Sjögren's syndrome

treatment and treatment evaluation

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Contents

Chapter 1

General introduction

General introduction

The historical development of what currently is defined as Sjögren syndrome (SS) begins with the description of Hadden in 1888 who noted an association between the presence of a dry mouth and dry eyes in a 65-year old female patient who also suffered from loss of taste and smell. When she was treated with a tincture of jaborandi (pilocarpine) three times a day, her mouth became much more moist.(1) Also in 1888, Mickulicz described a 42-year old farmer with painless, extensive bilateral swelling of parotid and lacrimal glands. The swelling disturbed his vision and interfered with eating. Mickulicz removed the greater part of the swollen lacrymal glands. Unfortunately, a few months after surgery the patient suddenly died, probably due to appendicitis. At that time, the diagnosis was not conclusive. (2) However, the original woodcuts and colour plate of the drawing of a microscopical field of the submandibular gland have been reviewed with our current knowledge and a diagnosis of MALT lymphoma was made, a condition that rather frequently is observed in patients with SS.(3)

In 1925 the French physician Gougerot related dry eyes and dry mouth to an exocrine gland abnormality.(4) However, in 1933 Henrik Sjögren was the first to give a complete description of the clinical and histological findings in patients with rheumatoid arthritis, dry eyes and a dry mouth. In his thesis entitled 'Zur Kentniss der Keratoconjuntivitis sicca' he presented clinical and pathological information of 19 cases of patients with such complaints.(5) Sjögren stated that his major contribution has been the recognition of the sicca syndrome as a systemic disease. At first there was a lot of criticism on his thesis and only years later he received more credit for his work. His thesis was translated in English by Hamilton in 1943.(6) The eponym Gougerot-Sjögren's disease appeared in the literature in the 1930-ies and was reduced to Sjögren's disease a decade later due to the many cases reported by Sjögren.

In 1965 Bloch described the same condition as a triad of keratoconjunctivitis sicca, xerostomia and a connective tissue disease.(7) Based on this triad several sets of criteria have been introduced in the eighties of the previous century,(8-11) but none of these classification criteria were validated and universally accepted. In 1988 the European Study Group on Classification Criteria for SS began a multicentre study in order to develop a set of criteria.(12;13) This set of criteria received broader acceptance, although criticism was raised as well. Therefore, a joint study of the European Study Group on Classification Criteria for SS and a group of American experts was started. Presently, the revised American-European classification criteria for SS, which were proposed in 2002, are the most widely accepted and validated criteria (table 1).(14) These criteria combine subjective symptoms of dry eyes and dry mouth with objective signs of keratoconjunctivitis sicca and hyposalivation, and with serological and histopathological characteristics. It should be mentioned that the revised American-European classification criteria for SS have not been developed for clinical practice, but as a research tool for performing studies in patients with SS. Nevertheless, they are now widely accepted as diagnostic tools for SS. One should realize, however, that SS can be present in a patient who does not completely fulfil these criteria.

Table 1 Revised international classification criteria and revised rules for classification for SS (14).

I	Ocular symptoms: 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?
II	Oral symptoms: a positive response to at least one of the following questions: 1. Have you had a daily feeling of dry mouth for more than 3 months? 2. Have you had recurrently or persistently swollen salivary glands as an adult? 3. Do you frequently drink liquids to aid in swallowing dry food?
III	Ocular signs-that is, objective evidence of ocular involvement defined as a positive result for a least one of the following two tests: 1. Schirmer's I test, performed without anaesthesia (≤5 mm in 5 minutes) 2. Rose Bengal score or other ocular dye score (≥4 according to Van Bijsterveld's scoring system)
IV	Histopathology: In minor salivary glands (obtained through normal-appearing mucosa) focal lymphocytic sialoadenitis, evaluated by an expert histopathologist, with a focus score ≥1, defined as a number of lymphocytic foci (which are adjacent to normal-appearing mucous acini and contain more than 50 lymphocytes) per 4 mm^2 of glandular tissue
V	Salivary gland involvement: objective evidence of salivary gland involvement defined by a positive result for at least one of the following diagnostic tests: 1. Unstimulated whole salivary flow (≤ 1.5 ml in 15 minutes) 2. Sialectasia on parotid sialography 3. Abnormal salivary scintigraphy
VI	Autoantobodies: presence in the serum of the following autoantibodies: 1. Antibodies to Ro(SSA) or La(SSB) antigens, or both

For primary SS
In patients without any potentially associated disease, primary SS may be defined as follows:
a) The presence of any 4 of the 6 items is indicative of primary SS, as long as either item IV (histopathology) or VI (serology) is positive or
b) The presence of any 3 of the 4 objective criteria items (that is, items III, IV, V, VI)

For secondary SS
In patients with a potentially associated disease (for instance, another well defined connective tissue disease), the presence of item I or item II plus any 2 from among items III, IV, and V may be considered as indicative of secondary SS

Exclusion criteria
Past head and neck radiation treatment
Hepatitis C infection
Acquired immunodeficienty disease (AIDS)
Pre-existing lymphoma
Sarcoidosis
Graft versus host disease
Use of anticholinergic drugs (since a time shorter than 4-fold the half life of the drug)

Although the first description of SS was given in 1888 and although SS is the second autoimmune disease in prevalence (0.5-2%), only recently knowledge about SS has become more generally recognized and over the last decades an increasing number of studies is performed on SS. The first symptoms of SS usually develop gradually and are hard to recognize without specific knowledge about SS. First symptom in almost all patients is fatigue accompanied by one or more other symptoms such as oral and eye dryness, arthralgia and extraglandular manifestations. Fortunately, SS is diagnosed more and more in an early stage of the disease nowadays. Currently, more patients are within their working age at the time of diagnosis (mean age 45.7±15.7 years).(15) The influence of having SS on patients functioning and daily acitvity is still underestimated by both the general public and physicians. Most patients with SS report a large impact of the disease on their quality of life.(15) Moreover, related to the limitations patients experience in their daily life, there is a growing request for treatment options, both from doctors and patients. Although, as for other autoimmune diseases, the aetiopathogenesis of SS is still unknown, there are indications that treatment with biological agents applied for other autoimmune diseases might also be of benefit in the treatment of SS.(16) So far, B cell depletion showed the best results amongst the biologicals tested.(17-20)

Before implementation of treatment of SS with biological agents can be realized, approval should be obtained. Treatment with biological agents is expensive and positive impact on socioeconomic status of SS patients should be clear before implementation. Biological agents have to be investigated, first, in small open-label phase I trials to investigate safety and efficacy and, thereafter, in double-blinded placebo controlled phase II trials and larger phase III trials to confirm results found in the open-label trials. Also, research on the aetiopathogenesis of SS is very important to gain more knowledge on the disease.

Although many trials have been performed during the last decades regarding treatment of SS, including trials aimed at reducing disease activity and/or intervening with the progression of the disease, up to now most agents that have been shown to be of some use in the treatment of SS mainly exert a symptomatic effect. The assessment of the effect of biologicals, aimed at reducing disease activity and to slow down progression of SS, is still at a very early stage. Also, much remains unknown regarding the aetiopathogenesis of SS.

Therefore, the main objective of this thesis is the evaluation of existing and new therapeutic strategies for intervention in SS. Furthermore, the impact of SS on quality of life was assessed and a case report is described aiming to deepen the insight in the role of B cells in the aetiology of SS.

Outline of this thesis

This thesis contains the results of various studies concerning (a) quality of life of SS patients, (b) the applicability of tools to evaluate the efficacy of treatment in SS patients, (c) the evaluation of intervention therapy with anti-CD20, a therapy that is focussed on B cell depletion, and (d) a case series to gain more insight into the role of B cells in the aetiopathogenesis of SS.

The impact of SS on the quality of life and the socioeconomic status of SS patients is described in chapter 2 This study was done to explore whether treatment is necessary for SS patients and why research on this disease should be performed. Next, in chapter 3 a specific overview of the trials performed up to 2006 with biological agents as treatment for SS is given. The main conclusion from that overview is that anti-CD20 in particular seems to be promising. In chapter 4a, a general overview of tools applicable for treatment evaluation of diseases affecting salivary glands, in particular SS, is provided. On the basis of this overview tools to be used in treatment evaluation (chapter 5) were selected. The possibility of indentifying a genomic and proteonomic profile of SS patients as a new tool for evaluation is described in chapter 4b. Based on the data published in chapters 2 and 3 and using a selection of the tools provided in chapter 4, several trials with anti-CD20 (rituximab) as intervention treatment for SS were designed. First, an analysis of the efficacy of retreatment and long-term follow up after treatment (chapter 5a) is described. In chapter 5b a study is presented evaluating the effects of rituximab on the histopathology of parotid gland biopsies in patients with SS described in chapter 5a. Thereafter, a placebo controlled double blinded randomized clinical trial of rituximab treatment in SS (chapter 5c) is described. A study related to the direct scope of this thesis, is the description of a case series of 8 patients in which the combination of nodular cutaneous amyloidosis and SS is present. (chapter 6) The type of amyloid was probably AL amyloid in all 8 patients (immunoglobulin light chain-associated amyloid). Therefore, the combination of cutaneous amyloid and SS appeared to be a distinct disease entity reflecting a particular and benign part of the polymorphic spectre of B cell dysfunction in lymphoproliferative diseases related to SS. Chapter 7 contains the summary and general discussion and chapter 8 the Dutch summary.

