873 (139-2350)	*0.021*	691 (193-1855)	449 (162-1379)	0.98	0.10^	327 (166-839)	393 (314-837)	0.50	1178 (249-1702)	573 (354-1564)	0.58	0.38^^
CD3/CD20 segregation, n(%)*	8 (72.7)	8 (72.7)	1.00	8 (47.1)	6 (35.3)	0.69	0.66	2 (40.0)	1.0 (20.0)	1.00	6 (75.0)	4 (50.0)	1.00	*
CD45⁺cells (%)	17.1 (14.0-25.8)	30.3 (5.6-42.7)	0.29	19.8 (5.5-36.5)	17.0 (8.7-29.1)	0.62	0.84^	17.0 (11.8-34.4)	26.9 (25.3-35.0)	0.14	35.7 (13.2-44.2)	28.8 (10.8-37.9)	0.21	0.10^^
IgA/IgG plasma cell shift, n(%)*	7 (63.6)	8 (72.7)	1.00	10 (58.8)	7 (41.2)	0.45	0.23	3 (60.0)	4 (80.0)	1.00	8 (100.0)	7 (87.5)	1.00	*
IgA plasma cells/mm²	326 (130-499)	296 (214-466)	0.48	110 (56-304)	252 (121-439)	*0.049*	0.43^	1655 (998-2168)	1378 (732-1695)	0.08	611 (316-1166)	656 (186-1907)	0.48	*0.034^^*
IgG plasma cells/mm²	205 (74-457)	446 (89-548)	0.25	97 (28-316)	121 (54-283)	0.29	0.47^	692 (362-898)	553 (290-866)	0.50	550 (259-1025)	804 (138-1172)	0.67	0.78^
IgM plasma cells/mm²	49 (12-106)	71 (32-134)	0.48	11 (3-43)	9 (2-45)	0.98	0.35^	566 (301-1445)	316 (210-457)	0.14	619 (242-804)	624 (213-1308)	1.00	0.13^^

[a] GEE analysis could not be performed due to non-normally distributed data

*GEE analysis could not be performed or was not reliable due to lack of power (groups consisting <5).

^ log transformation was applied to histopathological variables to obtain a normal distribution of residuals

^^Square root transformation was applied to histopathological variables to obtain a normal distribution of residuals

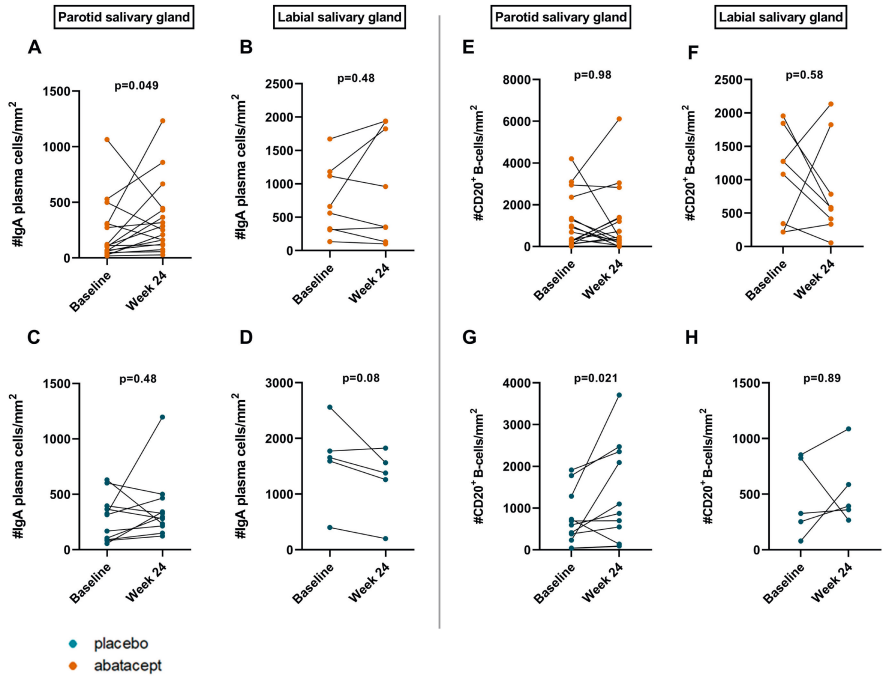


Figure 1. Number of IgA⁺ plasma cells/mm² (A-D) and CD20⁺ B-cells/mm² (E-H) in parotid and labial salivary gland sections of Sjögren's disease patients at baseline and after 24 weeks of abatacept and placebo treatment.

Only the change in number of IgA plasma cells/mm² in labial salivary glands (but not parotid salivary glands) differed significantly between the abatacept and placebo group over time (p=0.034).

Analysis of the effect of abatacept after 24 weeks of treatment revealed that there were no clear changes in histopathological parameters compared to baseline in both types of glands (see table 2). The only observed significant difference was an increase in the number of IgA plasma cells/mm² (p=0.049) in the parotid gland (but not the labial gland) (figure 1 A-B). Numbers of IgG and IgM plasma cells remained stable over time. The rise in IgA plasma cells was, however, not reflected by a concomitant altered IgA/IgG plasma cell ratio.

When analysing changes in the placebo group, a significant increase in infiltrating CD20⁺ B-cells in parotid glands (p=0.021) was observed (figure 1G). This increase was not associated by an increase in higher FS, percentage of infiltrated area, nor by other histopathological features. Furthermore, this rise

in B-cell numbers was not seen in labial gland biopsies (figure 1H). In labial gland biopsies, placebo treatment revealed only a trend towards decrease in the number of IgA plasma cells/mm^2 (figure D) (p=0.08).

Minor or major salivary gland histopathology could not predict abatacept treatment response

Twenty-two out of 35 patients (63%) who were treated with abatacept were classified as CRESS responders because they reached response on ≥3 of 5 items. The remaining 13 patients were classified as CRESS non-responders. Parotid salivary gland biopsies were available from 13 responders and 8 non-responders and labial salivary gland biopsies were available from 9 responders and 5 non-responders. No significant differences in baseline histopathological parameters were observed between CRESS-responders and non-responders, neither in labial nor parotid salivary gland biopsies (see supplementary table 3). When patients were classified as clinical responder based on ClinESSDAI MCII (67% responders), also no significant differences in histopathological parameters were observed on baseline between responders and non-responders. When response defined as maintenance or reaching ClinESSDAI low disease activity (62% responders), only the presence of IgA/IgG shift was higher in parotid (p=0.024) - but not labial gland biopsies – of responders at baseline compared to non-responders (data not shown).

DISCUSSION

In the work described here, we studied the effect of abatacept and placebo treatment over time on either parotid or labial gland biopsies of SjD patients. Overall, the effects of 24 weeks of abatacept treatment on parotid and labial gland tissue of SjD patients were limited, both when comparing differences over time between the abatacept and placebo groups as well as when the change within the abatacept group was compared to baseline. Furthermore, there were no differences in histopathological features at baseline between abatacept responders and non-responders based on the recently developed composite endpoint, the CRESS[11], nor on the ClinESSDAI.[18,19]

Although abatacept treatment showed in general no effect on histopathology, some subtle changes in absolute IgA plasma cell counts in salivary glands were observed after treatment. First, the change in number of IgA plasma cells in labial salivary glands differed in the abatacept treatment group compared to the placebo group. This seems to be largely attributed to a decrease in IgA plasma cell numbers in labial glands in patients receiving placebo. In this patient group there was a trend for a decline in IgA plasma cells, compared to baseline (p=0.08), while these numbers remained stable over time in the abatacept group. Second, a significant increase in the number of IgA plasma cells was seen in the parotid glands after abatacept treatment, compared to baseline, whereas IgA plasma cell numbers were stable in the placebo group. In both treatment arms and for both salivary gland types no effects were seen on IgG or IgM secreting plasma cells. Glandular IgA secreting plasma cells are part of the homeostatic mucosal immune system and IgA plasma cells are also normally present in healthy salivary glands.[20] Although labial and parotid salivary glands appear to respond differently, abatacept treatment may contribute to a (slight) normalisation of the glandular microenvironment that supports differentiation towards IgA plasma cells. The exact role of abatacept in this process is not known, but a possible explanation is that blockade of co-stimulation may inhibit T-cell dependent (CD28-dependent) class switch recombination towards IgG, but not T-cell independent (CD28-independent) class switch recombination towards IgA. Important cytokines for T-cell independent class switch recombination at mucosal sites towards IgA are BAFF and APRIL and these cytokines are abundantly expressed in serum, saliva and salivary glands of SjD patients. The absence of an effect on (T-cell dependent) IgG plasma cell numbers may be explained by the observation that plasma cells in salivary glands are long-lived cells, which form a relatively stable population of cells.[6] Moreover, according to Szysko et al. salivary glands of SjD patients provide niches rich in specific factors vital for survival of plasma cells[6]. The long-lived plasma cells in these niches are possibly not affected by 24 weeks of abatacept treatment. Further research is needed to gain more knowledge regarding plasma cell populations residing in salivary glands of SjD patients, in particular on long-term effects of abatacept treatment on these cells.

Besides changes in numbers of IgA plasma cells, histopathological evaluation revealed an increase in the number of infiltrating B-cells in parotid salivary glands of patients in the placebo group after 24 weeks. Christodoulou et al. observed that in labial gland biopsies the number of B-cells are associated with the Tarpley biopsy score, and thus likely with the progression of the disease.[21] In contrast to placebo, an increase in B-cell numbers was not seen in abatacept-treated patients, suggesting that further infiltration was halted by this immunomodulatory biological DMARD. Progression of B-cell infiltration was, however, not seen in labial glands of the placebo group. The reason for this is not clear, but untreated SjD patients harbour relatively more B-cells (but not T-cells) in parotid glands than in their labial glands, when paired glands are compared.[16] An increase in the numbers of B-cells (in placebo treated patients) might therefore preferably be seen in parotid glands. In line with these findings, the open-label extension phase of the ASAP-III trial revealed glandular function improvement after 48 weeks while this was not observed after 24 weeks of abatacept treatment.[22] Perhaps, glandular function and also histopathology, requires a longer treatment period to improve.

These findings are in line with our previous observations in parotid gland biopsies of abatacept treated patients in a smaller part of the study population (open label study; ASAP-II). In the previous ASAP-II open label study, GCs in parotid gland parenchyma were absent after abatacept treatment in those patients with GCs in their parotid glands on baseline.[14] While in most of the SjD patients treated with abatacept, the number of GCs/mm^2 in salivary gland parenchyma decreased (5 out of 7 patients, i.e., 71%), a similar pattern was observed in the placebo group (6 out of 8, i.e., 75%). Therefore, we can conclude that abatacept does not affect the formation of GC significantly.

A widely used tool to measure clinically meaningful improvement in SjD is change in ESSDAI or ClinESSDAI score of ≥3 points.[17] Despite the proven validity, reliability, and responsiveness of (clin)ESSDAI, several recent randomised controlled trials employing ESSDAI as the primary outcome measure were not able to discriminate in clinical response between the active treatment and placebo treatment groups. A main reason for this are the relatively large response rates in placebo groups.[9,23] The recently developed and validated composite endpoint CRESS was able to demonstrate superiority in clinical response of the active treatment compared to placebo treatment in

post-hoc analysis of several clinical trials, including the abatacept trials.[11] In the open-label study, presence of GCs at baseline predicted response to abatacept.[14] Here, we did not observe histopathological differences between responders and non-responders. This discrepancy may be explained by the fact that in the study by Haacke et al. treatment response was not defined by CRESS, or reaching ClinESSDAI MCII or LDA, but as an increase or decrease of ESSDAI in the glandular domain.

In this study, we combined labial and parotid gland biopsies derived from patients from three abatacept trials and analysed a wide range of histological parameters in order to obtain valuable insights into the effect of abatacept versus placebo treatment on salivary gland histology. However, this was an explorative analysis with multiple testing, which was not pre-powered to find significant differences, and especially the number of labial biopsies was relatively low. Another limitation might be that biopsies were taken 24 weeks after treatment, which might not be sufficient in order to find significant differences over time. Future studies may be needed to confirm the effects of (non)treatment.

To conclude, in this study, we showed that abatacept has a limited effect on salivary gland tissue in SjD, besides possibly affecting numbers of IgA plasma cells and preventing increases in B-lymphocyte infiltration. Additionally, we observed that salivary gland histopathology could not predict clinical response to abatacept in SjD patients.

REFERENCES

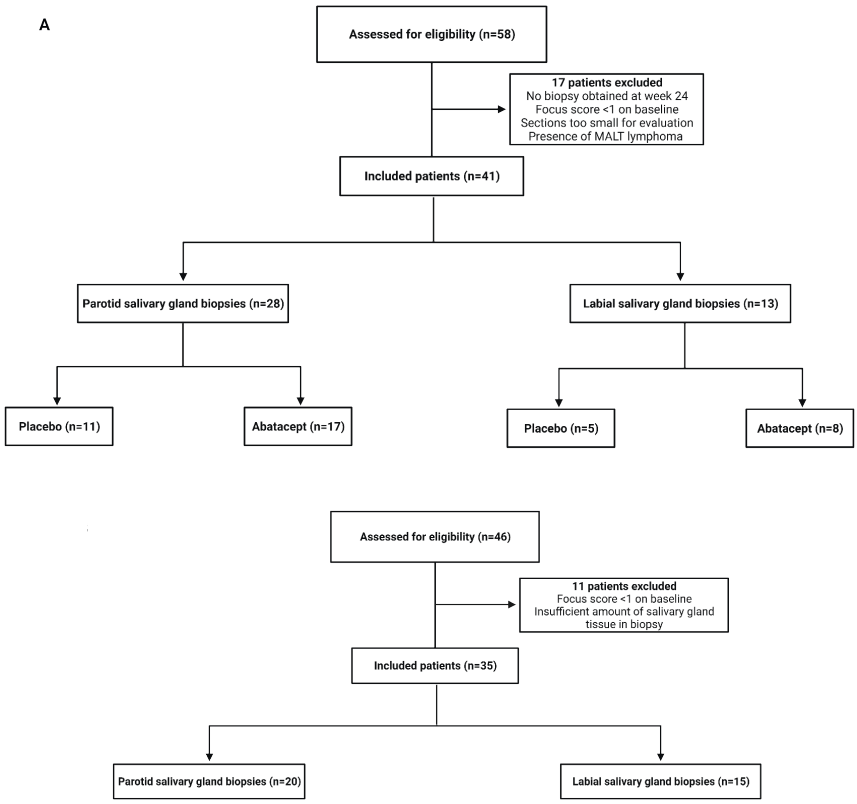
1. Mariette X, Criswell LA. Primary Sjögren's Syndrome. N Engl J Med. 2018 Mar;378(10):931–9.
2. Fox RI. Extraglandular Manifestations of Sjögren's Syndrome (SS): Dermatologic, Arthritic, Endocrine, Pulmonary, Cardiovascular, Gastroenterology, Renal, Urology, and Gynecologic Manifestations. In: Sjögren's Syndrome. New York, NY: Springer New York; 2011. p. 285–316.
3. Brito-Zerón P, Baldini C, Bootsma H, Bowman SJ, Jonsson R, Mariette X, et al. Sjögren syndrome. Nat Rev Dis Primers. 2016 Jul 7;2:16047.
4. Nakshbandi U, Haacke EA, Bootsma H, Vissink A, Spijkervet FKL, van der Vegt B, et al. Bcl6 for identification of germinal centres in salivary gland biopsies in primary Sjögren's syndrome. Oral Dis. 2020 Apr 28;26(3):707–10.
5. van Ginkel MS, Haacke EA, Bootsma H, Arends S, van Nimwegen JF, Verstappen GM, et al. Presence of intraepithelial B-lymphocytes is associated with the formation of lymphoepithelial lesions in salivary glands of primary Sjögren's syndrome patients. Clin Exp Rheumatol. 2019;37 Suppl 118(3):42–8.
6. Szyszko EA, Brokstad KA, Øijordsbakken G, Jonsson M V, Jonsson R, Skarstein K. Salivary glands of primary Sjögren's syndrome patients express factors vital for plasma cell survival. Arthritis Res Ther. 2011 Jan 7;13(1):R2.
7. Kroese FGM, Abdulahad WH, Haacke E, Bos NA, Vissink A, Bootsma H. B-cell hyperactivity in primary Sjögren's syndrome. Expert Rev Clin Immunol. 2014;10(4):483–99.
8. Westhovens R. Abatacept: the first-in-class costimulation blocker for the treatment of rheumatoid arthritis. Fut Rheumatol. 2006 Feb;1(1):15–22.
9. van Nimwegen JF, Mossel E, van Zuiden GS, Wijnsma RF, Delli K, Stel AJ, et al. Abatacept treatment for patients with early active primary Sjögren's syndrome: a single-centre, randomised, double-blind, placebo-controlled, phase 3 trial (ASAP-III study). Lancet Rheumatol. 2020 Mar;2(3):e153–63.
10. Baer AN, Gottenberg JE, St Clair EW, Sumida T, Takeuchi T, Seror R, et al. Efficacy and safety of abatacept in active primary Sjögren's syndrome: results of a phase III, randomised, placebo-controlled trial. Ann Rheum Dis. 2021 Mar;80(3):339–48.
11. Arends S, de Wolff L, van Nimwegen JF, Verstappen GMPJ, Vehof J, Bombardieri M, et al. Composite of Relevant Endpoints for Sjögren's Syndrome (CRESS): development and validation of a novel outcome measure. Lancet Rheumatol. 2021 Aug;3(8):e553–62.
12. Verstappen GM, Meiners PM, Corneth OBJ, Visser A, Arends S, Abdulahad WH, et al. Attenuation of Follicular Helper T Cell-Dependent B Cell Hyperactivity by Abatacept Treatment in Primary Sjögren's Syndrome. Arthritis Rheumatol. 2017 Sep;69(9):1850–61.
13. Corneth OBJ, Verstappen GMP, Paulissen SMJ, de Bruijn MJW, Rip J, Lukkes M, et al. Enhanced Bruton's Tyrosine Kinase Activity in Peripheral Blood B Lymphocytes From Patients With Autoimmune Disease. Arthritis Rheumatol. 2017 Jun;69(6):1313–24.
14. Haacke EA, van der Vegt B, Meiners PM, Vissink A, Spijkervet FKL, Bootsma H, et al. Abatacept treatment of patients with primary Sjögren's syndrome results in a decrease of germinal centres in salivary gland tissue. Clin Exp Rheumatol. 2017;35(2):317–20.
15. Meiners PM, Vissink A, Kroese FGM, Spijkervet FKL, Smitt-Kamminga NS, Abdulahad WH, et al. Abatacept treatment reduces disease activity in early primary Sjögren's syndrome (open-label proof of concept ASAP study). Ann Rheum Dis. 2014 Jul;73(7):1393–6.

16. Nakshbandi U, van Ginkel MS, Verstappen GMPJ, Spijkervet FKL, Arends S, Haacke EA, et al. Histopathological comparison of Sjögren-related features between paired labial and parotid salivary gland biopsies of sicca patients. Rheumatology. 2024 Oct 1;63(10):2670–7.
17. Seror R, Bootsma H, Saraux A, Bowman SJ, Theander E, Brun JG, et al. Defining disease activity states and clinically meaningful improvement in primary Sjögren's syndrome with EULAR primary Sjögren's syndrome disease activity (ESSDAI) and patient-reported indexes (ESSPRI). Ann Rheum Dis. 2016 Feb;75(2):382–9.
18. Seror R, Meiners P, Baron G, Bootsma H, Bowman SJ, Vitali C, et al. Development of the ClinESSDAI: a clinical score without biological domain. A tool for biological studies. Ann Rheum Dis. 2016 Nov;75(11):1945–50.
19. De Wolff L, Arends S, Pontarini E, Bombardieri M, Bowman SJ, Bootsma H. Development and performance of the Clinical Trials ESSDAI (ClinTrialsESSDAI), consisting of frequently active clinical domains, in two randomised controlled trials in primary Sjögren's syndrome. Clin Exp Rheumatol. 2021;39(6).
20. Brandtzaeg P. Mucosal immunity: induction, dissemination, and effector functions. Scand J Immunol. 2009 Dec;70(6):505–15.
21. Christodoulou MI, Kapsogeorgou EK, Moutsopoulos HM. Characteristics of the minor salivary gland infiltrates in Sjögren's syndrome. J Autoimmun. 2010;34(4):400–7.
22. de Wolff L, van Nimwegen JF, Mossel E, van Zuiden GS, Stel AJ, Majoor KI, et al. Long-term abatacept treatment for 48 weeks in patients with primary Sjögren's syndrome: The open-label extension phase of the ASAP-III trial. Semin Arthritis Rheum. 2022 Apr;53:151955.
23. Tsuboi H, Matsumoto I, Hagiwara S, Hirota T, Takahashi H, Ebe H, et al. Efficacy and safety of abatacept for patients with Sjögren's syndrome associated with rheumatoid arthritis: rheumatoid arthritis with orencia trial toward Sjögren's syndrome Endocrinopathy (ROSE) trial-an open-label, one-year, prospective study-Interim a. Mod Rheumatol. 2015 Mar;25(2):187–93.

ACKNOWLEDGEMENTS

This study was financially supported by Bristol Myers Squibb, who provided study medication and involved in data collection for the international abatacept trial (IM101603).

SUPPLEMENTARY MATERIAL



Supplementary figure 1. Flowchart of patient selection for the effect of abatacept on salivary gland biopsies of SjD patients (A) and the role of salivary gland biopsies to predict treatment response (B).

Supplementary table 1. Primary antibodies used for immunohistochemistry.

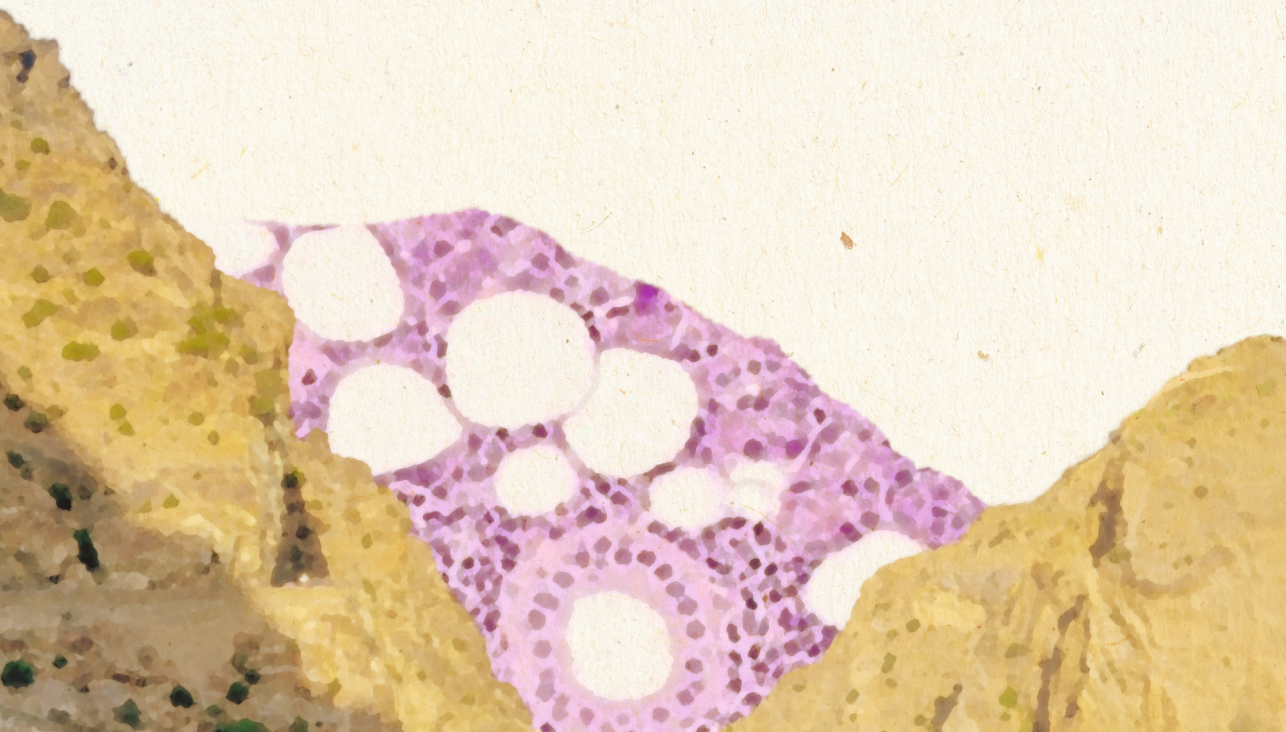
Antigen	Clone	Host	Company
CD3	2GV6	Rabbit	Ventana Roche
CD20	L-26	Mouse	Ventana Roche
CD21	2G9	Mouse	Ventana Roche
CD45	2B11 + PD7/26	Mouse	Ventana Roche
CK8/18	B22.1 + B23.1	Mouse	Ventana Roche
Bcl-6	GI19E1/A8	Mouse	Ventana Roche
IgA/IgG	Polyclonal	Rabbit	Ventana Roche
IgA	Polyclonal	Rabbit	Ventana Roche
IgG	Polyclonal	Rabbit	Ventana Roche
IgM	Polyclonal	Rabbit	Ventana Roche

Supplementary table 2. Histopathological and immunohistochemical data of parotid and labial salivary gland biopsies of SjD patients at baseline.

	Parotid salivary gland			Labial salivary gland		
	Placebo (n=11)	**Abatacept (n=17)**	**P-value**	**Placebo (n=5)**	**Abatacept (n=8)**	**P-value**
Focus score	1.8 (1.6-3.4)	4.3 (1.6-7.6)	0.11	2.0 (1.4-2.3)	2.0 (1.7-4.6)	1.00
LELs/mm²	0 (0-0.13)	0.27 (0-0.53)	0.09	0 (0-0.18)	0 (0-0.28)	0.94
LEL⁺ patients, n(%)*	5 (45.5)	10 (58.8)	0.70	2 (40.0)	2 (25.0)	0.70
FDC-networks/mm²	0.23 (0-0.49)	0.41 (0-0.57)	0.78	0.22 (0.07-0.86)	0.38 (0.04-0.67)	1.00
FDC-network⁺ patients, n(%)*	8 (72.7)	10 (58.8)	0.69	4 (80.0)	6 (75.0)	0.69
GCs/mm²	0.11 (0-0.33)	0 (0-0.29)	0.35	0 (0-0.10)	0 (0-0.09)	1.00
GC⁺ patients, n(%)*	6 (54.5)	5 (29.4)	0.25	1 (20.0)	2 (25.0)	0.25
CD3⁺cells/mm²	1212 (464-1904)	666 (277-1211)	0.33	528 (396-1054)	1317 (404-2088)	0.35
CD20⁺cells/mm²	596 (232-1285)	691 (193-1855)	0.71	327 (166-839)	1178 (249-1702)	0.17
CD3/CD20 segregation, n(%)*	8 (72.7)	8 (47.1)	0.25	2 (40.0)	6 (75.0)	0.25
CD45⁺cells (%)	17.1 (14.0-25.8)	19.8 (5.5-36.5)	0.85	17.0 (11.8-34.4)	35.7 (13.2-44.2)	0.44
IgA/IgG plasma cell shift, n(%)*	7 (63.6)	10 (58.8)	1.00	3 (60.0)	8 (100.0)	1.00
IgA plasma cells/mm²	326 (130-499)	110 (56-304)	0.15	1655 (998-2168)	611 (316-1166)	0.07
IgG plasma cells/mm²	205 (74-457)	97 (28-316)	0.35	692 (362-898)	550 (259-1025)	0.72
IgM plasma cells/mm²	49 (12-106)	11 (3-43)	0.20	566 (301-1445)	619 (242-804)	0.94

Supplementary table 3. Histopathological and immunohistochemical data of parotid and labial salivary gland biopsies of CRESS responders and non-responders at baseline.

	Parotid salivary gland biopsies			Labial salivary gland biopsies		
	CRESS responder (n=13)	**CRESS non-responder (n=8)**	**P-value**	**CRESS responder (n=9)**	**CRESS non-responder (n=6)**	**P-value**
Focus score	3.3 (1.6-8.7)	4.7 (1.9-5.2)	0.86	2.0 (1.4-2.6)	1.9 (5.0)	0.69
LELs/mm²	0.17 (0-0.33)	0.40 (0-0.56)	0.41	0 (0-0)	0 (0-0.38)	0.46
FDC networks/mm²	0.43 (0-0.74)	0.26 (0-0.86)	0.86	0.16 (0-0.28)	0.32 (0-0.82)	0.33
GCs/mm²	0 (0-0.33)	0 (0-0.38)	0.92	0 (0-0.03)	0 (0-0.03)	0.86
CD3⁺cells/mm²	433 (192-1704)	689 (455-3194)	0.34	428 (234-1160)	1182 (310-2266)	0.33
CD20⁺cells/mm²	360 (119-2655)	956 (262-1575)	0.80	279 (167-777)	781 (227-1446)	0.33
CD3/CD20 segregation, n(%)*	6 (46.2)	4 (50.0)	1.00	8 (88.9)	3 (50.0)	0.24
CD45⁺cells (%)	10.0 (4.4-48.6)	20.0 (5.9-51.3)	0.97	13.2 (8.5-32.7)	24.4 (13.0-40.8)	0.33
IgA/IgG plasma cell shift, n(%)*	6 (46.2)	6 (75.0)	0.37	7 (77.8)	6 (100.0)	0.34
IgA plasma cells/mm²	122 (64-404)	122 (54-769)	0.97	799 (515-1182)	488 (325-778)	0.30
IgG plasma cells/mm²	138 (28-384)	93 (20-287)	0.80	832 (272-1042)	228 (134-804)	0.24
IgM plasma cells/mm²	11 (5-43)	24 (2.3-83.3)	0.70	155 (57-564)	382 (135-730)	0.46



CHAPTER 10

General discussion

The development of periductal lymphocytic infiltrates in salivary glands is an important hallmark of Sjögren's disease (SjD). The pivotal role of salivary gland biopsy in SjD, based on the focus score (FS), is emphasised by its integration into the globally acknowledged ACR-EULAR classification criteria. When the serology is not positive (presence of anti-SSA autoantibody), a salivary gland biopsy is needed to classify a patients as having SjD.[1] The classification criteria have been validated for research purposes but are, incorrectly, also used for clinical diagnosis. In recent years, our collective understanding of histopathological changes characterising SjD has undergone substantial growth. Consequently, the potential role of the salivary gland biopsy has evolved within research from a diagnostic imperative to a multifaceted tool encompassing disease severity prediction and patient stratification. This thesis aims to offer insight into the effects of SjD on both minor and major salivary gland tissues. Moreover it focuses on the contribution of salivary gland histopathology to diagnosis, classification and patient stratification. This chapter discusses the most important findings of this thesis and highlights potential areas for future research.

Focus score: strengths and shortcomings

For diagnosis and classification of SjD the presence of focal lymphocytic sialadenitis (FLS) is assessed. FLS is characterized by the arrangement of one or more foci surrounded predominantly by unaffected salivary gland parenchyma. This histopathological pattern forms the foundation of the FS and other grading systems.[2] Examples of grading systems used for salivary gland evaluation of suspected SjD patients are the Chisholm and Mason system, the Greenspan and Daniels system, and the Tarpley system. In 1968, the Chisholm and Mason system was introduced, which was based on the presence of slight or moderate lymphocytic infiltration and/or lymphocytic foci in salivary gland.[3] As an extension, in 1974 Greenspan and Daniels introduced the FS by defining the number of foci per 4mm^2 area.[4] Subsequently Tarpley et al. incorporated additional considerations such as presence of diffuse infiltrates, acinar tissue destruction and fibrosis in salivary gland biopsies.[5] Compared to healthy individuals, SjD patients exhibit more acinar atrophy and fibrosis within their labial salivary glands, which is most likely a result of sustained

chronic inflammation. Progressive atrophy and fibrosis lead to a burnt-out biopsy, which even results in a negative FS.[6]

The FS and grading systems based on presence of foci, offer a standardised way to assess severity of lymphocytic infiltration in salivary glands. For accurate assessment of FS, it is recommended to evaluate a glandular surface area of at least 8mm^2 of multiple cutting levels.[7] FS is widely used and accepted, implemented and has proven to be a robust tool in the work-up of SjD. Moreover, FS is not only a diagnostic tool but also correlates with clinical manifestations such as presence of anti-SSA/B serology and presence of Sjögren related extra-glandular symptoms.[2,8] Also, several trials have used FS for evaluating the effectiveness of therapeutic interventions by obtaining pre- and post-treatment biopsies.[9–12] However, the FS is not without shortcomings. Few studies underscore the variability in observer agreement.[13,14] Vivino et al. revealed that upon re-examination 53% of biopsies referred to a tertiary centre for a second opinion, a revision of the initial diagnosis was necessary.[15] The main reason for revision was due to a failure to employ the focus score system. Moreover, the FS being a numeric average does not capture the complete area of infiltrate accurately. Consequently, reliance solely on the FS may result in a wrong diagnosis or misclassification of SjD. In sections where foci are confluent and should strictly be considered as a single focus, FS fails to represent glandular involvement as multiple small foci can result in a positive biopsy while a large focus can lead to a negative biopsy outcome.[16] In those cases for research purposes, at the UMCG, an arbitrary FS of 12 is assigned. To overcome this issue, the area of infiltrate can be analysed using anti-CD45 (staining all leukocytes), as exemplified in **chapters 3-5** and **9**. Future studies are necessary to establish a specific cut-off value and to assess the diagnostic accuracy of the area of lymphocytic infiltrate in SjD. However, the lack of specificity still poses a challenge, as the presence of lymphocytic infiltration is not exclusive to SjD and can be observed in healthy individuals as well as in other autoimmune or inflammatory conditions.[17–20] This becomes important when there are concurrent histopathological patterns such as different types of sialadenitis or increased areas of acinar atrophy, fibrosis and adipose tissue. Also, surprisingly, there is a poor correlation with the main function of the salivary glands, i.e. the production of saliva.[21] Thus, although lymphocytic

foci are characteristic of SjD, they do not solely define the histopathological landscape, emphasising the need for additional histopathological parameters.