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Chapter 2

Health related quality of life, employment and disability in patients with Sjögren's syndrome

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Abstract

Objective To compare health related quality of life (HR-QOL), employment and disability between primary (pSS) and secondary (sSS) Sjögren's syndrome (SS) patients and the general Dutch population.

Methods HR-QOL, employment and disability were assessed in SS patients regularly attending the University Medical Center Groningen (n=235). HR-QOL, employment and disability were evaluated with the Short Form-36 questionnaire (SF-36) and an employment and disability questionnaire. Results were compared with Dutch population data (matched for sex and age). Demographical and clinical data associated with HR-QOL, employment and disability were assessed.

Results Response rate was 83%. SS patients scored lower on HR-QOL than the general Dutch population. sSS patients scored lower on physical functioning, bodily pain and general health than pSS patients. Predictors for reduced HR-QOL were fatigue, tendomyalgia, articular involvement, use of artificial saliva, use of antidepressants, comorbidity, male sex, and eligibility for disability compensation (DC). Employment was lower and DC rates were higher in SS patients compared with the Dutch population.

Conclusions SS has a large impact on HR-QOL, employment and disability.

Introduction

Sjögren's syndrome (SS) is a chronic, systemic, lymphoproliferative autoimmune disease affecting the exocrine glands.(1) The salivary and lachrymal glands are most commonly affected, resulting in dry mouth and dry eyes. Extraglandular involvement can occur in SS, and includes, amongst others, pulmonary disease, renal disease and vasculitis. Moreover, almost all patients suffer from fatigue. SS can be primary (pSS) or secondary (sSS), the latter being associated with other autoimmune diseases such as rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE). The estimated prevalence of SS in the general population is between 0.5-2%, which makes SS, after RA, the most common systemic autoimmune disease. (2;3)

Rheumatologic conditions have a major impact on patients. Apart from the symptoms mentioned above, patients may be restricted in their activities and their participation in society, resulting in a reduced health related quality of life (HR-QOL) and an impaired socioeconomic status. The latter may result in lower employment rates and more disability as compared with the general population.(4)

SS is known to affect patients' physical, psychological and social functioning (5), but the impact of SS on HR-QOL, and especially on employment and disability, has not been studied extensively. Studies available were either performed in small series of SS patients (6;7) or mainly aimed at comparison with other rheumatic diseases (6-9), fatigue (9) and psychological status (8), or at developing new tools for measuring fatigue and general discomfort in pSS patients.(10) Comparison between pSS and sSS has occasionally been described for HR-QOL (7;9), but not for employment and disability. The aim of this study was, therefore, to evaluate HR-QOL, employment and disability in a large cohort of Dutch SS patients, to relate outcomes to clinical and demographic data in this patient cohort, and to compare these data with those available for the general Dutch population. In addition, HR-QOL, employment and disability were compared between pSS and sSS patients, since it was hypothesized that the disease burden of sSS might differ from that of pSS due to co-existing autoimmune disease(s).

Patients and methods

Patients

SS patients (185 pSS, 50 sSS) regularly attending the departments of Rheumatology, Clinical Immunology and Oral and Maxillofacial Surgery of the University Medical Center Groningen (UMCG), The Netherlands, were enrolled in this study. All patients were above the age of 18 years and fulfilled the European-American criteria for SS.(11) All patients participating in this study were followed according to protocol, and, therefore, data on extraglandular manifestations (EGM) were available for all patients. Ethical approval for this study was obtained from the local Institutional Review Board.

Methods

Demographical and clinical data were obtained by chart review. EGM were defined in accordance with previous studies.(12;13) Tendomyalgia, skin involvement other than cutaneous vasculitis, oesophageal involvement, bladder involvement and thrombocytopenia are commonly observed symptoms and signs, and, thus, were also considered as EGM. Moreover, at every visit the rheumatologists systematically evaluated the presence of EGMs.

Questionnaires were sent by regular mail to all patients. Six weeks after sending the questionnaires, patients who had not responded were approached by phone once, to ask for participation.

In the first questionnaire, patients were asked whether they suffered from arthralgia and/or tendomyalgia, fatigue, dry mouth and dry eyes. In addition, it was asked which symptom they considered to be their most severe complaint.

To evaluate HR-QOL, a validated Dutch translation of the Short Form 36 (SF-36) was used.(12;14) The SF-36 is a questionnaire consisting of 36 items, with eight scales assessing two dimensions, viz. physical and mental health functioning. Scales and summary scores vary from 0 to 100, with 0 being the worst possible health status and 100 representing the best possible health status.

The third questionnaire focused on level of education, employment and disability. In The Netherlands, an individual who is judged to be impaired by at least 80% is entitled to full disability compensation (DC). Individuals impaired by 15-80% are entitled to partial DC.

Age and sex matched data for the general Dutch population on the SF-36 were obtained from Aaronson et al.(14) Data regarding employment and DC were obtained from the Dutch Office of Statistics (Centraal Bureau voor Statistiek, CBS, Voorburg, The Netherlands).

Statistical analysis

The T-tests and χ^2 tests were used for the comparison of demographical data, HR-QOL, employment and receiving DC between responders and non-responders, between pSS and sSS patients, and between SS patients and the general Dutch population. Alpha was set at 5%. Correlation between disease duration and HR-QOL was evaluated with a Pearson correlation test.

To create effect models, univariate analyses were performed for each predictor variable on the outcomes (HR-QOL, employment and receiving DC). If variables were found to be significant, *P*-values were used in the further development of the model. Predictors with a *P*-value less than or equal to 0.2 were simultaneously entered into a multivariable model, after which backward elimination of predictors was used to remove non-significant

Table I Patients' characteristics.

Characteristics	All responding SS patients (n=195)	pSS (n=154)	sSS (n=41)	*P* pSS vs sSS
Age (years; mean±SD)	55.5±15.0	54.6±15.1	58.9±14.2	0.103
Age at diagnosis (years; mean±SD)	45.7±15.7	45.5±15.3	46.5±17.1	0.715
Female sex (n, %)	179 (91.8%)	143 (92.9%)	36 (87.8%)	0.197
Partner (n, %)	153 (78.5%)	121 (78.6%)	32 (78.0%)	0.769
Disease duration (years; mean±SD)	9.7±8.8	9.0±8.0	12.5±11.0	0.121
Immunological features				
Focus score (mean±SD)	2.7±1.8	2.7±2.0	2.5±2.0	0.716
ANA (n, %)	189 (96.9%)	151 (98.1%)	38 (92.7%)	0.109
Anti-Ro/SS-A (n, %)	155 (79.5%)	129 (83.8%)	26 (63.4%)	0.014
Anti-La/SS-B (n, %)	107 (54.9%)	90 (58.4%)	17 (41.5%)	0.077
IgG (g/L ; mean±SD)	18.6±7.2	18.8±6.8	17.7±8.3	0.405
IgA (g/L; mean±SD)	2.8±1.3	2.7±1.2	3.2±1.5	0.023
IgM (g/L; mean±SD)	1.4±1.0	1.4±1.1	1.3±0.8	0.629
RF (kIU/L; mean±SD)	106.2±190.2	99.5±195.6	131.2±168.7	0.343
Second auto immune disease (n, %)				
none	154 (79.0%)	154 (100%)	-	-
SLE	19 (9.7%)	-	19 (46.3%)	-
RA	16 (8.2%)	-	16 (39.0%)	-
Other	6 (3.1%)	-	6 (14.6%)	-
Extraglandular manifestations (n, %)	185 (94.9%)	144 (93.5%)	41 (100%)	0.112
Articular involvement*	110 (56.4%)	80 (51.9%)	30 (73.2%)	0.017
Raynaud's phenomenon	84 (43.1%)	67 (43.5%)	17 (41.5%)	0.789
Tendomyalgia	80 (41.0%)	64 (41.6%)	16 (39.0%)	0.746
Pulmonary involvement	33 (16.9%)	25 (16.2%)	8 (19.5%)	0.631
Lymphoproliferative disease	30 (15.4%)	24 (15.6%)	6 (14.6%)	0.869
Cutaneous vasculitis	28 (14.4%)	22 (14.3%)	6 (14.6%)	0.967
Peripheral neuropathy	26 (13.3%)	20 (13.0%)	6 (14.6%)	0.794
Skin involvement other than cutaneous vasculitis*	22 (11.3%)	13 (8.4%)	9 (22.0%)	0.047
Bladder involvement	22 (11.3%)	18 (11.7%)	4 (9.8%)	0.719
Lymphadenopathy	21 (10.8%)	19 (12.3%)	2 (4.9%)	0.168
Renal involvement	19 (9.7%)	14 (9.1%)	5 (12.2%)	0.560
Autoimmune thyroiditis	19 (9.7%)	16 (10.4%)	3 (7.3%)	0.548
Autoimmune hepatitis	12 (6.2%)	11 (7.1%)	1 (2.4%)	0.262
Oesophageal involvement	9 (4.6%)	7 (4.5%)	2 (4.9%)	0.872
Fever	8 (4.1%)	7 (4.5%)	1 (2.4%)	0.541
Serositis	6 (3.1%)	5 (3.2%)	1 (2.4%)	0.785
Myositis	5 (2.6%)	3 (1.9%)	2 (4.9%)	0.295
CNS involvement	5 (2.6%)	5 (3.2%)	-	0.241
Thrombocytopenia	2 (1.0%)	2 (1.3%)	-	0.337
Acute pancreatitis	1 (0.5%)	1 (0.6%)	-	-

This table continues on the next page.