Salivary gland histopathology in SjD beyond the focus score

Additional histopathological key-features for SjD include the presence of lymphoepithelial lesions, development of germinal centres and the presence of an IgA/IgG plasma cell shift.

Lymphoepithelial lesions (LELs)

Lymphoepithelial lesions (LELs) are characterized by the infiltration of lymphocytes into striated ducts along with concurrent hyperplasia of the ductal epithelium.[22] The current staging of LELs is based on the degree of hyperplasia: stage 1 (<50% epithelial hyperplasia), stage 2 (>50% epithelial hyperplasia), and stage 3 (circumferentially hyperplastic epithelium with occluded lumen).[12] Notably, the presence of intraepithelial B-lymphocytes was emphasised by van Ginkel et al., revealing that B-lymphocytes were exclusively found in 21% of striated ducts without hyperplasia and nearly all striated ducts with hyperplasia in SjD patients but not in controls. This suggests that intra-epithelial B-lymphocytes may drive the proliferation of the epithelium and that striated ducts without hyperplasia but with intraepithelial B-lymphocytes may represent a precursor stage of LELs, termed pre-LELs.[23]

While the precise mechanisms leading to the formation of (pre-)LELs are not fully understood, there is evidence pointing to the crucial role of epithelial cells, immune cells, and their interactions.[24] Initially, a limited number of B-lymphocytes respond to chemokines, such as CXCL10. CXCL10, possibly the driving force behind the recruitment and activation of immune cells, is produced by the inflamed ductal epithelium and is strongly upregulated by interferon. CXCL10 binds to its receptor CXCR3, which is expressed by a number of B-lymphocytes. Consequently, B-lymphocytes migrate into the ductal epithelium, leading to the formation of pre-LELs.[24] The connection between CXCL10 and (pre-)LEL development is also reflected by the observation that SjD patients exhibiting LELs express higher levels of CXCL10 in stimulated whole saliva and serum samples than SjD patients without LELs.[25] The phenotype of intra-epithelial B-lymphocytes appears to be different from B-lymphocytes in the periductal infiltrate.[24] Haacke et al. demonstrated that

a substantial proportion of intraepithelial B-lymphocytes express Fc receptor-like protein 4 (FcRL4) and are highly proliferative.[26] The communication between intra-epithelial B-lymphocytes and the ductal epithelium remains to be elucidated, but we speculate that pro-inflammatory cytokines, such as IL-6, may play a pivotal role by stimulating epithelial cells to proliferate. Subsequently, resulting in the loss of epithelial organisation and eventual loss of the ductal lumen. Further research is essential to unravel the intricate mechanisms underlying the formation of (pre-)LELs.[24]

Within the recommendations for the standardization of labial salivary gland assessment, LEL detection was not included. As the authors stated that LELs are predominantly found within parotid glands. In **chapter 3** our research revealed that (pre-)LELs occur as frequently in the labial gland as in the parotid gland, however less severe. Therefore we suggest to routinely assess the presence of (pre-)LELs within salivary glands of suspected SjD patients. The occurrence of LELs in the salivary glands of SjD patients is associated with various clinical parameters such as elevated levels of serum IgG, as well as an increased number of plasma cells and higher RF levels.[23,27]

Germinal centres (GCs)

Periductal infiltrates mostly consist of B-lymphocytes and $CD4^+$ T-lymphocytes, but also non-lymphoid cells such as dendritic cells and macrophages. Again, CXCL10 is presumed to play an important role in the initial stages of periductal infiltrate formation, given it is highly expressed by the ductal epithelium. Elevated levels of CXCL10 have also been identified in saliva, tears and serum of SjD patients. Over time, infiltrates may become organised forming ectopic lymphoid tissue characterised by compartmentalisation of T- and B-lymphocytes, the presence of follicular dendritic cell (FDC) networks and high endothelial venules. The organization of infiltrates is potentially due to homeostatic lymphoid chemokines such as CXCL13, CCL19, CCL21 and CXCL12.[6]

There is considerable heterogeneity in the reported prevalence of ectopic GCs within salivary glands of SjD patients which is due to the use of different staining techniques. In secondary lymphoid organs GCs can be identified by hematoxylin and eosin (HE) staining. A clear dark and light zone can be distinguished within the GC.[6] However, for ectopic GCs, detection by HE poses

a challenge, given the potential oversight of small GCs and the difficulty in distinguishing them from grade 3 LELs, that are also lightly stained and lack a clear lumen. Thus, additional immunohistochemical stainings are needed to aid GC identification. Some studies suggest to use CD3/CD20 to visualise T/B cell compartmentalisation or in combination with CD21 or CD35 to visualise FDC-networks.[28] However, next to expression by FDC-cells, CD21 and CD35 are also expressed by B-lymphocytes and therefore use of these markers can overestimate GC counts. Unfortunately, consensus guidelines to standardise the assessment of GCs are lacking.[29] Therefore, in the study described in **chapter 2** we compared H&E, CD21 and Bcl6 for the identification of GCs in salivary glands of SjD patients and clearly illustrated that the number of foci with $CD21^+$ FDC-networks was significantly higher than the number of foci with $Bcl6^+$ GCs. Furthermore, foci with $CD21^+$ FDC networks only showed $Bcl6^+$ clusters in 18% of labial and 32% of parotid gland foci. This shows that while FDC networks are a prerequisite for GC development, not all foci with $CD21^+$ FDC-networks also have GCs. Thus, we demonstrated that use of CD21 overestimates GC counts. While Fisher et al. suggest to use CD21 and CD3/CD20 for identification of GCs within clinical trials, we demonstrate Bcl6 should be used as an marker[30]

Traditionally GCs are found in secondary lymphoid organs like lymph nodes or tonsils. Within GCs mature B-lymphocytes undergo activation, proliferation, somatic hypermutation, and antigen-driven affinity maturation.[31,32] These processes give rise to high-affinity antibody-producing plasma cells and memory B-cells, with the enzyme activation-induced deaminase (AID) playing a pivotal role.[33] The presence of GCs in target tissues is a common histopathological feature in autoimmune diseases, exemplified by their observation in the synovium of rheumatoid arthritis patients.[34] Previous studies showed that presence of GCs in salivary glands of SjD patients is associated with higher prevalence and titres of RF, anti-SSA and anti-SSB antibodies, enhanced levels of local and systemic proinflammatory mediators. However, functional parameters such as ocular staining scores and salivary flow rates does not seem to correlate with the presence of GCs. Thus, patients with GCs appear to have more active disease in terms of serological parameters, but not functional parameters.[35]

The functionality of GCs within salivary glands of SjD patients has been questioned. Pottier et al. showed, by using reverse transcription-polymerase chain reaction to measure RNA expression after laser tissue dissection, expression of AID in only 36% of GC-containing patients.[36] Their finding lead to the suggestion that ectopic GCs within salivary glands of SjD patients are not functional. In their study CD21 and CD35, both markers which actually identify FDC-networks, were used to visualise GCs. Therefore, the lack of AID expression in $CD21^+$ and $CD35^+$ infiltrates is not surprising. As previously mentioned, we proposed to use Bcl6 as a marker for ectopic GC identification. In **chapter 3** we showed that almost all salivary gland biopsies in SjD which exhibited GCs as detected by Bcl6, also express AID and vice versa. In contrast to the study by Pottier et al. we demonstrated that GCs within salivary glands of SjD patients contain all elements necessary for driving the auto-immune process. Such methodological discrepancies underscore the importance of standardizing GC identification methods.[29,37]

Serum Tfr/Tfh ratio, a biomarker for GCs?

T follicular helper (Tfh) cells are essential for humoral immune responses and facilitate B-cell activation. Conversely T follicular regulatory cells (Tfr) are known to regulate and limit proliferation of Tfh cells and consequently B-cell activation (within GCs). An important mode of action of Tfr cells is through the expression of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), an inhibitory receptor.[38] In mouse models it has been illustrated that imbalance between these two types of T helper cells is associated with salivary gland inflammation, excessive GC responses and autoantibody production.[39] While multiple studies show that frequencies of Tfh are elevated in SjD patients, Verstappen et al. showed that frequencies of Tfr cells were even more elevated resulting in significantly higher Tfr/Tfh ratios in SjD. However, in SjD Tfr cells express lower levels of CTLA-4. This downregulation of CTLA-4 in Tfr cells may compromise their suppressive functions, potentially influencing the balance of immune responses and contributing to the dysregulated immune activity characteristic of SjD. Interestingly Fonseca et al. suggested that serum Tfr/Tfh ratio also correlates with ectopic GC formation in labial salivary glands, suggesting its potential as biomarker for GCs.[40] However, in their study the Tfr/Tfh ratio correlated with numbers of infiltrating lymphocytes and a pos-

itive biopsy, and not necessarily with GCs. In **chapter 5** we showed, in line with their study, elevated Tfr/Tfh ratios in blood of SjD patients compared to non-SjD sicca patients. Contrary to Fonseca et al., we did not find an association between this ratio in blood and parotid glandular inflammation, thus we were unable to confirm their findings.[40] This discrepancy might be due to the study population used. In their study the comparison was made between patients with focal sialadenitis and without focal sialadenitis, irrespective of the diagnosis of SjD. Most patients analysed were not SjD patients but non-SjD sicca patients.

Interestingly we did observe that levels of activated Tfh cells correlated with both ESSDAI and clinESSDAI (ESSDAI without the biological domain) scores and this could therefore be a sign of enhanced disease activity and supports the notion that Tfh and Tfr cells play a role in B-cell hyperactivity.

Ectopic GCs and MALT lymphoma development

Approximately 5-10% of SjD patients develop non-Hodgkin B-cell lymphomas, with MALT lymphomas being the most common subtype.[41] MALT lymphomas in SjD patients preferentially arise in the parotid salivary glands and have the potential to evolve into more aggressive diffuse large B-cell lymphoma. Some studies have suggested that GC development is associated with lymphomagenesis in SjD. However, a study performed by Haacke et al. reported no difference in the occurrence of GCs in labial gland biopsies of patients with SjD prior to developing parotid MALT lymphomas compared to matched SjD controls.[42] Discrepancy between results are most probably due to variations in patient cohorts and staining methods employed for GC identification. Theander et al. considered all NHLs rather than solely including MALT lymphomas.[43] Also, for GC identification HE staining was used and no additional immunohistochemical stainings were performed. In their study a biopsy was considered GC positive in cases in which a well-circumscribed chronic inflammatory cell infiltrate consisting of at least 50 mononuclear cells presented with features indicative of lymphoid organisation, such as a densely packed dark zone and a light zone within otherwise normal salivary gland epithelium.[43] In the study performed by Bombardieri et al. 75% of labial gland biopsies of SjD patients expressed 'GC-like structures' before parotid MALT lymphoma development.[44] Here, GCs-like structures were defined by presence of $CD3^+$ T-cells, $CD20^+$

B-lymphocytes and CD21⁺FDC-networks. Based on our findings in **chapter 2** we propose to use Bcl6 for the identification of GCs in all future clinical trials to ensure consistency and avoid discrepancies.

IgA/IgG plasma cell shift

IgA secreting plasma cells are a normal component of mucosal immunity within salivary glands.[45] However, in SjD patients there is a notable shift in the isotype composition, transitioning from IgA dominance to an increased frequency of IgG plasma cells, also referred to as plasma cell shift.[46] Elevated numbers of CD138⁺ plasma cells were observed in salivary glands of SjD patients with high FSs. Salivary gland epithelium, focal infiltrates and adipocytes express important plasma cell related survival factors such as CXCL12 and IL6.[40] Thus, the environment of salivary glands seems to provide factors essential for plasma cell survival.[47] The shift in plasma cell populations is not merely a quantitative change but seems to be intricately linked to the underlying immune dysregulation in SjD. In the study described in **chapter 3**, we observed a plasma cell shift in 60.2% of labial gland and 41.7% of parotid gland biopsies of SjD patients. Specificity of a plasma cell shift was noteworthy, as it was exclusively identified in SjD patients. The increase in IgG-producing plasma cells suggests a heightened B-cell activity and humoral immune response within the affected salivary glands. In our studies, we identified an IgA/IgG plasma cell shift through dual staining for IgA and IgG and employing an arbitrary cutoff of 70%. However, a noteworthy limitation of this technique lies in its subjective nature of assessment. More efforts should be put into improving its staining technique and applying (AI driven) image analysis techniques.

Major and minor salivary gland histopathology

Currently, worldwide the labial salivary gland biopsy is obtained for diagnosis and classification of SjD.[3] However, the parotid salivary gland is a good alternative with a comparable diagnostic potential.[48] Also, the parotid gland has the advantage of allowing for repeated biopsy from the same gland, offering a valuable tool for monitoring disease progression or treatment response. Notably, MALT lymphomas preferentially arise within the parotid gland. Therefore, parotid gland biopsies have a higher likelihood of detecting MALT lymphomas when compared to their labial gland counterparts, adding a layer of diagnostic

efficacy.[6,48] However, biopsies of the parotid gland have not become commonplace because of the fear of facial nerve damage, development of sialoceles and salivary fistulae.[49] The study performed by Pijpe et al. showed, however, no permanent sensory loss after parotid biopsy, while labial biopsy led to permanent sensory loss in 6% of patients. Moreover, level of pain was comparable, and no loss of motor function was observed.[48] These findings were partly confirmed by Delli et al. who found, that while changes in sensibility and pain in the biopsies area were significantly higher after parotid gland biopsy compared to labial gland biopsy at one week and six months post operatively, changes were comparable after 12 months.[50] Discrepancy between these two studies can be due to the fact that change of sensibility was assessed by physical testing in the study performed by Pijpe et al. while in the study by Delli et al. this was patient-reported and assessed based on the questionnaire. Nevertheless, in both studies, changes were minor and comparable.

In terms of histopathology, in the study described in **chapter 3** we observed that presence of histopathological key features, namely FS >1, (pre-) LELs, IgA/IgG shift and GCs, were largely comparable for paired labial and parotid gland sections of suspected SjD patients with an absolute agreement of 80-93%. Transcriptome analysis of paired parotid and labial salivary gland biopsies performed by our group also showed a high degree of overlap in immune pathway activity.[51] However, in parotid gland biopsies of SjD patients we observed more pronounced B-lymphocyte activity (e.g., higher numbers of B-lymphocytes/mm^2, more GCs/mm^2 and more severe LELs) compared to labial glands. Differences between the two types of glands in terms of B-cell hyperactivity remain unclear but we speculate that more pro-inflammatory cytokines result in more attraction and/or activation of B-cells, thereby creating an immunological environment favouring B-cell activity. Haacke et al. also observed more FcRL4$^+$ B-cells in parotid glands compared to labial glands.[26] The presence of more FcRL4$^+$ B-cells in parotid gland parenchyma may explain why in our study (**chapter 3**) LELs are found to be more severe in parotid glands compared to labial glands of SjD patients. Interestingly, in the same study Haacke et al. also revealed that almost all parotid MALT lymphomas of SjD patients express FcRL4 and that expression of FcRL4 mRNA in parotid gland tissue with MALT lymphoma is increased compared to MALT lymphoma negative SjD patients. Furthermore, the majority of malignant FcRL4$^+$ B-cells resided

nearby the ductal epithelium where LELs are formed. The potential of malignant transformation of FcRL4$^+$ B-lymphocytes, is further supported by the observation that clonal expansions were present among intra-ductal B-lymphocytes, as reported by Visser et al.[52] Taken together, these findings suggest that FcRL4$^+$ B-lymphocytes present nearby LELs are able to progress to neoplastic B-lymphocytes forming MALT-lymphomas in salivary glands of SjD patients.[24]

In **chapter 3** we also showed that labial glands have higher FSs, amount of infiltrates as well as number of T- and B-lymphocytes compared to paired parotid glands. Not only were more and larger infiltrates observed in labial glands of SjD patients but also in non-SjD sicca patients. The higher number of infiltrating lymphoid cells in labial glands of non-SjD sicca patients indicate that lymphocytic infiltrates are frequently present in these glands irrespective of the presence of SjD which can potentially result in false-positive biopsy results (see section below for further details).