Table 1 Patients' characteristics, continued.

Characteristics	All responding SS patients (n=195)	pSS (n=154)	sSS (n=41)	*P* pSS vs sSS
Comorbidity (n, %)**	75 (38.5%)	59 (38.3%)	16 (39.0%)	0.957
Osteoarthritis	15 (7.7%)	13 (8.4%)	2 (4.9%)	-
Cardiovascular disease	13 (6.7%)	9 (5.8%)	4 (9.8%)	-
Neurologic disease	10 (5.1%)	9 (5.8%)	1 (2.4%)	-
Diabetes mellitus	8 (4.1%)	5 (3.2%)	3 (7.3%)	-
Pulmonary disease	7 (3.6%)	5 (3.2%)	2 (4.9%)	-
Gastro-intestinal disease	6 (3.1%)	5 (3.2%)	1 (2.4%)	-
Eye disease	5 (2.6%)	4 (2.6%)	1 (2.4%)	-
Malignancy	3 (1.5%)	3 (1.9%)	0	-
Urologic disease	3 (1.5%)	2 (1.0%)	1 (2.4%)	-
Osteoporosis	2 (1.0%)	1 (0.6%)	1 (2.4%)	-
Depression	19 (9.7%)	15 (9.7%)	4 (9.8%)	-
Other	10 (5.2%)	6 (3.9%)	4 (9.8%)	-
Therapy (n, %)				
Artificial tears	151 (77.4%)	119 (77.3%)	32 (78.0%)	0.711
Oral moisturising gel	46 (23.6%)	37 (24.0%)	9 (22.0%)	0.840
Artificial saliva	20 (10.3%)	16 (10.4%)	4 (9.8%)	0.942
Pilocarpine	18 (9.2%)	15 (9.7%)	3 (7.3%)	0.663
NSAIDs	47 (24.1%)	31 (20.1%)	16 (39.0%)	0.012
Antimalarial drugs	31 (15.9%)	20 (13.0%)	11 (26.8%)	0.031
Oral corticosteroids	26 (13.3%)	20 (13.0%)	6 (14.6%)	0.783
Rituximab	20 (10.3%)	19 (12.3%)	1 (2.4%)	0.036
Other immunosuppressives	17 (8.7%)	9 (5.8%)	8 (19.5%)	0.006
Antidepressants	18 (9.2%)	14 (9.1%)	4 (9.8%)	0.769

n = number of patients; SLE = systemic lupus erythematosus; RA = rheumatoid arthritis; CNS = central nervous system; NSAIDs = non-steroidal anti-inflammatory drugs. *Extraglandular manifestation that affect sSS patients significantly more frequently than pSS patients. **Comorbidity unrelated to SS.

predictors (*P*-value to remove >0.10). Subsequently, predictors not included in the multivariable model were entered to determine whether they could now enter the model, after which the procedure of backward elimination of predictors was repeated. Variables in the final models were tested for possible interactions. All analyses were carried out using SPSS for Windows version 16.0.

Results

Patient characteristics (table 1)

196 patients (180 females, 16 males; mean age at diagnosis: 45.7±15.7 years) responded to the mail survey (83%). One patient returned the questionnaire incompletely and was therefore excluded. The mean age (±SD) at the time of completing the questionnaire was

55.5±15.0 years; the mean disease duration was 9.7±8.8 years. 154 patients (79%) were classified as pSS and 41 patients (21%) as sSS. Demographic data did neither differ between pSS and sSS patients nor between responders and non-responders.

The most frequently associated autoimmune disorders in sSS patients were SLE (46%) and RA (39%). Seventy-five patients (39%) suffered from at least one comorbid condition.

Artificial tears were used by 77% and antidepressants by 9% of patients. Non-steroidal anti-inflammatory drugs, antimalarial drugs and other immuno-suppressants were used more frequently by sSS patients, whereas rituximab was more frequently prescribed in pSS patients.

EGM were present in 185 patients (95%). The main EGM were articular involvement, Raynaud's phenomenon and tendomyalgia. sSS patients suffered from articular- and skin involvement more often than pSS patients. When restricting the EGM to the EGM defined in accordance with previous studies (21;22), EGM occurred in 177 patients (91%; pSS 137; sSS 40).

Current symptoms

Almost all patients suffered from dry mouth (n=183; 94%), dry eyes (n=183; 94%), and fatigue (n=166; 85%). Fatigue was the most severe symptom in 78 patients (40%). Arthralgia and/or tendomyalgia was present in 148 patients (76%). The prevalence of sicca symptoms, fatigue and arthralgia and/or tendomyalgia was comparable between pSS and sSS patients.

Health related quality of life

When compared with the general Dutch population, HR-QOL was significantly decreased in SS patients as demonstrated by reduced SF-36 scores on six out of the eight scales and for the summary scores for physical and mental functioning (table 2).

sSS patients experienced a significantly lower HR-QOL than pSS patients on three of the four physical scales (physical functioning, bodily pain and general health), however, no differences were observed on the psychological scales. HR-QOL was comparable between sSS patients with either RA or SLE as the associated autoimmune disorder. Disease duration was not significantly correlated with any of the SF-36 scales. Highly educated patients scored significantly better on physical functioning ($p=0.042$) and mental health ($p=0.005$) compared with non-highly educated patients.

Multivariate regression analysis showed that fatigue, tendomyalgia, comorbidity, male sex and receiving DC were associated with a reduced physical component summary score (PCS) (table 3). Confounders were disease duration, use of NSAIDs and antidepressants and employment. No significant effect modifiers (interaction terms) were found.

Multivariate regression analysis for the mental component summary score (MCS) demonstrated that fatigue, articular involvement, use of artificial saliva, use of antidepressants, and comorbidity were associated with a reduced MCS, whereas dry mouth was associated with a higher MCS (table 3). Receiving DC was a confounding factor for the determinants in the primary model for the MCS. No effect modifiers were found.

Socioeconomic status

135 patients (69%) were of working age (18-65 years) (table 4). SS patients were significantly less often employed ($p<0.001$), worked fewer hours ($p=0.015$) and were less frequently full time employed ($p<0.01$), compared with the Dutch population. In detail, approximately half of the SS patients (n=69) had paid employment. Only seven SS patients (10%) worked full-time (at least 36 hours). On average, SS patients worked 21.7±11.6 hours per week. The mean sick

Table 2 SF-36 scores for SS patients and the general Dutch population.

SF-36 scales and summary scores	GDP Mean (SD) n=195	RSS Mean (SD) n=195	*P* RSS vs GDP	pSS Mean (SD) n=154	sSS Mean (SD) n=41	*P* pSS vs sSS
PF	74.8 (25.8)	59.2 (26.0)	0.000	62.0 (25.1)	48.9 (27.0)	0.004
RP	70.3 (36.3)	41.0 (42.9)	0.000	44.0 (42.7)	29.1 (41.9)	0.058
BP	68.7 (25.6)	64.7 (24.4)	0.136	68.0 (23.0)	52.1 (25.7)	0.000
GH	65.7 (21.5)	40.3 (18.2)	0.000	41.9 (18.4)	34.2 (16.3)	0.018
VT	63.8 (21.0)	45.2 (20.1)	0.000	46.0 (20.4)	42.0 (18.9)	0.266
SF	81.3 (25.6)	63.1 (26.2)	0.000	64.5 (26.6)	57.9 (24.5)	0.152
RE	79.7 (34.4)	70.0 (41.4)	0.005	71.5 (41.5)	63.9 (40.9)	0.324
MH	73.3 (19.0)	70.3 (18.4)	0.055	70.6 (18.9)	69.0 (16.8)	0.627
PCS	73.0 (24.6)	51.7 (23.7)	0.000	53.3 (23.6)	44.7 (23.2)	0.055
MCS	74.5 (21.1)	63.3 (21.2)	0.000	64.0 (21.2)	60.5 (21.4)	0.385

n = number of patients; SF-36 = short form-36; GDP = general Dutch population; RSS = all responding SS patients; pSS primary Sjögren syndrome; sSS = secondary Sjögren syndrome; PF = physical functioning; RP = physical role functioning; BP = bodily pain; GH = general health; VT = vitality; SF = social functioning; RE = emotional role functioning; MH = mental health; PCS = physical component summary score; MCS = mental component summary score.

leave was 15.6±39.0 days during the past year (range 0-192 days). Highly educated patients were significantly more often employed than non-highly educated patients (p=0.001). No differences were found between pSS and sSS patients regarding employment variables.

Sixty-three working age patients (47%) received DC, because they were considered to be (partially) unfit for work (table 4). 28 of these patients (44%) were entitled to full DC. Moreover, 41 of the 63 patients receiving DC (65%) mentioned pSS, sSS or the associated rheumatic disease as the cause of receiving DC. No differences in DC were found between pSS and sSS patients or between highly educated and non-highly educated patients. A significantly higher percentage of SS patients received DC (47%) when compared with the general Dutch population (2%).

Multivariate regression analysis for employment (table 5) showed that a high level of education was associated with employment. Bladder involvement, use of oral moisturizing gel, NSAIDs and oral corticosteroids, comorbidity and age at diagnosis were all negatively associated with employment. Autoimmune thyroiditis, use of artificial tears and age were confounding factors for these determinants. No interaction terms were found. Multivariate regression analysis for receiving DC (table 5) demonstrated that the number of EGM, use of artificial saliva and antimalarial drugs, comorbidity, high level of education, and male sex were associated with receiving DC. Age at diagnosis was negatively associated with receiving DC. Fatigue, skin involvement other than cutaneous vasculitis and use of pilocarpine were confounding factors for the determinants in the primary model for receiving DC. No interaction terms were found.

Discussion

This study shows that SS has a large impact on HR-QOL, employment and disability as reflected by lower SF-36 scores and employment rates, and higher disability rates when compared with the general Dutch population. Moreover, analysis of HR-QOL revealed that sSS patients were more limited in physical activities than pSS patients. Although the results are obtained in a Dutch cohort of SS patients, the striking differences in HR-QOL, employment and disability suggest that the results of our study are not limited to the Dutch population, but probably are generally applicable to SS patients when compared with healthy subjects.

Reduced HR-QOL in SS patients compared with normative data has been reported before, but these studies were performed in smaller populations.(6;9;12;15) Overall, the SF-36 scores for pSS patients in our study were comparable to those mentioned in earlier literature. (8-10;15)

We observed more limitations in physical functioning in sSS than in pSS patients. This is in contrast to the results described by Sutcliffe et al. (7) and Tensing et al.(9) The latter studies were performed in smaller patient cohorts and mainly included sSS patients with SLE as second autoimmune disease. The associated rheumatic disease in our sSS patients was more diverse (RA, SLE and other). RA patients are considered to be more restricted in physical functioning than SLE patients (16), which might explain the difference in results. We, however, did not observe such a difference between sSS/RA and sSS/SLE patients; perhaps because of the relatively small sSS subgroups in our study.

In our regression analyses several demographic and clinical factors were found to be associated with HR-QOL. The unexplained variance probably reflects unmeasured, non-disease related psychosocial factors such as self-esteem, support and coping strategies (17), and other factors such as immunologic parameters, delay in diagnosis and untreated or undiagnosed depression.(15) Interestingly, fatigue was an important explanatory variable for reduced physical and mental HR-QOL. (5;9;18)This finding is in agreement with other studies. Furthermore, the importance of fatigue in SS was underscored by the fact that the majority of SS patients felt tired and 40% ranked fatigue as their most severe symptom. Fatigue should therefore be considered as an important treatment target.

Segal et al.(19) demonstrated that psychological variables such as depression are determinants for fatigue, but only partly account for it. Since depression could be of importance for our outcome measures as well, the use of antidepressants was scored in our population (9%). The regression analyses showed that antidepressants were a predictive factor for mental HR-QOL, as can be expected; but not for physical HR-QOL, employment or receiving DC.

We observed low employment and high disability rates in SS, which also have been reported for rheumatic diseases such as RA (17;20) and ankylosing spondylitis.(17) To our knowledge, these results have not previously been reported in SS patients.

A high level of education and comorbidity were the most significant predictors for having paid employment. One would expect, however, that fatigue and arthralgia would also have influenced the employment status. A possible explanation for the lack of this association could be that, with time, patients have gradually adapted their activities to these symptoms. This hypothesis is supported by the fact that only 10% of employed patients had a full-time job.

We found a higher frequency of EGM (95%) compared with other studies.(8;12;15) This can partly be explained by the extended definition of EGM used in this study. Interestingly,

Table 3 Linear multivariate regression analyses for the PCS and MCS of the SF-36.

PCS, model 1			**PCS, adjusted for confounding**		
Variable	***ß [95% CI]***	***P***	**Variable**	***ß [95% CI]***	***P***
Fatigue	-24.26 [-33.07 – -15.44]	0.000	Fatigue	-21.38 [-30.31 – -12.46]	0.000
Tendomyalgia	-9.18 [-15.22 – -3.13]	0.003	Tendomyalgia	-7.62 [-14.22 – -1.03]	0.024
Comorbidity	-18.51 [-24.97 – -12.06]	0.000	Comorbidity	-17.97 [-25.11 – -10.82]	0.000
			Male sex	-11.38 [-22.11 – -0.65]	0.038
			Receiving DC	-10.71 [-17.13 – -4.29]	0.001
Male sex	-12.69 [-23.47 – -1.92]	0.021	Disease duration (years)	0.15 [-0.27 – 0.56]	0.487
Receiving DC	-9.64 [-15.95 – -3.34]	0.003	NSAID use	-4.37 [-11.67 – 2.94]	0.239
			Antidepressant use	-6.76 [-18.19 – 4.67]	0.244
			Employment	-0.95 [-2.31 – 1.14]	0.217
MCS, model 1			**MCS, adjusted for confounding**		
Variable	***ß [95% CI]***	***P***	**Variable**	***ß [95% CI]***	***P***
Fatigue	-15.97 [-24.48 – -7.45]	0.000	Fatigue	-16.92 [-26.26 – -7.57]	0.000
Dry mouth	17.93 [5.94 – 29.91]	0.004	Dry mouth	16.75 [2.50 – 31.00]	0.022
Articular involvement	-7.63 [-13.65 – -1.60]	0.008	Articular involvement	-5.48 [-12.18 – 1.22]	0.108
Artificial saliva use	-9.33 [-18.46 – -0.21]	0.045	Artificial saliva use	-12.58 [-22.97 – -2.20]-	0.018
Antidepressant use	-9.57 [-20.47 – 1.32]	0.085	Antidepressant use	-11.32 [-24.18 – 1.54]	0.084
Comorbidity	-9.49 [-15.74 – -3.23]	0.003	Comorbidity	-11.91 [-18.92 – -4.89]	0.001
			Receiving DC	-2.11 [-8.68 – 4.45]	0.526

PCS = physical component summary score; MCS = mental component summary score; *ß* = regressioncoeficient; 95% CI = 95% confidence interval; DC = disability compensation; NSAIDs = non-steroidal anti-inflammatory drugs.

Table 4 Education level, employment characteristics and disability compensation (DC) in SS patients of working age.

Employment characteristics (n,%)	GDP n=135	SS patients n=135	*P SS patients vs GDP*	pSS patients n=109	sSS patients n=26	*P* pSS vs sSS
Level of education						
Low	31 (23.5%)	5 (3.7%)	<0.001	5 (3.8%)	0	0.800
Middle	57 (43.2%)	94 (69.6%)		75 (57.7%)	19 (57.6%)	
High	44 (33.3%)	33 (24.4%)		26 (20.0%)	7 (21.2%)	
Unknown		3 (2.2%)		3 (2.3%)	0	
Paid employment	109 (82.6%)	69 (51.1%)	<0.001	58 (53.2%)	11 (42.3%)	0.297
Full time paid job	26 (23.9%)	7 (10.1%)	<0.01	7 (12.1%)	0	0.237
Hours worked per week (mean±SD)	26.9±14.2	21.7±11.6	0.011	21.7±12.1	21.3±8.5	0.914
Days sick leave per year (mean±SD)	NA	15.6±39.0	NA	14.7±37.8	22.3±50.0	0.675
Receiving DC	2 (1.5%)	63 (46.7%)	<0.001	49 (45.0%)	14 (53.8%)	0.267
Full DC	NA	28 (44.4%)	NA	21(42.9%)	7 (50.0%)	0.434
Disability percentage (mean±SD)	NA	66.2±30.2	NA	63.6±30.0	75.8±30.0	0.246
Cause receiving DC						
pSS, sSS or associated rheumatic disease	NA	41 (65.1%)	NA	33 (67.3%)	8 (57.1 %)	
Other		7 (11.1%)		6 (12.2%)	1(7.1%)	
Unknown		15 (23.8%)		10 (20.4%)	5 (35.7%)	

GDP = general Dutch population; n = number of patients; DC = disability compensation; NA = not available.

we found a higher frequency of Raynaud's phenomenon (43%), as compared with the study performed by García-Carrasco et al. (16%).(12) This may be explained by different weather circumstances in The Netherlands. The observed higher prevalence of lymphoproliferative disease (15% vs. 2%) may be related to the use of parotid gland biopsies in the diagnostic work-up of our patients.(21) Parotid biopsies are more suited for (early) detection of lymphoproliferative disease than labial biopsies as mucosa associated lymphoid tissue (MALT) and non-Hodgkin lymphomas are rarely found in labial glands.