Additional value of other histopathological characteristics

Studies have assessed the diagnostic accuracy of both the labial and parotid salivary gland biopsy in SjD. Existing studies report sensitivity percentages ranging from 63.5% to 93.7% and specificity from 61.2% to 100%.[53] Notably many studies employ criteria such as the AECG or ACR-EULAR classification criteria. As a result circular reasoning occurs, as a positive biopsy based on FS ≥1 is an item within these criteria sets. In **chapters 6** and **7** expert opinion was used to define patient and control groups, in order to limit circular reasoning. In the study described in **chapter 6,** it was shown that the diagnostic accuracy of the *labial gland biopsy* improves significantly when all earlier mentioned histopathological key features, namely presence of FS ≥1, (pre-)LELs, IgA/IgG plasma cell shift and GCs, are taken into account. The specificity of the labial gland biopsy improved with 12% to 100% indicating that no non-SjD sicca patients were misclassified. Also, the performance of the ACR-EULAR criteria improves when the labial gland biopsy item in the criteria is replaced by the item presence of ≥2 of the 4 histopathological features. The importance of assessing histopathological features beyond the FS was further illustrated in **chapter 8**, where we observed that the presence of at least one of the four different histopathological key features was a stronger predictor of expert diagnosis than FS ≥1 alone.

In **chapter 7** we assessed the diagnostic accuracy of the *parotid gland biopsy* when additional histopathological key features were taken into account. For the parotid gland specificity of a FS ≥1 is already high (98%), however, sensitivity is rather low when only focusing on the FS (64%). We demonstrate that by taking the presence of the other histopathological key features into account, sensitivity increased with 8% from 64% to 72%. Some studies defined a positive parotid biopsy by taking both FS ≥1 and presence of other histopathological features such as LELs into account.[48,51,54] In our study (**chapter 7**), the addition of only (pre-)LELs to the evaluation of parotid salivary gland biopsies had minor impact on the sensitivity of the parotid gland biopsy. Only one SjD patient presented with a (pre-)LEL but without a FS ≥1, while no non-SjD sicca patients revealed (pre-)LELs. Thus, for the standard histopathological evaluation of both the labial and the parotid gland biopsies we recommend to include all four parameters.

The cut off-value of the FS for SjD has only been validated for labial gland biopsies. In **chapter 3** we demonstrated that labial gland biopsies revealed more non-SjD related periductal infiltrates compared to paired parotid glands. In line with this observation, in **chapter 7** we show that a value of 0.9 resulted in a slightly higher sensitivity than 1.0 and comparable specificity. Larger studies are necessary to validate our observation.

Thus, in contrast to the labial gland biopsy, the parotid gland biopsy has an excellent specificity for predicting SjD patients based on FS ≥1. The sensitivity of a parotid gland biopsy can be improved if the presence of other histopathological key features are taken into account as well.

In the study described in **chapter 8,** it was assessed to what extent key histopathological features (FS ≥1, (pre)LELs, IgA/IgG plasma cell shift and GCs) correlated with each other in labial gland biopsies of suspected SjD patients. We observed fair to moderate inter-feature agreement between FS ≥1, (pre) LELs, and IgA/IgG plasma cell shift, whereas poor agreement was observed for these features with the presence of GCs. In this study, only 12/262 patients expressed GCs within their labial gland biopsy. This low prevalence of GCs may explain the low agreement between GCs and other key features observed. Overall, there is agreement between the different histopathological features associated with SjD, but further research is needed to determine whether these features develop in a specific sequence over the course of the disease.

Multiple studies illustrated the association between the presence of histopathological features and serological abnormalities, as mentioned before.[23,35] This association was confirmed in the study described in **chapter 8** where we observed that in patients that tested positive for anti-SSA, anti-SSB, RF or elevated IgG plasma cells, significantly more histopathological key features were observed compared to those without serological abnormalities. The relation between SjD specific serological abnormalities and salivary gland histopathology, suggest that histopathological changes potentially reflect auto-immune activity. Whether salivary glands are the location where auto-immune processes are driven and perhaps auto-antibodies are secreted, remains to be elucidated.

Salivary gland ultrasonography as an alternative for the salivary gland biopsy

While improving the assessment of salivary gland biopsies is important, there is also a subgroup of patients who do not have a positive biopsy (i.e. FS <1.0). 18-40% of SjD patients have a FS <1 in their labial salivary gland biopsy.[48,55,56] These patients may be misdiagnosed as non-SjD sicca patient according to the classification criteria. This raises the question whether there are other, less invasive, techniques available that may potentially replace the salivary gland biopsy. One of the diagnostic modalities is the salivary gland ultrasonography (SGUS), which has become popular for the diagnostic work-up of suspected SjD patients. SGUS is easy to perform, low in costs and, importantly, not invasive. Mossel et al. assessed whether the salivary gland biopsy could completely replace the salivary gland biopsy.[21] A good absolute agreement was observed between SGUS and labial (79%) and parotid (83%) salivary gland biopsy outcomes. However, another study performed by Mossel et al. revealed that labial gland biopsies had a positive biopsy in 26% of patients with a negative SGUS and 22% of SGUS positive patients FS <1 in their parotid gland biopsy.[57] The discrepancy between labial gland biopsy and SGUS outcomes may arise from the fact that SGUS is not performed on labial glands. This makes comparisons challenging, as the labial gland contains significantly more non-SjD-related infiltrates (**chapter 3**). When taking other histopathological features ((pre-) LELs, plasma cell shift and GCs) into account, not all patients with additional features also showed SjD related SGUS abnormalities.[21] In line with these findings, other studies demonstrated that replacement of salivary gland biopsy

with SGUS resulted in a significant decrease in sensitivity and specificity of ACR-EULAR classification criteria.[58,59] These results indicate that SGUS is valuable as an addition for the diagnostic work-up of suspected SjD patients but not necessarily as an alternative for the salivary gland biopsy.

Another less invasive diagnostic technique that can be applied is ultrasound-guided core needle biopsy (CNB). In contrast to the fine needle aspiration cytology, CNB provides biopsy samples with preserved architecture which can be stained with immunohistochemically. While the CNB is already widely applied for the diagnosis of lymphoma in SjD, it is not routinely employed in the diagnostic work-up of SjD.[60] A recent proof-of-concept study performed by Deroo et al. compared the incisional biopsy with the CNB by comparing paired biopsies.[61] For paired biopsies >4mm2 a concordance of 90% was observed for FS >1 and presence of LELs and GCs showed 100% concordance. These findings illustrate that US-guided CNB of the parotid gland is comparable in terms of these histopathological features. Therefore, US-guided CNB seems to be very promising. Nevertheless, as also mentioned by Deroo et al., inherent to the technique, smaller amount of salivary gland tissue is harvested and can complicate reliable FS assessment. These results need to be verified in larger cohorts to assess whether the US-guided CNB can be implemented in diagnosis but also to evaluate treatment response. Additionally, a comprehensive comparison between incisional biopsy and US-guided CNB warrants the assessment of additional histopathological parameters.

Pediatric SjD

Classification criteria for SjD, such as the AECG or ACR-EULAR criteria, are worldwide in use for classification of disease. These criteria have been validated for research purposes and are well-suited as entry criteria for clinical trials in adults.[62,63] However, within these criteria, glandular dysfunction is taken into account, such as decreased tear and saliva production. However, multiple studies showed that children may not present with sicca symptoms especially early in the disease, but rather with recurrent parotid gland swelling and fever.[64–66] These differences in clinical presentation pose the question whether these classification criteria are suitable for pediatric SjD (pedSjD) patients. A large retrospective study performed by Basiaga et al. showed that the majority of pedSjD patients (232/300, 77%) did not meet the ACR-EULAR

classification criteria.[64] However, for the majority of patients not all five items of the classification criteria were tested.

In terms of histopathology, limited studies are available that compare adult and pedSjD patients. While for adult SjD (adSjD) patients, a FS ≥1 is considered as a positive biopsy, Yokogawa et al. observed that only 8/12 pedSjD had a labial gland biopsy with a FS ≥1 while in all samples 12/12 one or more foci were found.[67] Also, in age-matched controls only one patient had a focus in their labial gland section. These results were confirmed by two other studies.[68,69] Potentially a FS ≥1 is not suitable for pedSjD patients and just the mere presence of a focal lymphocytic sialadenitis might indicate SjD. The importance of a parotid gland biopsy, as a potential alternative to a labial gland biopsy, is touched upon by McGuirt Jr et al., who illustrated in a case series review of six pedSjD patients that only 2/6 reported with a labial gland biopsy consistent with SjD.[70] The other four patients went on to have parotid biopsies which were all positive.[70] However, to our knowledge, the study described in **chapter 4** is the first study to quantitatively compare all key histopathological features of salivary gland biopsies between paediatric- and adult-onset SjD patients. In this study we demonstrated that PedSjD patients exhibit all the characteristic histopathological features of SjD. Moreover, histopathological findings seem to be more severe and are accompanied by increased B-lymphocyte involvement. Our results emphasise the need for a different approach in pedSjD patients as the onset of disease is likely to be different compared to adSjD patients and might begin within the parotid gland. The study described in **chapter 4** was important to demonstrate that SjD related key features exhibited by adults are also exhibited by pedSjD patients. Nevertheless, we did not take acinar atrophy, fibrosis or the amount of adipose tissue into account. Acinar atrophy and fibrosis might be a result of continuous salivary gland inflammation, and thus perhaps detectable over a longer period of time. Similarly, the amount of adipose tissue has, by some studies, been associated with age. Thus, potentially these histopathological items might be less notable in pedSjD patients. While extensive histopathological analysis of salivary glands of pedSjD patients is interesting for research purposes, it is questionable whether it is of clinical relevance.

Salivary gland biopsies for treatment response and stratification of patients

Until now there are no approved biological DMARDs for the treatment of SjD. However, there is a high number of clinical trials. Analysis of pre- and post-treatment biopsies provides more insight in the pathogenesis of SjD and involvement of specific pathways, cells and molecules. In this section we discuss the evidence that might support the role of histopathology as a response to therapy and as a biomarker for stratification.

As an example: Abatacept

Abatacept, a CTL4-Ig, blocks the CD28-mediated co-stimulation of T-cells.[71] While in phase II trials efficacy was shown with respect to ESSDAI[72], this was not confirmed by phase III trials.[73,74] Also histopathologically, no significant differences were observed in parotid gland biopsies of SjD patients after 24 weeks of abatacept treatment.[75] However, limitations of the study were the lack of a placebo group, analysis of only parotid gland biopsies and the limited number of patients. In the study described in **chapter 9** we analysed histopathological parameters before and after 24 weeks of abatacept or placebo treatment. In line with the ASAP-II study, we found that abatacept did not alter FS or other histopathological parameters significantly within the labial and parotid gland biopsy.

The ASAP-II study led to the suggestion that abatacept affects GC formation which might have been expected given the mode of action.[75] However, in our study, with both labial and parotid gland biopsies before and after treatment, a similar pattern was observed in the placebo group. Therefore, we can conclude that abatacept does not affect the formation of GC significantly and differences over time are perhaps random. Slight changes were observed in number of IgA plasma cell counts, indicating restoration of the glandular plasma cell population. Interestingly, these differences did not concurrently occur with decreased numbers of IgG plasma cells. This can be due to the fact that salivary glands express factors vital for plasma cell survival (e.g., CXCL12 and IL-6).[76] As a result long-lived IgG plasma cells remain within the glandular tissue. We also found that the number of $CD20^+$ B-lymphocytes in parotid glands significantly increased after 24 weeks in the placebo group but remained stable in the abatacept group. This finding indicates that abatacept potentially halts further B-cell hyperactivity through inhibition of full T

cell activation and T-cell dependent B-cell activation. An explanation for not seeing a significant difference in $CD20^+$ B-lymphocyte counts in labial gland biopsies can be due to our observation that the number of B-cells is higher in parotid glands compared to paired labial glands (**chapter 3**). Therefore, a significant difference in B-lymphocyte numbers is sooner observed in parotid salivary gland sections. A pilot study performed by Adler et al. showed that the number of Treg cells within foci significantly decreased after abatacept treatment.[77] Treg cells can be found in sites of inflamed tissue in auto-immune diseases and compensate for autoimmune related inflammation. As the number of Treg cells is positively correlated with more salivary gland inflammation in SjD, a decrease of Treg cells after abatacept treatment might imply better immunological control. In line with this observation, Adler et al. concluded that abatacept treatment resulted in significant reduction of glandular inflammation in SjD patients. In their study, histopathological analysis of labial salivary glands, showed a significant decrease of absolute numbers of lymphocytic foci. Importantly, the number of lymphocytic foci per mm^2 and number of T- and B-lymphocytes did not change after 24 weeks of treatment. Therefore, overall abatacept seems to have a limited effect on salivary glands in SjD.

The open-label extension phase of the ASAP-III trial revealed glandular function improvement after 48 weeks while this was not observed after 24 weeks of abatacept treatment.[78] Perhaps, glandular function and also histopathology, requires a longer treatment period to improve. However, this assumption remains questionable as multiple studies have shown that the correlation between glandular function and histopathology is poor. It would be interesting if further research will be performed to assess long-term effects of abatacept treatment and non-treatment on salivary glands.

Salivary gland biopsies for stratification of patients and the role of endpoints

The various histopathological features might also be used for patient stratification for treatment, and precision medicine. For example, Delli et al. showed that high absolute number of B-cells in the baseline parotid gland biopsy predicts better responsiveness of patients with SjD to rituximab treatment.[12] In the ASAP II open-label study, presence of GCs at baseline predicted response to abatacept.[75] This finding was not confirmed in the study described

in **chapter 9**, where we concluded that salivary gland histopathology could not predict treatment response**.** This discrepancy may be explained by the fact that in the study by Haacke et al.[79] a clinically meaningful improvement was defined as an increase or decrease of ESSDAI in the glandular domain while we used the newly developed Composite of Relevant Endpoints for Sjögren's Syndrome (CRESS)[80] and minimal clinically important improvement (MCII) and low disease activity (LDA) for the ClinESSDAI.[81,82] Importance of using the right endpoint is highlighted by the observation reported by Arends et al., that RCTs that previously showed negative primary endpoint results, post-hoc analysis using the CRESS resulted in higher response rates in patients treated with abatacept and rituximab than in those given placebo. Given the heterogenous nature of SjD our research group developed the CRESS.[80] The CRESS consists of five complementary, clinically relevant items: systemic disease activity, patient-reported symptoms, tear gland item, salivary gland item, and serology. Another highly comparable recently developed composite endpoint for SjD is the Sjögren's Tool for Assessing Response (STAR).[83] In RCTs that previously showed negative primary endpoint results, post-hoc analysis of the trial data using composite endpoints resulted in higher response rates in patients treated with abatacept, rituximab and tocilizumab than in those given placebo.[84] While prospective RCTs are necessary in order to validate these composite endpoints, outcomes seem very promising.

Conclusions

This thesis aimed to gain more knowledge about the histopathology of minor and major salivary glands in SjD patients. We aimed to identify differences between the two gland types but also to improve its assessment for diagnostic and research purposes. Our findings demonstrated that while histopathological changes are present in both labial and parotid glands, the labial glands exhibited fewer signs of B-cell hyperactivity and more non-SjD related inflammation. Therefore, relying solely on the FS in labial slang biopsies carries a risk of false-positive results. We established that incorporating additional histopathological key features besides a FS ≥1, such as the presence of (pre-) LELs, plasma cell shift, and GCs, enhances the accuracy of diagnosis and classification in both types of glands. Furthermore, we showed that presence of histopathological key features (FS ≥1, (pre-)LELs, plasma cell shift, and GCs)

are associated with each other and with serological abnormalities such as anti-SSA, anti-SSB, RF and elevated IgG plasma cells. Importantly, our results showed that the presence of multiple histopathological key features serves as a strong predictor for SjD diagnosis. Therefore, we suggest that these features should be assessed routinely. Furthermore, our research highlighted the utility of applying Bcl6 in clinical practice for detecting ectopic GCs within salivary glands, as opposed to various staining methods currently in use. Assessment of parotid gland biopsies of pedSjD patients revealed that similar characteristics were detectable in adSjD patients but were more severe. Obtaining pre- and post-treatment biopsies provides more insight in mode of action of treatments. As effects on salivary gland histopathology was limited in SjD patients and no significant differences could be identified in responders compared to non-responders, abatacept does not seem to have promising results in SjD.

Future perspectives

Salivary gland involvement stands out as a defining characteristic of SjD. Beyond the FS, which serves as a pivotal parameter in evaluating lymphocytic infiltration, it is important to consider additional histopathological features such as the presence of (pre-)LELs, plasma cell shift, and GCs when conducting diagnostic assessments for both labial and parotid gland biopsies. There is a pressing need for greater standardisation in evaluating salivary gland histopathological parameters. The establishment of globally uniform methods would not only ensure reliable result comparisons but also facilitate a comprehensive assessment of clinical relevance and correlations with other disease characteristics. Consensus guidelines should be formulated and specifically take different salivary gland types into account.

Inter-observer discrepancies in histopathological evaluation pose a significant challenge and contribute to variations in salivary gland results. To address this issue, an increasing number of studies are adopting digital image analysis and other program-driven methods for biopsy assessments, aiming to ensure uniformity in interpretations. Looking toward the future, artificial intelligence (AI) is most probably going to play a transformative role. Although currently limited to a select number of studies, AI applications in SjD are emerging. AI-driven initiatives include the development of classification and diagnostic models, as well as predicting subgroup of SjD patients that

might respond to therapy. Within histopathology of SjD AI is also of interest. AI-driven apps can enable analysis of large datasets in a shorter period of time, thereby increasing efficiency. Moreover, predefined algorithms ensure uniformity in the interpretation of tissue characteristics across different pathologists and laboratories.

While incisional salivary gland biopsies provide a world of information other diagnostic modalities are emerging. SGUS is non-invasive, low in costs and repeatable. Nevertheless, SGUS outcomes only seem to be partly related to biopsy results. More research is needed to understand how US images correspond to biopsy results. To overcome the invasive nature of the incisional biopsy recently the application of CNB was assessed and seem to be comparable. Nevertheless, larger studies are necessary to validate these findings and specific guidelines should be formulated to further guide assessment of biopsies.

REFERENCES

1. Shiboski CH, Shiboski SC, Seror R, Criswell LA, Labetoulle M, Lietman TM, et al. 2016 American College of Rheumatology/European League Against Rheumatism Classification Criteria for Primary Sjögren's Syndrome: A Consensus and Data-Driven Methodology Involving Three International Patient Cohorts. Arthritis & rheumatology. 2017 Jan;69(1):35–45.
2. Daniels TE, Cox D, Shiboski CH, Schiødt M, Wu A, Lanfranchi H, et al. Associations between salivary gland histopathologic diagnoses and phenotypic features of Sjögren's syndrome among 1,726 registry participants. Arthritis Rheum. 2011;63(7):2021–30.
3. Chisholm DM, Mason DK. Labial salivary gland biopsy in Sjögren's disease. J Clin Pathol. 1968 Sep;21(5):656–60.
4. Greenspan JS, Daniels TE, Talal N, Sylvester RA. The histopathology of Sjögren's syndrome in labial salivary gland biopsies. Oral Surgery, Oral Medicine, Oral Pathology. 1974 Feb;37(2):217–29.
5. Tarpley TM, Anderson LG, White CL. Minor salivary gland involvement in Sjögren's syndrome. Oral Surgery, Oral Medicine, Oral Pathology. 1974;37(1):64–74.
6. Kroese FGM, Haacke EA, Bombardieri M. The role of salivary gland histopathology in primary Sjögren's syndrome: promises and pitfalls. Clin Exp Rheumatol. 2018;36 Suppl 1(3):222–33.
7. Fisher BA, Jonsson R, Daniels T, Bombardieri M, Brown RM, Morgan P, et al. Standardisation of labial salivary gland histopathology in clinical trials in primary Sjögren's syndrome. Ann Rheum Dis. 2017;76(7):1161–8.
8. Carubbi F, Alunno A, Cipriani P, Bartoloni E, Baldini C, Quartuccio L, et al. A retrospective, multicenter study evaluating the prognostic value of minor salivary gland histology in a large cohort of patients with primary Sjögren's syndrome. Lupus. 2015 Mar;24(3):315–20.
9. Haacke EA, van der Vegt B, Meiners PM, Vissink A, Spijkervet FKL, Bootsma H, et al. Abatacept treatment of patients with primary Sjögren's syndrome results in a decrease of germinal centres in salivary gland tissue. Clin Exp Rheumatol. 2017;
10. Mariette X, Ravaud P, Steinfeld S, Baron G, Goetz J, Hachulla E, et al. Inefficacy of infliximab in primary Sjögren's syndrome: results of the randomized, controlled Trial of Remicade in Primary Sjögren's Syndrome (TRIPSS). Arthritis Rheum. 2004 Apr;50(4):1270–6.
11. Seror R, Nocturne G, Lazure T, Hendel-Chavez H, Desmoulins F, Belkhir R, et al. Low numbers of blood and salivary natural killer cells are associated with a better response to belimumab in primary Sjögren's syndrome: results of the BELISS study. Arthritis Res Ther. 2015 Sep;17(1):241.
12. Delli K, Haacke EA, Kroese FGM, Pollard RP, Ihrler S, van der Vegt B, et al. Towards personalised treatment in primary Sjögren's syndrome: baseline parotid histopathology predicts responsiveness to rituximab treatment. Ann Rheum Dis. 2016 Nov;75(11):1933–8.
13. Fisher BA, Brown RM, Bowman SJ, Barone F. A review of salivary gland histopathology in primary Sjögren's syndrome with a focus on its potential as a clinical trials biomarker. Ann Rheum Dis. 2015;74(9):1645–50.
14. Lucchesi D, Pontarini E, Donati V, Mandarano M, Sidoni A, Bartoloni E, et al. The use of digital image analysis in the histological assessment of Sjögren's syndrome salivary glands improves inter-rater agreement and facilitates multicentre data harmonisation. Clin Exp Rheumatol. 2020;38 Suppl 126(4):180–8.
15. Vivino FB, Gala I, Hermann GA. Change in final diagnosis on second evaluation of labial minor salivary gland biopsies. J Rheumatol. 2002 May 1;29(5):938–44.

16. Kroese FGM, Haacke EA, Bombardieri M. The role of salivary gland histopathology in primary Sjögren's syndrome: promises and pitfalls. Clin Exp Rheumatol. 2018;36 Suppl 1(3):222–33.

17. Lindahl G, Hedfors E. Focal lymphocytic infiltrates of salivary glands are not confined to Sjögren's syndrome. Scand J Rheumatol Suppl. 1986;61:52–5.

18. Radfar L, Kleiner DE, Fox PC, Pillemer SR. Prevalence and clinical significance of lymphocytic foci in minor salivary glands of healthy volunteers. Arthritis Care Res (Hoboken). 2002 Oct 15;47(5):520–4.

19. Lindahl G, Hedfors E. Lymphocytic infiltrates and epithelial HLA-DR expression in lip salivary glands in connective tissue disease patients lacking sicca: a prospective study. Br J Rheumatol. 1989 Aug;28(4):293–8.

20. Maldonado JO, Beach ME, Wang Y, Perez P, Yin H, Pelayo E, et al. HCV Infection Alters Salivary Gland Histology and Saliva Composition. J Dent Res. 2022 May;101(5):534–41.

21. Mossel E, van Ginkel MS, Haacke EA, Arends S, Liefers SC, Delli K, et al. Histopathology, salivary flow and ultrasonography of the parotid gland: three complementary measurements in primary Sjögren's syndrome. Rheumatology (Oxford). 2022 May 30;61(6):2472–82.

22. Ihrler S, Zietz C, Sendelhofert A, Riederer A, Löhrs U. Lymphoepithelial duct lesions in Sjögren-type sialadenitis. Virchows Archiv. 1999 Apr 22;434(4):315–23.

23. van Ginkel MS, Haacke EA, Bootsma H, Arends S, van Nimwegen JF, Verstappen GM, et al. Presence of intraepithelial B-lymphocytes is associated with the formation of lymphoepithelial lesions in salivary glands of primary Sjögren's syndrome patients. Clin Exp Rheumatol. 2019;37 Suppl 118(3):42–8.

24. Verstappen GM, Pringle S, Bootsma H, Kroese FGM. Epithelial-immune cell interplay in primary Sjögren syndrome salivary gland pathogenesis. Nat Rev Rheumatol. 2021 Jun;17(6):333–48.

25. Pringle S, Verstappen GM, van Ginkel MS, Nakshbandi U, Girigoria Z, Bootsma H, et al. Lymphoepithelial lesions in the salivary glands of primary Sjögren's syndrome patients: the perfect storm? Clin Exp Rheumatol. 2022 Dec;40(12):2434–42.

26. Haacke EA, Bootsma H, Spijkervet FKL, Visser A, Vissink A, Kluin PM, et al. FcRL4(+) B-cells in salivary glands of primary Sjögren's syndrome patients. J Autoimmun. 2017 Jul;81:90–8.

27. Steinmetz TD, Verstappen GM, Suurmond J, Kroese FGM. Targeting plasma cells in systemic autoimmune rheumatic diseases – Promises and pitfalls. Immunol Lett. 2023 Aug 1;260:44–57.

28. Salomonsson S, Jonsson M V., Skarstein K, Brokstad KA, Hjelmström P, Wahren-Herlenius M, et al. Different operators and histologic techniques in the assessment of germinal center-like structures in primary Sjögren's syndrome minor salivary glands. PLoS One. 2003;48(1):e0211142.

29. Delli K, Haacke EA, Ihrler S, Van Der Vegt B, Vissink A, Bootsma H, et al. Need for consensus guidelines to standardise the assessment of germinal centres and other histopathological parameters in salivary gland tissue of patients with primary Sjögren's syndrome. Ann Rheum Dis. 2016;75(6):2016–8.

30. Fisher BA, Jonsson R, Daniels T, Bombardieri M, Brown RM, Morgan P, et al. Standardisation of labial salivary gland histopathology in clinical trials in primary Sjögren's syndrome. Ann Rheum Dis. 2017;76(7):1161–8.

31. Allen CDC, Okada T, Cyster JG. Germinal-Center Organization and Cellular Dynamics. Immunity. 2007 Aug;27(2):190–202.

32. MacLennan IC. Germinal centers. Annu Rev Immunol. 1994;12:117–39.

33. Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell. 2000 Sep;102(5):553–63.

34. Weyand CM, Goronzy JJ. Ectopic Germinal Center Formation in Rheumatoid Synovitis. Ann N Y Acad Sci. 2003 Apr 24;987(1):140–9.

35. Risselada AP, Looije MF, Kruize AA, Bijlsma JWJ, Van Roon JAG. The Role of Ectopic Germinal Centers in the Immunopathology of Primary Sjögren's Syndrome: A Systematic Review. Semin Arthritis Rheum. 2013;42(4):368–76.

36. Le Pottier L, Devauchelle V, Fautrel A, Daridon C, Saraux A, Youinou P, et al. Ectopic germinal centers are rare in Sjogren's syndrome salivary glands and do not exclude autoreactive B cells. J Immunol. 2009 Mar;182(6):3540–7.

37. Haacke EA, van der Vegt B, Vissink A, Spijkervet FKL, Bootsma H, Kroese FGM. Standardisation of the detection of germinal centres in salivary gland biopsies of patients with primary Sjögren's syndrome is needed to assess their clinical relevance. Ann Rheum Dis. 2018 Jun 22;77(6):e32.

38. Sage PT, Sharpe AH. T follicular regulatory cells in the regulation of B cell responses. Trends Immunol. 2015 Jul;36(7):410–8.

39. Fu W, Liu X, Lin X, Feng H, Sun L, Li S, et al. Deficiency in T follicular regulatory cells promotes autoimmunity. J Exp Med. 2018 Mar 5;215(3):815–25.

40. Fonseca VR, Romão VC, Agua-Doce A, Santos M, López-Presa D, Ferreira AC, et al. The Ratio of Blood T Follicular Regulatory Cells to T Follicular Helper Cells Marks Ectopic Lymphoid Structure Formation While Activated Follicular Helper T Cells Indicate Disease Activity in Primary Sjögren's Syndrome. Arthritis Rheumatol. 2018 May;70(5):774–84.

41. Giannouli S, Voulgarelis M. Predicting progression to lymphoma in Sjögren's syndrome patients. Expert Rev Clin Immunol. 2014 Apr 1;10(4):501–12.

42. Haacke EA, van der Vegt B, Vissink A, Spijkervet FKL, Bootsma H, Kroese FGM. Germinal Centers in Diagnostic Biopsies of Patients With Primary Sjögren's Syndrome Are Not a Risk Factor for Non-Hodgkin's Lymphoma but a Reflection of High Disease Activity: Comment on the Article by Sène et al. Arthritis & Rheumatology. 2019 Jan 24;71(1):170–1.

43. Theander E, Vasaitis L, Baecklund E, Nordmark G, Warfvinge G, Liedholm R, et al. Lymphoid organisation in labial salivary gland biopsies is a possible predictor for the development of malignant lymphoma in primary Sjögren's syndrome. Ann Rheum Dis. 2011 Aug;70(8):1363–8.

44. Bombardieri M, Barone F, Humby F, Kelly S, McGurk M, Morgan P, et al. Activation-induced cytidine deaminase expression in follicular dendritic cell networks and interfollicular large B cells supports functionality of ectopic lymphoid neogenesis in autoimmune sialoadenitis and MALT lymphoma in Sjögren's syndrome. J Immunol. 2007 Oct;179(7):4929–38.

45. Brandtzaeg P. Mucosal Immunity: Induction, Dissemination, and Effector Functions. Scand J Immunol. 2009 Dec 11;70(6):505–15.

46. de Wilde PC, Vooys GP, Baak JP, van Houwelingen JC, Slootweg PJ, Hené RJ, et al. Quantitative immunohistologic and histomorphometric diagnostic criteria for Sjögren's syndrome. Pathol Res Pract. 1989 Nov;185(5):778–80.

47. Szyszko EA, Brokstad KA, Oijordsbakken G, Jonsson M V, Jonsson R, Skarstein K. Salivary glands of primary Sjögren's syndrome patients express factors vital for plasma cell survival. Arthritis Res Ther. 2011 Jan 7;13(1):R2.

48. Pijpe J, Vissink A, Kallenberg CGM, Spijkervet FKL. Parotid gland biopsy compared with labial biopsy in the diagnosis of Sjogren's syndrome: reply. Rheumatology. 2006 Jul 28;46(2):371–371.

49. Spijkervet FKL, Haacke E, Kroese FGM, Bootsma H, Vissink A. Parotid Gland Biopsy, the Alternative Way to Diagnose Sjögren Syndrome. Rheumatic Disease Clinics of North America. 2016;42(3):485–99.

50. Delli K, Dagal EF, Bootsma H, Vissink A, Spijkervet FKL. Patient-reported change of sensibility and pain after parotid and labial gland biopsy applied for primary Sjögren's syndrome diagnostics: one-year follow-up study. Clin Exp Rheumatol. 2018;36 Suppl 112(3):173–6.

51. Verstappen GM, Gao L, Pringle S, Haacke EA, van der Vegt B, Liefers SC, et al. The Transcriptome of Paired Major and Minor Salivary Gland Tissue in Patients With Primary Sjögren's Syndrome. Front Immunol. 2021 Jul 6;12.

52. Visser A, Verstappen GM, van der Vegt B, Vissink A, Bende RJ, Bootsma H, et al. Repertoire Analysis of B-Cells Located in Striated Ducts of Salivary Glands of Patients With Sjögren's Syndrome. Front Immunol. 2020;11:1486.

53. Guellec D, Cornec D, Jousse-Joulin S, Marhadour T, Marcorelles P, Pers JO, et al. Diagnostic value of labial minor salivary gland biopsy for Sjögren's syndrome: a systematic review. Autoimmun Rev. 2013 Jan;12(3):416–20.

54. Leroy JP, Pennec YL, Letoux G, Youinou P. Lymphocytic infiltration of salivary ducts: a histopathologic lesion specific for primary Sjögren's syndrome? Arthritis Rheum. 1992 Apr;35(4):481–2.

55. Vitali C, Moutsopoulos HM, Bombardieri S. The European Community Study Group on diagnostic criteria for Sjögren's syndrome. Sensitivity and specificity of tests for ocular and oral involvement in Sjögren's syndrome. Ann Rheum Dis. 1994 Oct;53(10):637–47.