Although the percentage of patients with EGM did not differ between pSS and sSS patients, it should be noted that part of the EGM in sSS patients could be attributed to the associated autoimmune disease and not only to SS. EGM and EGM related treatment were predictive for HR-QOL, employment and receiving DC. Therefore, there is a need for accurate follow-up and treatment aimed at EGM.

The response rate of 83% in our study is very reasonable. As such, the risk of a sampling bias of certain categories of patients to be preferentially included in this study is considered negligible. Furthermore, we did not observe any significant differences for age, sex and pSS/sSS ratio between responders and non-responders. We, therefore, conclude that our results are representative for SS patients regularly attending a Medical Center specialized in SS patient care.

Table 5 Logistic multivariate regression analyses for employment and receiving disability compensation (DC) in SS patients.

Employment, model 1			**Employment, adjusted for confounding**		
Variable	***Odds ratio [95% CI]***	***P***	**Variable**	***Odds ratio [95% CI]***	***P***
Bladder involvement	0.19 [0.05 – 0.75]	0.017	Bladder involvement	0.20 [0.05 – 0.81]	0.024
Oral moisturising gel use	0.32 [0.11 – 0.94]	0.038	Oral moisturising gel use	0.37 [0.12 – 1.15]	0.084
NSAID use	0.30 [0.12 – 0.81]	0.017	NSAID use	0.25 [0.09 – 0.70]	0.008
Oral corticosteroids use	0.16 [0.04 – 0.59]	0.006	Oral corticosteroids use	0.14 [0.04 – 0.56]	0.005
Comorbidity	0.13 [0.05 – 0.36]	0.000	Comorbidity	0.14 [0.05 – 0.39]	0.000
Age at diagnosis (years)	0.95 [0.92 – 0.97]	0.000	Age at diagnosis	0.97 [0.92 – 1.02]	0.261
High level of education	4.39 [1.69 – 11.44]	0.002	High level of education	4.21 [1.59 – 11.16]	0.004
			Autoimmune thyroiditis	0.46 [0.09 – 2.54]	0.376
			Artificial tears use	0.50 [0.18 – 1.37]	0.177
			Age	0.97 [0.92 – 1.02]	0.250
Receiving DC, model 1			**Receiving DC, adjusted for confounding**		
Variable	***Odds ratio [95% CI]***	***P***	**Variable**	***Odds ratio [95% CI]***	***P***
Number of EGM	1.37 [1.04 – 1.80]	0.026	Number of EGM	1.28 [0.96 – 1.70]	0.099
Artificial saliva use	6.89 [1.92 – 24.76]	0.003	Artificial saliva use	6.21 [1.66 – 23.18]	0.007
Antimalarial drug use	3.41[1.19 – 9.74]	0.022	Antimalarial drug use	2.79 [0.94 – 8.32]	0.065
Comorbidity	2.70 [1.08 – 6.79]	0.034	Comorbidity	2.73 [1.05 – 7.11]	0.039
Age at diagnosis (years)	0.93 [0.90 – 0.97]	0.000	Age at diagnosis (years)	0.94 [0.90 – 0.97]	0.000
Male sex	23.11 [4.40 – 121.24]	0.000	Male sex	32.21 [5.23- 198.42]	0.000
High level of education	2.86 [1.09 – 7.50]	0.032	High level of education	2.66 [1.00 – 7.06]	0.050
			Fatigue	3.33 [0.67 – 16.57]	0.142
			Skin involvement other than cutaneous vasculitis	1.35 [0.41 – 4.42]	0.625
			Pilocarpine use	2.72 [0.76 – 9.74]	0.124

95% CI = 95% confidence interval; UTI = urinary tract infections; NSAIDs = non-steroidal anti-inflammatory drugs; EGM = extraglandular manifestations.

Since many SS patients suffer from reduced HR-QOL and are restricted in social and work related activities, there is a great need for developing adequate treatment modalities to reduce SS related complaints and to intervene in the progression of SS. Currently, no causal systemic treatment is available in SS and, therefore, only symptomatic treatment can be given. Recently, some studies reported good results of treatment with biologicals, especially anti-CD20 treatment.(22-25) Therefore, further development and evaluation of systemic treatment options should be stimulated.

Conclusion

SS has a large impact on HR-QOL, employment and disability as reflected by lower SF-36 scores and employment rates, and higher disability rates in SS patients as compared with the general Dutch population. Several demographical and clinical factors were associated with HR-QOL, employment and receiving disability compensation. Physical functioning, bodily pain and general health were worse in sSS than in pSS patients.

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Chapter 3

The future of biologic agents in the treatment of Sjögren's syndrome

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Abstract

The gain in knowledge regarding the cellular mechanisms of T and B lymphocyte activity in the pathogenesis of Sjögren's syndrome (SS) and the current availability of various biological agents (anti-TNF-α, IFN-α, anti-CD20, and anti-CD22) have resulted in new strategies for therapeutic intervention. In SS, various phase I and II studies have been performed to evaluate these new strategies. Currently, B cell-directed therapies seem to be more promising than T cell-related therapies. However, large, randomized, placebo-controlled clinical trials are needed to confirm the promising results of these early studies. When performing these trials, special attention has to be paid to prevent the occasional occurrence of the severe side effects.

Introduction

Sjögren's syndrome (SS) is a chronic lymphoproliferative autoimmune disease with disturbances of T lymphocytes, B lymphocytes and exocrine glandular cells.(1) SS can be primary (pSS) or secondary SS (sSS), the latter being associated with another autoimmune disease (e.g. rheumatoid arthritis, systemic lupus erythematosus (SLE)).

Lymphocytic infiltrates are a characteristic histopathological finding in SS. These infiltrates consist of T and B cells. The expression of different cytokines, such as tumor necrosis factor-α (TNF-α) and interferon-α (IFN-α), during the formation and proliferation of these infiltrates has been investigated. There is an over expression of TNF-α, which is secreted by CD4+ T lymphocytes, mononuclear cells and epithelial cells.(2) The intraglandular synthesis of TNF-α causes destruction of acini by up-regulation of Fas at the surface of the glandular epithelial cells, stimulation of secretion of type 2 and 9 matrix metalloproteases by epithelial cells, and over expression of different chemokines.(3-5) IFN-α is produced by activated plasmacytoid dendritic cells in primary SS (pSS) and numerous IFN-α producing cells have been detected in labial salivary glands.(6) IFN-α promotes the autoimmune process by increasing autoantibody production and through the formation of endogenous IFN-α inducers. IFNs have potent immunomodulating properties and are thought to trigger a systemic biological response.(7)

Besides the presence of proinflammatory cytokines, described in the previous paragraph, recent studies have shown an important role for B cells in the pathogenesis of SS. Presence of autoantibodies and hypergammaglobulinemia are both considered to reflect B cell hyperactivity. Systemic complications of SS are associated with this B cell hyperactivity.(8) Moreover, about 5 % of SS patients develop malignant B cell lymphoma.(9) B cell activating factor (BAFF), also known as B lymphocyte stimulator (BLyS), is an important factor in local and systemic autoimmunity.(1) Dysregulated BAFF expression is implicated in disease progression and perpetuation of humoral autoimmunity. Overproduction of BAFF in transgenic mice has been shown to result in B cell proliferation and antibody production resulting in inflammation and destruction of the salivary glands, as well as kidney failure similar to observations seen in SLE.(10) In humans, circulating BAFF levels are increased in patients with pSS and correlate with disease activity.(11)

Recent insights in the cellular mechanisms of T and B lymphocyte activity in the pathogenesis of SS and the current availability of various biological agents have resulted in new strategies for therapeutic intervention. The use of these biological agents in the treatment of SS will be discussed in this review.

Biological agents

Currently, biological agents have been introduced in various systemic autoimmune diseases, as rheumatoid arthritis and SLE. Biological agents most frequently applied in autoimmune diseases are monoclonal antibodies, soluble receptors and molecular imitators.(12) These biological agents enhance or replace conventional immunosuppressive therapy. In contrast to rheumatoid arthritis and SLE, no biological agent has been approved yet for the treatment of SS, but several phase II and III studies have been or are currently conducted. The biological agents used in SS trials are IFN-α and agents targeting TNF-α and B cells (anti-CD20, anti-CD22). Although no trials have yet been performed with BAFF antagonists, this might be a promising therapy(13) and will be discussed in this review, as well.

Anti-TNF-α monoclonal antibodies

There are three main biological agents targeting TNF-α: the chimeric monoclonal IgG1 antibody infliximab, the receptor fusion protein etanercept, and the fully humanized monoclonal antibody adalimumab.

In an open-label study, short-term treatment with infliximab was reported to be very effective in active pSS over a 3-month period.(14) Sixteen patients received 3 infusions (3mg/kg) at weeks 0, 2 and 6, which led to significant improvement in all clinical and functional parameters, including global assessments, erythrocyte sedimentation rate, whole salivary flow rate, tear secretion (Schirmer test), tender joint count, fatigue score, and sensation of dry eyes and dry mouth. Three patients, all with short disease duration (< 3 years), were considered to be in complete remission up till 1 year. In 10 out of the 16 patients, SS symptoms, particularly mouth dryness, relapsed after a median of 9 weeks. In a follow-up study, a maintenance regimen of one infusion every 12 weeks was evaluated in these 10 patients. Retreatment induced an improvement of signs related to SS that was comparable with the effects from the three loading infusions.(15) To confirm these promising results from an uncontrolled study, the Trial of Remicade In Primary Sjögren's Syndrome study was designed. In this multicenter, double-blinded, placebo-controlled randomized clinical trial, 103 patients with active pSS were included and treated with infliximab infusions (5 mg/kg) or placebo at weeks 0, 2 and 6. Follow-up was 22 weeks. Primary endpoint was an improvement of >30% of two of three VAS scores measuring joint pain, fatigue and dry eyes. There were several secondary endpoints of which one was the basal salivary flow rate. In contrast to the previously mentioned uncontrolled studies, no evidence of efficacy of infliximab treatment on all clinical and functional parameters could be demonstrated in this randomized controlled clinical trial.(2)

A trial on 15 pSS patients (mean disease duration 3.6 years) with 25-mg etanercept, subcutaneously twice a week for 12 weeks, did not reveal a reduction of sicca symptoms and signs, neither did the repeated treatment for up to 26 weeks. Only in the subset of 4 patients with severe fatigue, a decrease of fatigue was observed.(16) Another trial evaluating subcutaneous administration of etanercept versus placebo for 12 weeks (28 patients) also showed no clinical efficacy.(17)

No trials of adalimumab treatment in pSS have been reported in the literature yet.

In conclusion, TNF-targeting treatment could not be proven to be of benefit in reducing the complaints of pSS patients.

IFN-α

IFNs are proteins with antiviral activity and potent immunomodulating properties. SS patients have an activated type I IFN system.(6) Such a role for IFN-α appears to contradict the reports described below, that low doses of IFN-α administered via the oromucosal route increase the unstimulated salivary output. However, it is hypothesized that oral IFN-α treatment may act by increasing saliva secretion by upregulation of aquaporin 5 transcription without significantly influencing the underlying autoimmune process.(6;7)

In a phase II study, treatment of pSS patients with IFN-α administered via the oromucosal route (by dissolving lozenges) was demonstrated to be effective (improvement of salivary output, decreased complaints of xerostomia) and safe.(18) Based on these promising results, a randomized, parallel group, double-blinded, placebo-controlled clinical trial (497 pSS patients) was designed. Patients were randomized into two groups and received a 24-week daily treatment with either 450 IU IFN-α (150IU three times per day) or placebo in a ratio 3:2, administered by the oromucosal route. This randomized, controlled clinical trial failed to demonstrate a significant effect on the primary endpoints (VAS score for oral dryness and stimulated whole salivary flow) in the IFN-α group relative to the placebo group. There was a significant increase in unstimulated whole saliva in the patients treated with IFN-α, which correlated positively and significantly with improvement in seven of eight symptoms associated with oral and ocular dryness. No adverse events were observed.(7)

In conclusion, no clinical evidence for the efficacy of IFN-α treatment in pSS patients has been shown yet; however an improvement of unstimulated whole saliva was observed. Further research is needed to objectify the effect of IFN-α on salivary gland tissue.

Anti-CD20 monoclonal antibodies

Anti-CD20 (rituximab) is a chimeric humanized monoclonal antibody specific for the B cell surface molecule CD20, which is expressed on the surface of normal and malignant pre-B and mature B lymphocytes. CD20 mediates B cell proliferation and differentiation. This antibody has been demonstrated to prevent B cells from proliferating and to induce lysis of B cells by complement-dependent and antibody-dependent cytotoxicity mechanisms as well as by direct induction of apoptosis.(19)

Rituximab is currently used for the treatment of low-grade B cell lymphomas.(20) In controlled studies, it was shown to be safe and effective in the treatment of rheumatoid arthritis.(21-23) Moreover, open-label studies in SLE patients are promising.(24)

In an open-label phase II study, 15 patients with pSS were treated with 4 infusions of rituximab (375 mg/m^2 once weekly) and followed up for a 3-month period. Eight of the 15 patients were early pSS patients (mean disease duration 28 months, all had residual salivary gland function at baseline), and 7 patients had a concomitant mucosa associated lymphoid tissue (MALT) lymphoma (mean disease duration 79 months).

In the early pSS patients, rituximab treatment resulted in significant improvement of subjective symptoms and an increase in salivary gland function. All patients showed a rapid depletion of peripheral B cells within a few weeks, accompanied by a decrease in IgM-RF levels.(8) Repeated parotid gland biopsies in five of the early patients after treatment, showed redifferentation of the lymphoepithelial duct lesions into normal striated ducts, possibly indicating regeneration of salivary gland tissue. (Unpublished data)

Five of the eight pSS patients without a MALT lymphoma received a second course of rituximab (after 9-11 months) due to recurrence of symptoms. Retreatment resulted in the same significant improvement of the salivary flow rate and subjective symptoms compared

Table 1 Adverse events after treatment with biological agents in SS.

	Agent/dose	Number of patients in trial (number treated with the agent)	Premedication/ concommitant immunosuppressive therapy	Infusion reaction	Ìnfections	Serum sickness	HACA / HAHA formation	Other
Anti-TNF-α monoclonal antibodies								
Steinfeld(14)	Infliximab Intravenous, 3 mg/kg	16 (16)	n.r. /no	1 (6%)	2 (13%) (respiratoy tract)	-	n.r.	-
Steinfeld(15)	Infliximab Intravenous, 3 mg/kg	10 (10)	n.r./no	4 (40%)	2 (20%) (enteritis, tonsillitis)	-	n.r.	-
Marriette(2)	Infliximab Intravenous, 5 mg/kg	103 (54)	n.r./continuation of hydroxychloroquine and corticosteroids (≤ 15 mg/day)	2 (4%)	2 (4%) (1 cutaneous, 1 respiraotry tract)	-	n.r.	2 (breast cancer, autoimmune hepatitis) †
Zandbelt(16)	Etanercept subcutaneously, 25 mg	15 (15)	n.r./pilocarpine at a constant dose	-	1 (7%) (parotitis)	-	n.r.	-
Sankar(17)	Etanercept subcutaneously, 25 mg	28 (14)	n.r./allowed to use long-term medication	1 (7%)	1 (7%) (skin lesion) ‡	-	n.r.	-
IFN-α								
Ship(18)	IFN-α oromucosal, 150 IU, 450 IU	109 (87)	n.r./no	n.a.	-	-	n.r.	- ¶
Cummins(7)	IFN-α oromucosal, 450 IU	497 (300)	n.r./no	n.a.	-	-	-	23 (7.7%) § (34% gastrointestinal, 25% musculoskeletal)
Anti-CD20								
Pijpe(8)	Rituximab Intravenous, 375 mg/m²	15 (15)	25 mg prednisolon intravenously/ patients with severe extraglandular manifestations (n=3) received immunosuppressive therapy	2 (13%)	1 (7%) (zoster)	4 (27%) #	4 (27%)	-
Devauchelle-Pensec(25)	Rituximab Intravenous, 375 mg/m²	16 (16)	n.r./no	-	-	1 (6%)	n.r.	-
Gottenberg(26)	Rituximab Intravenous, 375 mg/m²	6 (6)	n.r./ hydroxychloroquine (n=1), methylprednisolone (n=3)	1 (17%)	-	1 (17%)	n.r.	-
Seror(27)	Rituximab Intravenous, 375 mg/m²	12 (12)	n.r./ cyclophosphamide (n=1), hydroxychloroquine (n=1), leflunomide (n=1)	1 (8%)	-	2 (17%)	n.r.	-
Anti-CD22								
Steinfeld(29)	Epratuzumab Intravenous, 360 mg/m²	16 (16)	0.5-1 g acetominophen, 25-50 mg antihistamine./ no	2 (13%)	2 (13%) (sinusitis, dental abcess)	-	3 (19%)	6 (38%) (TIA, osteoporotic fracture, diarrhea, dyspepsia, palpitations, paresthesia)

to the results of the first treatment, together with a decrease in B cells and IgM-RF levels.