56. Vitali C, Tavoni A, Simi U, Marchetti G, Vigorito P, d'Ascanio A, et al. Parotid sialography and minor salivary gland biopsy in the diagnosis of Sjögren's syndrome. A comparative study of 84 patients. J Rheumatol. 1988 Feb;15(2):262–7.

57. Mossel E, Delli K, van Nimwegen JF, Stel AJ, Kroese FGM, Spijkervet FKL, et al. Ultrasonography of major salivary glands compared with parotid and labial gland biopsy and classification criteria in patients with clinically suspected primary Sjögren's syndrome. Ann Rheum Dis. 2017 Nov;76(11):1883–9.

58. van Nimwegen JF, Mossel E, Delli K, van Ginkel MS, Stel AJ, Kroese FGM, et al. Incorporation of Salivary Gland Ultrasonography Into the American College of Rheumatology/European League Against Rheumatism Criteria for Primary Sjögren's Syndrome. Arthritis Care Res (Hoboken). 2020 Apr;72(4):583–90.

59. Geng Y, Li B, Deng X, Ji L, Zhang X, Zhang Z. Salivary gland ultrasound integrated with 2016 ACR/EULAR classification criteria improves the diagnosis of primary Sjögren's syndrome. Clin Exp Rheumatol. 2020;38(2):322–8.

60. Giovannini I, Lorenzon M, Manfrè V, Zandonella Callegher S, Pegolo E, Zuiani C, et al. Safety, patient acceptance and diagnostic accuracy of ultrasound core needle biopsy of parotid or submandibular glands in primary Sjögren's syndrome with suspected salivary gland lymphoma. RMD Open. 2022 Feb;8(1).

61. Deroo L, Genbrugge E, Dochy F, Creytens D, Achten H, De Boeck K, et al. Ultrasound-guided core needle biopsy and incisional biopsy of the parotid gland are comparable in diagnosis of primary Sjögren's syndrome. Rheumatology. 2023 Aug 1;62(8):2765–72.

62. Guerrero MR, Herranz LS, Morales CM, Ubiaga CI, Bufort MG, Golmar AC, et al. AB0518 New 2016 ACR/EULAR classification criteria for sjÖgren syndrome: usefulness and applicability in clinical practice. Ann Rheum Dis. 2017 Jun;76:1232.

63. Shiboski CH, Shiboski SC, Seror R, Criswell LA, Labetoulle M, Lietman TM, et al. 2016 American College of Rheumatology/European League Against Rheumatism Classification Criteria for Primary Sjögren's Syndrome: A Consensus and Data-Driven Methodology Involving Three International Patient Cohorts. Arthritis & Rheumatology. 2017 Jan 26;69(1):35–45.

64. Basiaga ML, Stern SM, Mehta JJ, Edens C, Randell RL, Pomorska A, et al. Childhood Sjögren syndrome: features of an international cohort and application of the 2016 ACR/EULAR classification criteria. Rheumatology (Oxford). 2021 Jul 1;60(7):3144–55.

65. Yokogawa N, Lieberman SM, Sherry DD, Vivino FB. Features of childhood Sjögren's syndrome in comparison to adult Sjögren's syndrome: considerations in establishing child-specific diagnostic criteria. Clin Exp Rheumatol. 2016;34(2):343–51.

66. Legger GE, Erdtsieck MB, de Wolff L, Stel AJ, Los LI, Verstappen GM, et al. Differences in presentation between paediatric- and adult-onset primary Sjögren's syndrome patients. Clin Exp Rheumatol. 2021;39 Suppl 133(6):85–92.

67. Yokogawa N, Lieberman SM, Alawi F, Bout-Tabaku S, Guttenberg M, Sherry DD, et al. Comparison of labial minor salivary gland biopsies from childhood Sjögren syndrome and age-matched controls. J Rheumatol. 2014 Jun;41(6):1178–82.

68. Stiller M, Golder W, Döring E, Biedermann T. Primary and secondary Sjögren's syndrome in children--a comparative study. Clin Oral Investig. 2000 Sep;4(3):176–82.

69. Saad Magalhães C, de Souza Medeiros PB, Oliveira-Sato J, Custódio-Domingues MA. Clinical presentation and salivary gland histopathology of paediatric primary Sjögren's syndrome. Clin Exp Rheumatol. 2011;29(3):589–93.

70. McGuirt WF, Whang C, Moreland W. The role of parotid biopsy in the diagnosis of pediatric Sjögren syndrome. Arch Otolaryngol Head Neck Surg. 2002 Nov;128(11):1279–81.

71. Westhovens R. Abatacept: the first-in-class costimulation blocker for the treatment of rheumatoid arthritis. Fut Rheumatol. 2006 Feb;1(1):15–22.

72. Meiners PM, Vissink A, Kroese FGM, Spijkervet FKL, Smitt-Kamminga NS, Abdulahad WH, et al. Abatacept treatment reduces disease activity in early primary Sjögren's syndrome (open-label proof of concept ASAP study). Ann Rheum Dis. 2014 Jul;73(7):1393–6.

73. van Nimwegen JF, Mossel E, van Zuiden GS, Wijnsma RF, Delli K, Stel AJ, et al. Abatacept treatment for patients with early active primary Sjögren's syndrome: a single-centre, randomised, double-blind, placebo-controlled, phase 3 trial (ASAP-III study). Lancet Rheumatol. 2020 Mar;2(3):e153–63.

74. Tsuboi H, Matsumoto I, Hagiwara S, Hirota T, Takahashi H, Ebe H, et al. Effectiveness of abatacept for patients with Sjögren's syndrome associated with rheumatoid arthritis. An open label, multicenter, one-year, prospective study: ROSE (Rheumatoid Arthritis with Orencia Trial toward Sjögren's syndrome Endocrinopathy) trial. Mod Rheumatol. 2016 Nov;26(6):891–9.

75. Haacke EA, van der Vegt B, Meiners PM, Vissink A, Spijkervet FKL, Bootsma H, et al. Abatacept treatment of patients with primary Sjögren's syndrome results in a decrease of germinal centres in salivary gland tissue. Clin Exp Rheumatol. 2017;35(2):317–20.

76. Szyszko EA, Brokstad KA, Øijordsbakken G, Jonsson M V, Jonsson R, Skarstein K. Salivary glands of primary Sjögren's syndrome patients express factors vital for plasma cell survival. Arthritis Res Ther. 2011 Jan 7;13(1):R2.

77. Adler S, Körner M, Förger F, Huscher D, Caversaccio MD, Villiger PM. Evaluation of histologic, serologic, and clinical changes in response to abatacept treatment of primary Sjögren's syndrome: a pilot study. Arthritis Care Res (Hoboken). 2013 Nov;65(11):1862–8.

78. de Wolff L, van Nimwegen JF, Mossel E, van Zuiden GS, Stel AJ, Majoor KI, et al. Long-term abatacept treatment for 48 weeks in patients with primary Sjögren's syndrome: The open-label extension phase of the ASAP-III trial. Semin Arthritis Rheum. 2022 Apr;53:151955.

79. Haacke EA, Kroese FGM, Meiners PM, Van Der Vegt B, Vissink A, Spijkervet FKL, et al. 2014 ACR/ARHP Annual Meeting Abstract Supplement. Arthritis & Rheumatology. 2014 Oct 23;66(S10):S1114–5.

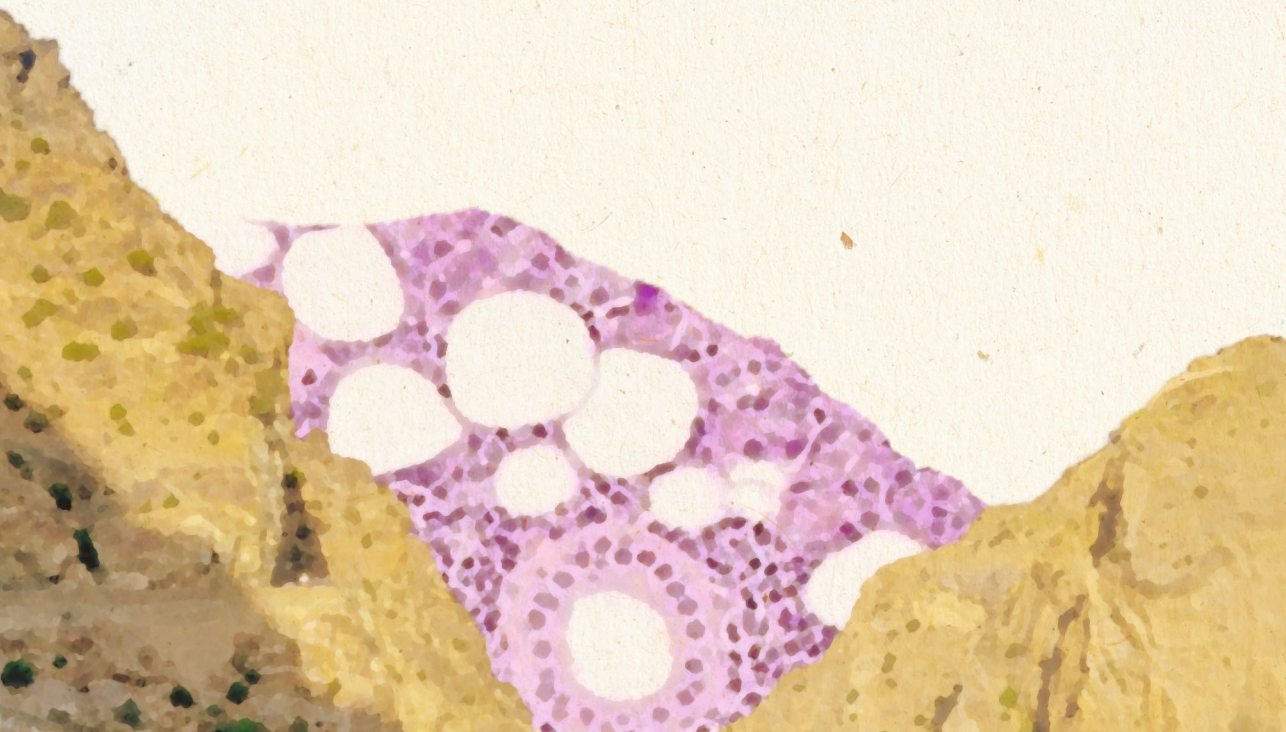
80. Arends S, de Wolff L, van Nimwegen JF, Verstappen GMPJ, Vehof J, Bombardieri M, et al. Composite of Relevant Endpoints for Sjögren's Syndrome (CRESS): development and validation of a novel outcome measure. Lancet Rheumatol. 2021 Aug;3(8):e553–62.

81. Seror R, Meiners P, Baron G, Bootsma H, Bowman SJ, Vitali C, et al. Development of the ClinESSDAI: a clinical score without biological domain. A tool for biological studies. Ann Rheum Dis. 2016 Nov;75(11):1945–50.

82. de Wolff L, Arends S, Pontarini E, Bombardieri M, Bowman SJ, Bootsma H. Development and performance of the Clinical Trials ESSDAI (ClinTrialsESSDAI), consisting of frequently active clinical domains, in two randomised controlled trials in primary Sjögren's syndrome. Clin Exp Rheumatol. 2021;39 Suppl 133(6):100–6.

83. Seror R, Baron G, Camus M, Cornec D, Perrodeau E, Bowman SJ, et al. Development and preliminary validation of the Sjögren's Tool for Assessing Response (STAR): a consensual composite score for assessing treatment effect in primary Sjögren's syndrome. Ann Rheum Dis. 2022 Jul;81(7):979–89.

84. Isenberg DA. CRESS: improving the assessment of disease activity in Sjögren's syndrome. Lancet Rheumatol. 2021 Aug 1;3(8):e534–5.



APPENDICES

SUMMARY

Sjögren's disease, often referred to as Sjögren's syndrome, is a chronic systemic auto-immune disease characterised by, a.o., inflammation and dysfunction of the salivary and lacrimal glands. As a result, typical sicca complaints such as dry mouth and dry eyes are mostly present. Patients may also experience extra glandular manifestations such as fatigue, joint pain and Raynaud's phenomenon. In addition, SjD patients have an increased risk of developing non-Hodgkin's lymphoma, mostly of the Mucosa Associated Lymphoid Tissue (MALT) type. This type of lymphoma occurs in 5-10% of SjD patients and preferentially develops in parotid glands.

Currently, for classification of disease the American College of Rheumatology – European League against Rheumatism (ACR-EULAR) classification criteria are used but for diagnosis of disease, expert opinion remains the gold standard. Within these classification criteria a salivary gland biopsy plays an important role as it is one of the two leading criteria. A positive biopsy is currently solely based on the presence of a focus score (FS) ≥1. The FS is calculated as the number of foci per 4 mm^2 glandular parenchyma. A focus is defined as ≥50 lymphocytes. However, the FS has some important limitations such as the lack of specificity and difficulty of assessing the presence of foci when other features such as acinar atrophy and fibrosis are present. Besides importance for classification and diagnosis of disease, the salivary gland biopsy can be used to assess treatment effects and patient stratification.

Worldwide the labial salivary gland biopsy is considered as the standard of care in SjD. Nevertheless, a parotid gland biopsy is a good alternative and has certain advantages such as the possibility to obtain a repeated biopsy from the same gland, comparison of histopathology with other diagnostic modalities (e.g. salivary gland ultrasound, parotid salivary flow, sialography, scintigraphy, CT, MRI and PET). Moreover, a parotid gland biopsy is more suitable for diagnosing MALT-lymphoma. Within the University Medical Centre Groningen parotid gland biopsies are routinely taken as part of the diagnostic work-up. Therefore, these biopsies are commonly available in the UMCG and used in the studies within this thesis.

The aim of this thesis was to get more insight into the role of the histopathology of salivary glands of SjD patients for diagnosis, classification and

patient stratification. Histopathological features additional to the FS were analysed in several patient cohorts and effects of immunomodulatory therapy vs placebo were assessed.

PART 1: SALIVARY GLAND HISTOPATHOLOGY IN SJÖGRENS DISEASE

Although foci are characteristic of SjD they are not the only feature. In addition to the FS we also examined the presence of (pre-)lymphoepithelial lesions (LELs), an IgA/IgG plasma cell shift, and germinal centres (GCs) in salivary gland biopsies – all representing manifestations of B-cell hyperactivity. LELs are defined as ducts in which epithelial cells proliferate, leading to hyperplasia accompanied by the presence of lymphocytes. In the case of the IgA/IgG plasma cell shift, there is a relative decrease in IgA and a relative increase in IgG plasma cells. Furthermore, the presence of GCs – the sites where memory B cells are formed – appears to be specific to SjD.

In haematoxylin and eosin (H&E) stained sections, ectopic GCs are difficult to recognise, and therefore additional immunohistochemical staining is required to correctly identify these structures. Currently, the methods used to identify these germinal centres vary considerably between studies. Bcl6 is a transcription factor that is highly expressed in GC B cells. In the study described in **chapter 2** we show that Bcl6 is an easy to use and appropriate marker for the identification of ectopic GCs within salivary glands. Moreover, we demonstrate that staining for follicular dendritic cell networks such as CD21 overestimate GC counts and thus are not suitable as a marker for ectopic GCs.

In the study described in **chapter 3,** the labial salivary gland histopathology was compared to paired parotid gland biopsies of suspected SjD patients. Histopathological key features, specific for SjD, besides a FS ≥1, namely the presence of (pre)LELs, IgA/IgG plasma cell shift and GCs were measured in both type of glandular biopsies of SjD patients. Labial salivary glands biopsies contain more non-SjD related inflammation than parotid gland biopsies, in both non-SjD sicca and SjD patients. Thus, purely focusing on the FS in labial salivary gland biopsies may result in false positive biopsy results and therefore a higher risk of incorrect classification. In parotid glands more evident histopathological signs of B-cell hyperactivity were observed.

The peak incidence of SjD is around the 4th to 5th decade of life. Nevertheless, SjD may also develop in children. The clinical presentation in children is different compared to adult SjD patients. Paediatric SjD, patients present more often with symptoms of major salivary gland involvement such as recurrent parotid gland swelling, decreased stimulated whole salivary flow rate and higher ultra sound scores. In the study described in **chapter 4** we compared the parotid salivary gland histopathology of paediatric SjD patients with adult SjD patients. Here we showed that SjD specific key features found in adult salivary glands of SjD patients were also observed in salivary gland sections of paediatric patients. However, B-lymphocyte related parameter were significantly more severe in paediatric SjD patients.