Six of the seven MALT/pSS patients were initially effectively treated with rituximab. The remaining MALT/pSS patient had progressive MALT disease and severe extraglandular SS disease within three months after the start of rituximab treatment. Cyclophosphamide was added, which led to stable disease of both MALT and SS. One of the six patients initially responding had a recurrence of MALT lymphoma after 9 months and was successfully retreated with rituximab. The other patients are still in remission. (Unpublished data)

In another open label study, 16 pSS patients received only two weekly rituximab infusions (375 mg/m^2), with a follow-up of 36 weeks. Again, treatment resulted in rapid complete depletion of peripheral B cells. At week 12, a significant improvement of VAS scores for fatigue and dryness was recorded, and at week 36, a significant improvement for VAS scores for global disease, fatigue, dry mouth, dry eyes and dry vagina, but also in the number of tender joint and tender point counts was seen.(25) Both in the study of Pijpe et al.(8) and the study of Devauchelle-Pensec et al.(25) patients with a short disease duration showed more improvement than patients with longer disease duration.

Two trials retrospectively evaluated the effect of rituximab (4 infusions of 375 mg/m^2) in 18 pSS patients (mean disease duration 10 years) with systemic features. Self-reported dryness improved in six patients (VAS scores not known for three patients, no improvement in the other nine patients). Both studies reported good efficacy of the treatment on systemic features.(26;27)

In conclusion, in phase II trials, it has been shown that rituximab seems to be effective for at least 6-9 months in pSS patients with active disease, improving both subjective and objective complaints. Retreatment with rituximab resulted in a similar good clinical response. In pSS patients with longer disease duration, without residual salivary gland function, rituximab treatment seems to be effective for systemic features. To confirm these promising results, randomized placebo-controlled clinical trials are needed.

Anti-CD22 monoclonal antibodies

Epratuzumab is a fully humanized monoclonal antibody specific for the B cell surface molecule CD22. CD22 is expressed on the surface of normal mature and malignant B lymphocytes. CD22 appears to be involved in the regulation of B cell activation through B cell receptor signaling and cell adhesion.(28) In an open label phase I/II study, safety and efficacy of epratuzumab were investigated in 16 pSS patients. Follow up was 6 months. These pSS patients received four doses of 360 mg/m^2 epratuzumab intravenously. Mean disease duration before therapy was 2.9 years, and none of the patients had received prior B cell-targeted therapy. Most improvements occurred in the Schirmer test, unstimulated

† 1 patient in the placebo group developed benign lymph node enlargement
‡ 1 patient in the placebo group developed a prolonged upper respiratory tract infection
¶ In this study there were mild adverse events, however there were no significant differences between the groups. Adverse events were not specified
§ 8 patients (4.1%) in the placebo group developed adverse events
# 1 of these 4 patients developed serum sickness after retreatment (*8*)
n.a. not applicable
n.r. not reported
HACA human anti-chimeric antibodies
HAHA human anti-human antibodies

whole salivary flow and the VAS score for fatigue. The new developed disease activity score consisted of the four domains: dryness of the eyes, dryness of the mouth, fatigue and laboratory parameters. Based on this score 53% achieved at least 20% improvement in at least two domains at 6 weeks. Corresponding rates for 10, 18, and 32 weeks are 53, 47 and 67%. Remarkably, the number of responders was higher 6 months after the treatment administration than earlier. Peripheral B cells decreased with a median decrease of 54 and 39% at 6 and 18 weeks, respectively.

In conclusion, epratuzumab seems to be an effective treatment. Randomized, placebo-controlled clinical trials are needed before epratuzumab can be advised for general treatment in pSS patients.(29)

Anti-BAFF

BAFF is a B cell-activating factor that acts as a positive regulator of B cell function and expansion. BAFF levels were found elevated in serum and saliva in SS patients, but no correlation could be shown between serum and saliva levels.(30) However, circulating levels of BAFF in pSS patients were shown to be a marker for disease activity.(11)

To the best of our knowledge, no trials have been performed with anti-BAFF treatment in SS yet, but such an approach might be considered for future trials. Currently, 2 human BAFF antagonists have been developed, a human antibody (anti-BLyS) that binds to soluble BAFF and a fusion protein of one of the BAFF receptors.(31;32) Especially SS patients with elevated BAFF levels, hypergammaglobulinemia, elevated levels of auto antibodies, and associated B cell lymphoma might be candidates for anti-BAFF treatment. (33)

Safety and tolerability of biological agents

The most important side effects of treatment with biological agents are direct mild infusion reactions. Several patients developed a serum sickness-like disease a few days after the second infusion that might be related to the formation of antibodies against the biological agent (human anti-chimeric antibodies (HACA's) or human anti-human antibodies). A few patients developed infections during treatment with a biological agent; however, some patients concomitantly used other immunosuppressive therapies. Therefore, the direct relation between the biological agent and the infection is unsure. All adverse events reported in the trials described in this review are reported in table 1. According to this table, the most frequent side effects of treatment with biological agents are mild infusion reactions. The most severe side effect of the various treatments used in SS patients was the development of a serum sickness-like disease. This adverse effect of treatment occurred in 16% (8 of 49) of the patients treated with rituximab. HACA formation was observed in patients developing a serum sickness-like disease and occurred only in patients receiving low-dose corticosteroids and no other immunosuppressive drugs. It is assumed that higher doses of corticosteroids during treatment might prevent the occurrence of serum sickness.

Future perspectives

Biological agents are promising therapies for SS. Randomized studies failed to show a clinical effect of anti-TNF-α and IFN-α in the treatment of SS. Notwithstanding the unfortunate

results of anti-TNF-α and IFN-α, B cell depletion (both anti-CD20 and anti-CD22) seems very promising. Again, this promising effect, as was previously also assumed for anti-TNF-α and IFN-α, must be confirmed in larger randomized controlled clinical trials.

HACA's have been reported to occur at a higher rate in patients with an autoimmune disease. It seems that monoclonal antibodies are more immunogenic in active autoimmune disease, independent of the type of disease. Additional use of immunosuppressive therapy in these patients might be mandatory to prevent serious side effects. These unwanted side effects might also be prevented by the use of fully humanized antibodies. The currently available humanized antibodies are promising, but need further study. Moreover, there is still a need for improved assessment parameters to monitor treatment effects, both subjectively and objectively. For studies on intervention of SS, evaluation of the parotid gland might be of use because function, composition of saliva and histology can be evaluated on the same gland at different time-points. Activity scores are currently under development by Bowman and Vitali.*(*34;35*)* Finally, as soon as effective intervention treatments have been established, the cost-effectiveness of these currently very expensive antibodies needs to be analyzed to select those patients that might benefit the most from this kind of treatment.

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Chapter 4a

Progression and treatment evaluation in diseases affecting salivary glands

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Abstract

A general overview of existing tools for evaluation of treatment for diseases affecting salivary glands is given. Assessments of salivary gland function (sialometry, sialochemistry) and histopathological examination of salivary gland biopsies provide powerful tools to diagnose diseases affecting the salivary glands, to assess disease progression and to evaluate treatment. More general tools are subjective questionnaires (e.g. visual analogue scale (VAS) scores, Multidimensional Fatigue Inventory (MFI) score and SF-36) and serological parameters.

Introduction

Many diseases and conditions can affect salivary glands resulting in a reduced or increased salivary flow. Treatment for these and other disorders can affect salivary secretion as well. Frequent causes of long-lasting reduced salivary flow are drugs, systemic conditions like Sjögren's syndrome (SS) and radiation injury to salivary gland tissue. The sensation of a dry mouth (xerostomia) is not always accompanied by a reduced salivary secretion (hypo-salivation). In about one third of the patients with xerostomia there is no good correlation between actual mouth dryness and level of salivary secretion. The discrepancy between salivary secretion status and level of complaints is even more striking in drooling. Usually, salivary secretion is normal or even reduced, but swallowing of saliva is impaired. Well-known causes of the inability to empty the mouth of saliva are an infantile swallowing pattern, a disturbed sensibility of the oral tissues and anatomic limitations due to trauma and ablative surgery. Thus, many factors have to be considered when selecting a salivary evaluation tool for the subset of patients or healthy subjects.

Notwithstanding the above, salivary research provides powerful tools to diagnose diseases affecting salivary glands, to assess disease progression, and to evaluate treatment. In progressive diseases like SS, salivary secretion generally diminishes with time. (figure 1) This progression is not so obvious when monitoring whole saliva, but becomes much clearer when measuring gland specific saliva.(1) While sialometry is a robust tool for evaluating disease progression, analysis of salivary composition (sialochemistry) differentiates between salivary gland diseases, and measures the disease activity (table 1)(2) and the effect of intervention treatment.(3) Additional tools are sialography (imaging of the extent of destruction of the ductal system), salivary scintigraphy (imaging of the glandular secretory activity), salivary gland biopsy (glandular pathology underlying the observed changes), and the imaging of anatomical structures with CT, MRI, or ultrasound.

The six above-mentioned variables (sialometry, sialochemistry, sialography, salivary scintigraphy, biopsy, and imaging) are gland-specific and measure disease progression and/or activity. Other essential information might come from the pattern of complaints, medical history, the clinical picture, serology and questionnaires. Serological parameters and subjective questionnaire responses can add important information on the disease progression and treatment outcome.