An important subset of B cell helper T cells has been identified to play an important role in SjD. In the study described in **chapter 5** the prevalence of T follicular helper (Tfh) cells and their regulatory counterparts, T follicular regulatory (Tfr) cells, was assessed in SjD patients and compared to non-SjD sicca patients. Compared to non-SjD sicca patients, both subsets were increased in SjD patients. However, Tfr cells were even more increased resulting in significantly higher Tfr/Tfh ratios in SjD patients. As both subsets correlated with serum IgG plasma cell and CXCL13 levels and systemic disease activity, frequencies can be useful as a biomarker for systemic disease activity.

PART 2: DIAGNOSIS, CLASSIFICATION AND TREATMENT

The next three chapters focused on the added value of additional histopathological key features besides a FS ≥1, namely the presence of (pre)LELs, IgA/IgG plasma cell shift and GCs, for the performance of salivary gland biopsies. In **chapter 6** and **7** we showed that the addition of three histopathological features that are characteristic for SjD (presence of: (pre)LELs, plasma cell shift, GCs) in the histopathological SjD work-up increases the diagnostic accuracy of both the labial and parotid salivary gland biopsy in SjD. For labial salivary gland biopsies the increased diagnostic accuracy was due to an increase in specificity while for parotid gland biopsies it was due to an increase in sensitivity. In **chapter 8,** we observed that the presence of histopathological key features was generally associated with each other. In addition, we observed that in patients with serological abnormalities (presence of anti-SSA, anti-SSB, RF, elevated IgG plasma cells) more histopathological key features were present

compared to those without serological involvement. Moreover, the presence of multiple histopathological key features was a better predictor of expert diagnosis of SjD, compared to FS ≥1 alone. Therefore, we recommend to look beyond the FS and also analyse the three other histopathological key features in the routine work-up of salivary gland biopsies of SjD patients.

Salivary gland biopsies can also be used to assess and unravel pathogenetic mechanisms involved in SjD and to aid patient stratification. Abatacept did not result in a significant difference in ESSDAI score compared with placebo treatment after 24 weeks, however post-hoc assessment of trial data using the CRESS resulted in higher response rates in abatacept treated patients. In the study described in **chapter 9** we analysed histopathological parameters before and after 24 weeks of abatacept treatment. Except for some differences in the number of IgA plasma cells, no significant differences were observed compared to the placebo group. In addition, we observed that there were no significant histopathological differences between abatacept responders and non-responders on baseline. Thus, salivary gland biopsy could not predict treatment effectiveness.

To conclude, salivary gland biopsies play an important role in the diagnosis and classification of SjD as well as in understanding treatment response and assist in stratification of patients. Standardised methods of analysis are essential for improving our understanding of the pathophysiology and for enhancing the reliability of outcomes in (pre)clinical studies.

SAMENVATTING

De ziekte van Sjögren (ZvSj), tot voor kort vaak aangeduid als het syndroom van Sjögren, is een systemische auto-immuunziekte die o.a. wordt gekenmerkt door ontsteking en disfunctie van de speeksel- en traanklieren. Als gevolg van de ontsteking en disfunctie zijn typische sicca-klachten zoals droge mond en droge ogen aanwezig. Daarnaast kan een veelheid aan extra-glandulaire symptomen optreden zoals vermoeidheid, gewrichtspijn en het fenomeen van Raynaud. Patiënten met de ZvSj hebben tevens een verhoogd risico op het ontwikkelen van een non-Hodgkin lymfoom, voornamelijk van het Mucosa Associated Lymphoid Tissue (MALT)-type. Dit type lymfoom komt voor bij 5-10% van de patiënten met de ZvSj gedurende het verloop van hun ziekte en ontwikkelt zich bij voorkeur in de parotisspeekselklier (oorspeekselklier).

Voor de classificatie van de ziekte worden momenteel de American College of Rheumatology - European League Against Rheumatism (ACR-EULAR) criteria gebruikt, maar voor de diagnose blijft het deskundigenoordeel de gouden standaard. Het speekselklierbiopt (van de speekselkliertjes uit de lip of parotisspeekselklier) speelt een cruciale rol binnen deze classificatiecriteria, waarbij een positief biopt wordt gedefinieerd door een focus score (FS) ≥1. De FS wordt berekend door het aantal foci per 4 mm² speekselklierparenchym te berekenen. Een focus is een cluster ≥50 lymfocyten. Echter, de FS heeft beperkingen, zoals gebrek aan specificiteit en uitdagingen bij de beoordeling van foci in aanwezigheid van acinaire atrofie en fibrose. Naast de classificatie en diagnose kan het speekselklierbiopt ook worden gebruikt om het behandeleffect te beoordelen en ter ondersteuning van het stratificeren van patiënten.

Het biopteren van de speekselkliertjes uit de lip wordt wereldwijd beschouwd als onderdeel van de diagnostische work-up van de ZvSj. Het biopt van de parotisspeekselklier is een goed alternatief gebleken en biedt bepaalde voordelen boven het biopt van de lipspeekselkliertjes. Een belangrijk voordeel is de mogelijkheid om herhaaldelijk van dezelfde klier te biopteren, evenals het vergelijken van de histopathologie met resultaten van andere diagnostische methoden (bijv. zoals echografie, het verzamelen van parotisspeeksel, sialografie, scintigrafie, CT, MRI en PET). Ook is het parotisbiopt geschikter voor het diagnosticeren van een MALT-lymfoom, vooral als er sprake is van een zwelling van de parotiden. Binnen het Universitair Medisch Centrum

Groningen worden bij patiënten, verdacht voor de ZvSj, daarom routinematig parotisbiopten afgenomen als onderdeel van het diagnostisch traject. In diverse studies van dit proefschrift worden deze speekselklierbiopten gebruikt en geanalyseerd.

Het doel van het in dit proefschrift beschreven promotieonderzoek was om meer inzicht te krijgen in de histopathologie van de speekselklieren van patiënten met de ZvSj om de rol hiervan bij de diagnose, classificatie en stratificatie van patiënten te evalueren. Histopathologische kenmerken naast de FS werden geanalyseerd in verschillende patiënt cohorten. Tevens werden de effecten van immunomodulerende therapie versus placebo beoordeeld.

DEEL 1: HISTOPATHOLOGIE VAN SPEEKSELKLIEREN BIJ DE ZIEKTE VAN SJÖGREN

Hoewel foci kenmerkend zijn voor de ZvSj, is dat niet het enige kenmerk. Naast de FS hebben wij ook de aanwezigheid van (pre-)lymphoepitheliale laesies (LELs), van IgA/IgG plasma cel shift en van kiemcentra – allen een uiting van B-cel hyperactiviteit - onderzocht in speekselklierbiopten. LELs worden gedefinieerd als ducten waarvan de epitheelcellen delen, wat leidt tot hyperplasie met daarbij ook de aanwezigheid van lymfocyten. Bij de IgA/IgG plasma cell shift is er sprake van een relatieve afname van IgA en relatieve toename van IgG plasma cellen. Daarnaast blijkt is de aanwezigheid van kiemcentra, de plek waar geheugen B-cellen worden aangemaakt, specifiek voor de ZvSj.

In hematoxyline-eosine gekleurde coupes zijn ectopische kiemcentra moeilijk te herkennen en daarom zijn aanvullende immunohistochemische kleuringen nodig om deze structuren correct te identificeren. Momenteel verschillen de methoden voor identificatie van deze kiemcentra aanzienlijk tussen studies. Bcl6 is een transcriptiefactor die sterk tot expressie komt in kiemcentrum B-cellen. In de studie beschreven in **hoofdstuk 2** tonen we aan dat Bcl6 een eenvoudig te gebruiken en geschikte marker is voor de identificatie van ectopische kiemcentra in speekselklieren. Bovendien laten we zien dat het gebruik van kleuringen voor folliculair dendritische cel-netwerken, zoals CD21, het aantal biopten met kiemcentra overschat en daarom niet geschikt zijn als marker voor kiemcentra.

In de studie beschreven in **hoofdstuk 3** is de histopathologie van lipspeekselklierbiopten vergeleken met gepaarde parotisspeekselklierbiopten

van patiënten die verdacht zijn voor het lijden aan de ZvSj. Belangrijke en Sjögren-specifieke kenmerken zoals FS≥1, (pre-)LELs, IgA/IgG plasmacelshift en kiemcentra werden in zowel lip- als parotisspeekselklieren gevonden. Biopten van de speekselkliertjes uit de lip vertoonden meer niet-auto-immuun gerelateerde ontsteking dan parotisbiopten, zowel bij niet-ZvSj patienten met sicca klachten als ZvSj patiënten. Hierdoor kan het gebruik van alleen de FS leiden tot fout-positieve bioptresultaten en onjuiste classificatie van de ZvSj. In parotisbiopten werden meer uitgesproken histopathologische tekenen van B-cel hyperactiviteit waargenomen.

De piekincidentie van de ZvSj ligt rond het 4e tot 5e decennium van het leven. De ZvSj kan echter ook voorkomen bij kinderen. De klinische presentatie bij kinderen is wel anders dan bij volwassen Sjögren patiënten. Bij kinder-Sjögren patiënten komen vaker symptomen van betrokkenheid van grote speekselklieren voor, zoals terugkerende zwelling van de parotisspeekselklier, verminderde gestimuleerde totale speekselproductie en hogere echografie scores. In de studie beschreven in **hoofdstuk 4** werd de histopathologie van de parotisspeekselklier tussen volwassen- en kinder-Sjögren patiënten vergeleken. Uit deze studie kwam naar voren dat de typerende histopathologische kenmerken in speekselklierbiopten van zowel volwassen- als kinder-Sjögren patiënten voorkomen. De B-cel gerelateerde kenmerken waren wel significant meer uitgesproken in biopten van het kindercohort.

Een subset van B-cel helper T-cellen speelt een belangrijke rol in de pathofysiologie van de ZvSj. In de studie beschreven in **hoofdstuk 5** werd de prevalentie van T folliculaire helper (Tfh) cellen en hun regulerende tegenhangers, T folliculaire regulerende (Tfr) cellen, beoordeeld bij patiënten met de ZvSj en vergeleken met niet-Sjögren sicca patiënten. In vergelijking met niet-Sjögren sicca patiënten waren beide subsets verhoogd bij Sjögren patiënten. Echter, Tfr-cellen waren in verhouding meer verhoogd, wat resulteerde in een significant hogere Tfr/Tfh-ratio bij Sjögren patiënten. Aangezien beide subsets correleerden met serum IgG plasmacellen en CXCL13-niveaus en systemische ziekteactiviteit, kunnen absolute waarden nuttig zijn als een biomarker voor systemische ziekteactiviteit.

DEEL 2: DIAGNOSE, CLASSIFICATIE EN BEHANDELING

De volgende drie hoofdstukken richtten zich op de toegevoegde diagnostische waarde van aanvullende histopathologische kenmerken naast een FS≥1, namelijk de aanwezigheid van (pre-)LELs, IgA/IgG plasmacelshift en kiemcentra bij het analyseren van speekselklierbiopten. In **hoofdstuk 6** en **7** worden studies beschreven waarin we aantonen dat de toevoeging van drie histopathologische kenmerken die typerend zijn voor de ZvSj (aanwezigheid van: (pre-)LELs, IgA/IgG plasmacelshift, kiemcentra) aan de histopathologische evaluatie, de diagnostische nauwkeurigheid van zowel de lip- als parotisspeekselklierbiopt bij de ZvSj verhoogt. Voor de biopten van de lipspeekselkliertjes werd de toegenomen diagnostische nauwkeurigheid veroorzaakt door een toename in specificiteit, terwijl voor parotisspeekselklierbiopten dit werd veroorzaakt door aan een toename in sensitiviteit. In de studie beschreven in **hoofdstuk 8** observeerden we dat de aanwezigheid van histopathologische kenmerken vaak geassocieerd is met elkaar. Bovendien zagen we dat bij patiënten met serologische afwijkingen (aanwezigheid van anti-SSA, anti-SSB, RF, verhoogde IgG-plasmacellen) meer histopathologische kenmerken aanwezig zijn dan bij patiënten zonder serologische betrokkenheid. De aanwezigheid van meerdere histopathologische kenmerken bleek een betere voorspeller van een expert-diagnose van SjD dan een FS ≥1 alleen. Daarom raden wij aan om verder te kijken dan de FS bij de diagnostiek van de ZvSj. Het is beter ook de drie andere histopathologische kenmerken te analyseren in de routinematige evaluatie van speekselklierbiopten in patiënten die verdacht zijn te lijden aan de ZvSj.

Speekselklierbiopten kunnen ook worden gebruikt om de effecten van immunomodulerende therapieën te beoordelen en te begrijpen, en voor het stratificeren van patiënten met de ZvSj. In de studie beschreven in **hoofdstuk 9** zijn histopathologische parameters geanalyseerd vóór en na 24 weken een behandeling met abatacept. Afgezien van enkele verschillen in het aantal IgA-plasmacellen, werden geen significante verschillen waargenomen in vergelijking met de placebogroep. Bovendien hebben we vastgesteld dat er geen significante histopathologische verschillen voorafgaand aan de behandeling waren tussen abatacept-responders en niet-responders. Derhalve werd geconcludeerd dat het speekselklierbiopt de effectiviteit van de behandeling niet kon voorspellen.

Samenvattend, het speekselklierbiopt speelt een belangrijke rol bij de diagnose en classificatie van de ZvSj, evenals bij het begrijpen van de behandeleffecten en de stratificatie van patiënten. Gestandaardiseerde analysemethoden zijn essentieel voor het verbeteren van ons begrip van de pathofysiologie en voor een grotere betrouwbaarheid van de uitkomsten (pre)klinische studies.

DANKWOORD

Achievement is never the work of one, but the result of many.

De afgelopen jaren heb ik met veel toewijding en plezier aan dit proefschrift gewerkt. Dit werk is echter niet alleen het resultaat van mijn eigen inspanningen, maar ook van de waardevolle begeleiding, inspirerende samenwerking en onmisbare steun van de mensen om mij heen. Zonder hun bijdrage, in welke vorm dan ook, was dit proefschrift niet tot stand gekomen. Graag wil ik de volgende personen bedanken.

Allereerst, mijn bijzondere dank gaat uit naar **alle patiënten** die hebben deelgenomen aan de onderzoeken beschreven in dit proefschrift.

Prof. Dr. Kroese, eerste promotor, beste Frans, hartelijk dank voor jouw onmisbare begeleiding gedurende het hele promotietraject. Vanaf het allereerste moment was je enthousiast over het idee om, naast Marthe, nog een tweede onderzoeker te hebben die zich zou richten op de histopathologie van speekselklierbiopten bij Sjögren-patiënten. Er lagen immers nog zoveel vragen open en projecten te wachten om opgepakt te worden. De identificatie van kiemcentra was het startpunt van dit proefschrift—een onderwerp dat jou na aan het hart ligt, niet in de laatste plaats omdat je zelf op dit thema bent gepromoveerd. Vanaf dat eerste project en gedurende alle studies die volgden, heb jij een grote en waardevolle bijdrage geleverd. Jouw passie voor onderzoek en immunologie is inspirerend te noemen. Je weet abstracte materie met energie en helderheid over te brengen en anderen te enthousiasmeren. Je leerde mij hoe belangrijk het is om correct en gestructureerd wetenschappelijk te schrijven. Wat ik daarnaast enorm heb gewaardeerd, is jouw toegankelijkheid. Je deur stond—letterlijk en figuurlijk—altijd open. Dank voor je begeleiding, je betrokkenheid en de fijne samenwerking.

Prof. Dr. Vissink, tweede promotor, beste Arjan, mijn onderzoeksavontuur begon met een e-mail aan jou, waarin ik mijn interesse voor de MKA-chirurgie en onderzoek kenbaar maakte. Vanaf het eerste moment toonde je je open, enthousiast en bereid om mee te denken over de mogelijkheden. Niet

lang daarna introduceerde je me bij de Sjögren-groep, waar ik de afgelopen jaren met veel plezier onderzoek heb mogen doen. Dankzij jouw introductie en vertrouwen kreeg ik de kans om me verder te ontwikkelen, iets waar ik je enorm dankbaar voor ben. Jouw scherpe blik en ongekende precisie heb ik ontzettend op prijs gesteld. Geen spelfout, komma of punt ontging je, en je wist altijd in no-time waardevolle feedback te geven op artikelen, abstracts en aanvragen. Wat ik misschien nog wel het meest heb gewaardeerd, is jouw vertrouwen in mijn kunnen en ambities. Dank je wel voor je begeleiding, je kritische blik en je steun!

Dr. van der Vegt, co-promotor, beste Bert, ik had mij geen betere co-promotor kunnen wensen. Bedankt voor alles dat jij mij hebt geleerd over de histopathologie. Ondanks je volle agenda wist je altijd tijd te maken wanneer er weer coupes beoordeeld moesten worden voor de verschillende studies. En vaak was er dan ook nog ruimte om even bij te praten, iets wat ik ontzettend heb gewaardeerd. Het plezier dat jij haalt uit het begeleiden van (promotie)studenten was voor mij duidelijk voelbaar. Je betrokkenheid, toegankelijkheid en histopathologische input waren onmisbaar voor mijn proefschrift. Mede dankzij jouw kritische oog zijn alle artikelen binnen dit proefschrift naar een hoger niveau getild. Hartelijk dank voor je begeleiding en de fijne werksfeer.

I would like to thank the members of the reading committee: **Prof. Dr. I.M. Bajema, Prof. Dr. F.J. Bikker** and **Prof. Dr. B. Fisher**. Thank you for reading and reviewing this PhD thesis.

Prof. Dr. Bootsma, beste Hendrika, in mijn vierde jaar van de Geneeskunde opleiding kreeg ik de gelegenheid om een praatje te houden op de EULAR - tevens het allereerste congres wat ik bijwoonde. Ik herinner mij nog goed hoe jij mij op sleeptouw nam en introduceerde aan de (internationale) wetenschappelijke wereld. Dat gaf mij niet alleen vertrouwen, maar ook inspiratie om verder te groeien. Wat volgde waren meerdere leermomenten, die ik stuk voor stuk mee zal dragen in mijn verdere loopbaan. Hartelijk dank voor alle wijsheden die je mij hebt meegegeven tijdens mijn tijd op de afdeling.

Prof. Dr. Spijkervet, beste Fred, hartelijk dank voor het afnemen van de vele biopten die de basis waren voor dit proefschrift. Daarnaast dank voor je vertrouwen in mijn kunnen waardoor ik de opleiding tot MKA chirurg kan volgen in het UMCG.

Next, I would like to thank my collaeges from the departments of Rheumatology and Clinical Immunology, Oral and Maxillofacial Surgery and Pathology, especially the Sjögren and SLE group: **Sarah, Konstantina, Annie, Janneke, Tobit, Abel, Hannie, Berber, Juan, Lu** and **Svenja**. A special thanks to a few individuals that played an important role in this journey.

Dear fellow-PhD'ers **Marthe, Liseth, Ting, Noa, Rick, & Mark**, even though I wasn't always full-time present at the office due to my Dentistry studies, thank you for all the moments we shared at the office. Whether it was sharing laughs, venting about the frustrating moments of our PhD journey, or just enjoying the little breaks, your company made it all so much better. From our coffee moments (featuring Ting's not-so-secret stash of chocolate) to our questionable dart-playing skills at the office, every moment was memorable. And of course, I'll always cherish our trips to Rome, Philadelphia, and Copenhagen!

Beste **Silvia**, hartelijk dank voor je hulp bij de verschillende onderzoeken beschreven in dit proefschrift. Dank voor alle uren die je hebt gestoken in het verzamelen, op orde brengen en analyseren van biopten.

Gwenny en Sarah, dank voor de gezelligheid op de afdeling de afgelopen jaren. Ik heb veel van jullie mogen leren over onderzoek doen. Mede dankzij jouw geweldige begeleiding en input, Gwenny, staat hoofdstuk 8 erin!

Beste **Suzanne**, dank dat je altijd even mee wilde kijken naar de statistische analyses die zijn uitgevoerd. Jouw uitleg tijdens diverse teams meetings en presentaties over statistiek hebben mij veel geleerd. Het was ontzettend fijn om zo laagdrempelig vragen te kunnen stellen. Het is een kunst hoe je binnen een artikel hoofd- en bijzaak weet te onderscheiden waardoor het artikel in de juiste vorm gegoten wordt. Dank voor je input!

Beste **Erlin**, terwijl jij in de afrondende fase van jouw proefschrift zat, kwam ik net om de hoek kijken op de afdeling. Dank dat je mij hebt helpen opstarten en voor het histologisch voorwerk waarop een deel van de studies zijn gebaseerd.

Collega's van de digitale pathologie groep, **Henk**, **Jacko**, **Lorian**, **Michel**, **Mieke**, **Marian** en **Tineke**, dank voor jullie hulp bij de verschillende studies en uiteraard voor de gezelligheid tijdens de maandagochtend meetings!

Beste **Elizabeth**, dank voor de samenwerking en succes bij het afronden van jouw eigen proefschrift.

Dear **prof. dr. A. Rasmussen, prof. dr. C. Lessard** and **prof. dr. D. Farris**, dear Astrid, Chris and Darris, thank you for your collaboration and contributions to chapter 8.

Next, I would also like to express my heartfelt gratitude to all my family and friends, here and abroad. While they may not have directly influenced my thesis, their support, genuine interest, and, perhaps most importantly, the highly welcome distractions they provided were invaluable during this journey.

Lieve **Kelly**, dank je voor je onvoorwaardelijke vriendschap. Ondanks dat we elkaar niet zo vaak zien als ik zou willen, verandert dat niets aan onze band. Al sinds ons 12e levensjaar heb ik het voorrecht om jouw vriendin te zijn, en daar ben ik nog steeds ontzettend dankbaar voor.

Lieve **Friederike, Alex** en **Faisal**, dank voor jullie warme vriendschap in de afgelopen jaren. Ik ben dankbaar dat ik zulke fijne vrienden heb mogen maken tijdens de Geneeskunde opleiding. Jullie vriendschap is mij dierbaar!

Lieve **mede VBT'ers**, **Date, Yang, Sanne, Niels, Melany** en **Bjorn**, dank jullie wel voor de gezellige tijd tijdens de opleiding Tandheelkunde. De vele uren op skills, in de CMB en de collegebanken werden een stuk gezelliger door hier samen over te lachen en te klagen. Ik kijk dan ook met ontzettend veel plezier terug op deze tijd. Date, Yang, Sanne en Niels ik kan niet wachten om

ook jullie promoties bij te wonen en samen de opleiding tot MKA chirurg te volgen. Ook dank aan **alle (pre-)AIOS'en van de MKA chirurgie**, die ons met bemoedigende woorden hebben toegesproken, vooral op momenten dat we het gevoel hadden tekort te schieten in onze onderzoeksbezigheden tijdens de Tandheelkunde-opleiding.

Lieve **Saïda** en **Marjan**, dank jullie wel voor jullie oprechte betrokkenheid bij alles wat ik doe, en voor de fijne, gezellige afleiding precies op het juiste moment. Gizal en ik prijzen ons gelukkig met jullie — als familie én als echte vriendinnen.

Lieve **schoonouders**, *kakajaan en khalajaan*, lieve **Omar,** dank jullie wel voor het warme welkom in jullie gezin. Dank voor de oprechte interesse die jullie tonen in mijn studies en werk en de liefde en steun die ik van jullie krijg. Ik hoop nog veel mooie momenten samen te beleven en herinneringen te maken die een leven lang mee zullen gaan.

Lieve **Javad**, *bradarem*, ik ben Gizal ontzettend dankbaar voor het feit dat jij in ons leven bent gekomen en daarmee onze familie groter is geworden. Jouw oprechtheid, doorzettingsvermogen en nuchterheid zijn kwaliteiten waar een voorbeeld aan te nemen is. Om maar niet te spreken over je toewijding en passie voor het medische vak. Ik heb de kunst vorig jaar bij jou, tijdens jouw eigen promotie, af kunnen kijken. Veel succes met je verdere opleiding, ik twijfel er geen moment aan dat je een fantastische kinderarts zult worden.

Lieve **Gizal**, mijn grote zus, vriendin, steun en toeverlaat. Ik geloof dat ik nog 100 andere titels voor je kan bedenken, want zoveel verschillende rollen speel jij in mijn leven. Het was voor mij vanzelfsprekend dat jij mijn paranimf moest zijn. Van jongs af aan nam je mij bij de hand om mij alles te leren wat jij al wist. Iets wat tot op heden niet anders is. Nog steeds kom ik met ieder dilemma waar ik zelf niet uit kom naar jou. Jouw luisterend oor en kijk op het leven zorgen er vrijwel altijd voor dat ik weer anders kijk naar situaties die zich voordoen. Ik heb bewondering voor hoe jij de verschillende ballen in het leven hoog-houdt. Zo ben je een fantastische (schoon)dochter, zus, echtgenote en sinds de komst van **Abbas**, mijn aller liefste neefje, ook een geweldige moeder. Dit

alles combineer je met jouw eigen promotie onderzoek en baan als AIOS bij de Longgeneeskunde. Ik kan mij geen betere zus en beter voorbeeld wensen in het leven. *Tashakor*.

Abbas, mijn lieve neefje, al kan je nog niet lezen, ik hoop dat je later dit boekje erbij pakt en terugleest hoeveel er van jou gehouden wordt. Voor mij ben jij nu al de liefste, meest eigenwijze en slimste driejarige die er bestaat. Ik ben je dankbaar voor alle vreugde en liefde die jij onze familie schenkt. Ik prijs mij de gelukkigste tante.

Lieve **Sohrab**, vanaf het moment dat jij in mijn leven bent gekomen, heb je altijd naast mij gestaan, zo ook tijdens mijn verdediging. Wanneer ik 's avonds of in het weekend door moest werken aan mijn onderzoek of aan Tandheelkunde, was jij daar: om me moed in te spreken, om me gezelschap te houden, om het net iets lichter te maken. Als het even niet liep zoals ik voor ogen had, was jouw luisterend oor daar. Ik bewonder hoe je iedere mijlpaal – hoe klein ook – met me weet te vieren, alsof het de grootste overwinning is. Dank je wel, mijn lief, voor je liefde, je vriendschap en je vertrouwen. Ik ben dankbaar voor wie jij bent, en voor alles wat je voor mij doet – elke dag weer. Ik kijk uit naar onze toekomst samen. *Dostet darom*.

Lieve **mama en papa,** *mamojaan wa agha saib*, woorden schieten te kort om mijn dankbaarheid aan jullie te uiten maar ik ga toch een poging wagen. Al van jongs af aan hebben jullie mij en Gizal geleerd wat het belang van onderwijs is. Niet alleen door dit belang uit te spreken maar ook door daad bij woord te voegen. Zo grepen jullie iedere kans die er was om een stabiele toekomst te creëren. Het gedwongen achterlaten van jullie eigen land, familie en positie om mij en Gizal veiligheid en een stabiele toekomst te bieden is een moeilijke keuze geweest. De leeftijd, toentertijd gebrekkige taal, en culturele verschillen weerhielden jullie er niet van om opnieuw de studiebanken in te gaan in Nederland. Jullie zijn een onuitputtelijke inspiratiebron, zonder wie ik niet heb kunnen doen wat ik doe. De mate van betrokkenheid bij onze studies is uitzonderlijk te noemen. Zo was jij, *Agha Saib*, altijd op de hoogte van ieder tentamen, iedere deadline of presentatie in mijn agenda en heb je nooit een moment gemist om mij succes te wensen. *Mamojaan*, jij leerde mij alle mo-

menten in het leven te vieren en als iets niet meezat dit te relativeren, weer op staan en door te gaan. Je bent de sterkste vrouw die ik ooit heb gezien of waar ik van heb gehoord. Ik ben dankbaar dat jullie mij hebben geleerd om kansen te creëren en te grijpen wanneer die zich voordoen. Dankbaar voor alle wijsheden en inzichten die ik van jullie krijg. Dankbaar dat jullie ons inspireren om het beste uit ons te halen en daarbij altijd je eigen persoon te blijven. Bovenal, dankbaar voor jullie onvoorwaardelijke liefde en steun. *Yak dunja sepas wa tashakor.*

ABOUT THE AUTHOR

Uzma Nakshbandi was born on the 13th of January 1995, during a time when her parents were fleeing the war in Afghanistan. Within her first year of life, their journey led them to settle in the Netherlands. After initially living in Haren, Uzma and her family later moved to Hengelo. She obtained her Gymnasium diploma at Grundel Lyceum in Hengelo and continued her academic journey at the University of Groningen by studying Pharmacy (*Propaedeutic diploma obtained*).

In 2014 she started studying Medicine at the University of Groningen. During medical school she was involved in various extracurricular activities, including research projects. She obtained her Bachelor's degree in Medicine in 2017 and completed the Junior Scientific Masterclass honours program. Subsequently she performed her master thesis at the department of Rheumatology & Clinical Immunology, University Medical Centre Groningen under the supervision of prof. dr. F.G.M. Kroese and dr. B. van der Vegt.

In 2021 she was accepted for the (D)MD-PhD program by the Gradual School of Medical Sciences (supervisors prof. dr. F.G.M. Kroese, prof. dr. A. Vissink and dr. B. van der Vegt) and obtained her Master's degree in Medicine. This was followed by being accepted for the Oral and Maxillofacial Surgery residency program. In September that year she started studying Dentistry at the University of Groningen. The (D)MD-PhD trajectory enabled her to combine her research projects with Medicine and with Dentistry. She presented her results at several (inter)national conferences (*EULAR 2018, NVR 2018, ISSS 2023, NVR 2023, NVMKA 2023, EULAR 2023, ACR 2023*) and was awarded the *''Vereniging voor medisch tandheelkundige interacties (VMTI)''*-publication award. After finishing Dentistry, she began her residency in Oral and Maxillofacial Surgery at the University Medical Centre Groningen in May this year.

LIST OF PUBLICATIONS

U. Nakshbandi, S.C. Liefers, S. Arends, F.K.L. Spijkervet, G.M.P.J. Verstappen, A. Vissink, L. de Wolff, B. van der Vegt, H. Bootsma, F.G.M. Kroese. Abatacept use for 24 weeks has a limited effect on salivary gland inflammation in sjögren's disease patients. *Clin Exp Rheumatol* 2024 Dec;42(12):2362-2368.

U. Nakshbandi, M.S. van Ginkel, G.M.P.J. Verstappen, F.K.L. Spijkervet, S. Arends, E.A. Haacke, S.C. Liefers, A. Vissink, H. Bootsma, F.G.M. Kroese, B. van der Vegt. Histopathological comparison of sjögren-related features between paired labial and parotid salivary gland biopsies of sicca patients. *Rheumatology* 2024;63(10): 2670–2677.

G.E. Legger, **U. Nakshbandi**, M.S. van Ginkel, S.C. Liefers, L. de Wolff, A.J. Stel, W. Armbrust, F.K.L.Spijkervet, A. Vissink, S. Arends, H. Bootsma, B. van der Vegt, G.M. Verstappen, F.G.M. Kroese. More severe parotid gland histopathology in paediatric-onset than in adult-onset sjögren's disease. *RMD Open* 2024;10(3):e004201.

M.S. van Ginkel, **U. Nakshbandi**, S. Arends, E.A. Haacke, S.C. Liefers, G.M.P.J. Verstappen, J.F. van Nimwegen, E. Brouwer, A.J. Stel, F.K.L. Spijkervet, A. Vissink, H. Bootsma, B. van der Vegt, F.G.M. Kroese. Increased diagnostic accuracy of the labial gland biopsy in primary sjögren's syndrome when multiple histopathological features are included. *Arthritis & Rheumatology* 2024 76(3):421-428.

S. Pringle, G.M.P.J. Verstappen, M.S. van Ginkel, **U. Nakshbandi**, Z. Girigoria, H. Bootsma, B. van der Vegt, F.G.M. Kroese. Lymphoepithelial lesions in the salivary glands of primary Sjögren's syndrome patients: the perfect storm? *Clin Exp Rheumatol* 2022; 40: 2434-2442.

S.M.H. Yeung, M. van Londen, **U. Nakshbandi**, M.Y. Said, M.F. Eisenga, B.G. Hepkema, I.M. Nolte, S.P. Berger, M.H. de Borst, S.J.L. Bakker. Pretransplant NT-proBNP, dialysis vintage, and posttransplant mortality in kidney transplant recipients. *Transplantation* 2020; 104(10):2158-2165.

U. Nakshbandi, E.A. Haacke, H. Bootsma, A. Vissink, F.K.L. Spijkervet, B. van der Vegt, F.G.M. Kroese. Bcl6 for identification of germinal centres in salivary gland biopsies in primary sjögren's syndrome. *Oral Diseases* 2020;26:707-710.

G.M.P.J. Verstappen, **U. Nakshbandi**, E. Mossel, E.A. Haacke, B. van der Vegt , A. Vissink, H. Bootsma, F.G.M. Kroese. Is the T Follicular Regulatory:Follicular Helper T cell ratio in blood a biomarker for ectopic lymphoid structure formation in sjögren's syndrome? *Arthritis & Rheumatology* 2018;70(8):1354-1355.