This chapter discusses the main tools for evaluation of disease progression and treatment including applications to clinical research and practice.

Tools to measure salivary gland function and disease activity

Sialometry

Saliva collection provides sound clinical information. Accurate measures of salivary flow rate and composition are essential for many diagnostic, therapeutic and research protocols. Saliva collection is a noninvasive tool of assessing a variety of disease characteristics and levels of certain drugs and hormones. Whole saliva is a mixture of not only salivary secretions, but also fluids, debris, and cells not originating in the salivary glands. Therefore, the analysis of individual gland saliva is usually a more reliable procedure for diagnosing diseases of the salivary glands than analysis of whole saliva. However, for certain diagnostic procedures whole saliva might be more useful, for example, when assessing specific roles of saliva in the oral cavity or when whole saliva is used as a diagnostic fluid for conditions relying on leakage of serum products or gingival crevicular fluid into saliva.

In healthy subjects and patients in whom both glands are affected simultaneously (e.g. SS) flow rates of the left and right parotid gland are similar. Therefore, sorting out discrepancies between the observed flow of the left and right parotid gland assures the reliability of the samples collected. This is a very powerful internal control of the reliability of the saliva sample collected and outweighs the effect of repeated sampling of a parotid gland to get a reliable baseline sample. Increasing the number of collections has been shown to have a negligible effect on the reliability of baseline parotid flow rates for clinical trials. Consequently, one reliable baseline sample is sufficient for clinical studies evaluating the progression of disease or the effect of a therapy.(4) Moreover, salivary flow rates are not constant and exhibit a considerable amount of variability. Therefore, salivary collections should be performed under well-defined conditions and, for repeated collections, at the same time of the day to minimize intrapatient variability. Nevertheless, even if the circadian rhythm is ruled out and the samples are indeed collected under well-defined conditions, the measured increase or decrease of salivary flow has to exceed about one-quarter to one-third of the parotid flow rate at baseline before an observed effect related to a given therapy can be assessed as a 'real' effect in an individual patient. This information is additional to subjective assessments of such an effect.(4)

Sialochemistry

Saliva is an attractive diagnostic fluid because salivary testing provides several key advantages including low cost, noninvasiveness, and easy sample collection and processing. Human saliva collection is less invasive than phlebotomy and is clinically relevant because many, if not all, blood components are reflected in saliva. Amongst others, sodium, potassium, chloride, calcium, phosphate, urea, total protein and a number of enzymes (e.g., amylase, lysozyme and lactoferin) can be detected in saliva and have diagnostic potential. (table 1) In addition, a large range of more or less disease-related changes in protein composition of saliva have been reported. A new method to assess the protein composition in health and disease is salivary proteonomics - the identification of the entire spectrum of proteins in human saliva. Saliva also harbours diagnostic RNA biomarkers (detection of RNA biomarkers).

Sialography

Through retrograde infusion of oil- or water-based iodine contrast, the architecture of the salivary duct system is visualized radiographically. It is a low morbidity, well-accepted technique. Sialography should not, however, be performed in patients with a history of iodine allergy. The sialographic procedure can be performed in 10 -15 minutes.

Inflammation appears on sialograms as diffuse collections of contrast fluid at the terminal acini of the ductal tree. This condition, known as sialectasia can be classified into punctate (less than 1 mm), globular (uniform and 1-2 mm), cavitary (coalescent and >2 mm) and destructive (normal ductal structures are no longer visible). Sialectasia is thought to result from progressive acinar atrophy and dilatation, which, in turn, is caused by increasing intraluminal pressure resulting from the presence of periductal lymphocytic infiltrates with secondary duct narrowing. So, these four grades of sialectasia are thought to represent increasing glandular damage, caused by chronic salivary gland inflammation.

Figure 1

Relationship between disease duration (time from first complaints induced by or related to oral dryness until referral) and mean (SEM) salivary flow rates in patients with (A) primary SS(pSS) and in those with (B) secondary SS(sSS). Normal values are derived from historic controls (n=36). SM/SL, submandibular/sublingual glands; UWS, unstimulated whole saliva. *Significant difference versus patients with early-onset SS(< 1 year oral complaints; p<0.005) by the Mann-Whitney U test. †Significant difference versus patients with early-onset SS(p<0.05) by the Mann-Whitney U test (Pijpe et al. (1), reprinted with permission).

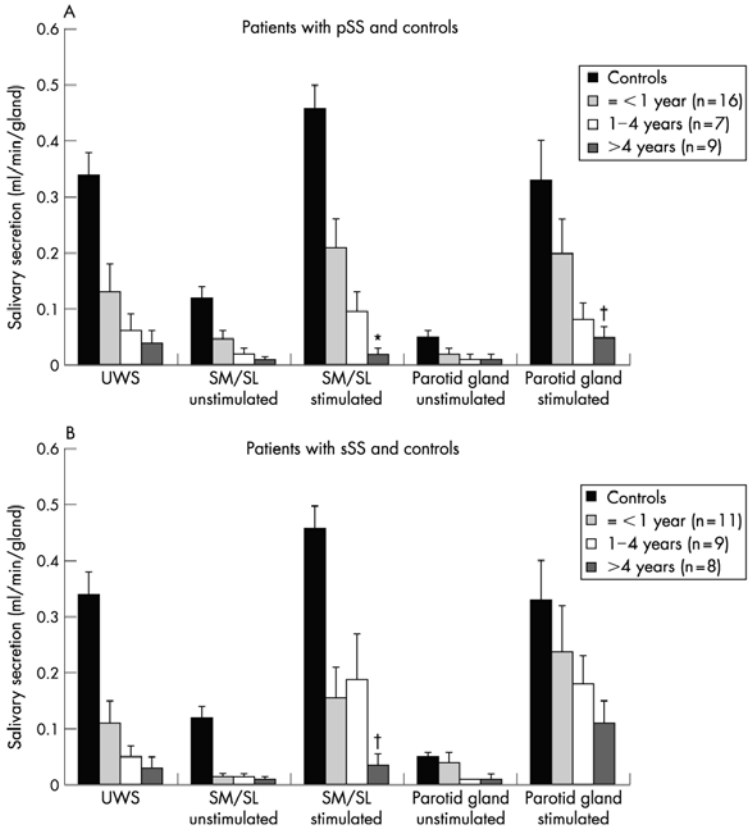


Table 1 Salivary gland parameters and clinical data of some disorders affecting the salivary glands (Van den Berg et al., 2007) (2). SS is an autoimmune disorder affecting the exocrine glands including the salivary glands. Sialosis is a salivary condition characterized by persistent swelling of the parotid glands related to a metabolic disorder as diabetes, alcohol abuse, anorexia and bulimia. Sodium retention syndrome is characterized by mostly unilateral, incidental, short-lasting (hours) swelling of the parotid gland often related to cardiovascular disorders (hypotension, hypertension).

	SS (pSS/sSS)	Sialosis	Sodium retention syndrome	Medication induced xerostomia
Sialometry	UWS ≤1.5 ml in 15 min	Normal, increased or decreased	Normal or decreased	UWS decreased; SWS (sub) normal
Sialochemisty	Na and Cl increased	K increased	Na decreased	Normal
Sialography	Sialectasia	Thin duct system, enlarged gland	Usually normal, but a thin duct system and enlarged gland may be present	Normal
Complaints	Mouth dryness in rest and during eating or speaking Need for drinks to swallow (dry) food Eye dryness Swelling of the salivary glands	Persistent, bilateral swelling of the parotid glands	Often mouth dryness. Recurrent, short lasting (usually at most some hours), mostly unilateral swellings of the parotid gland	Mouth dryness in rest
Schirmer's test	≤5mm/5min	Unknown, but reduction is not uncommon	Unknown, but reduction is not uncommon	Unknown, but reduction is not uncommon
Associated Diseases	sSS: associated with another connective tissue/auto-immune disease	Endocrine disorder Metabolic disorder Dysfunction ANS	Cardiovascular disease Disorder of the fluid or electrolyte balance	Use of xerogenic medication

SS: Sjögren's syndrome; pSS: primary Sjögren's syndrome; sSS: secondary Sjögren's syndrome; UWS: unstimulated whole saliva; SWS: stimulated whole saliva; SM/SL: saliva from sublingual/-mandibular gland; ANS: autonomic nervous system.

Salivary scintigraphy

Salivary scintigraphy is based on the ability of parotid and submandibular glands to trap the radionuclide isotope technetium-sodium (Tc99m) pertechnetate. This ability is due to the fact that Tc99m substitutes for chloride in the active sodium/potassium/chloride cotransport in the striated ducts. After intravenous injection of Tc99m, scintigraphy may reveal functional abnormality of the salivary glands through photographically recording with a gamma scintillation camera, the radiation from salivary isotope accumulation and excretion.

Improvements of salivary scintigraphy include salivary single-photon emission computed tomography (SPECT) and human immunoglobulin G (HIG) scintigraphy. Salivary SPECT creates a three-dimensional image with a rotating gamma camera without marking an ROI (region of interest) as it uses a single pixel as the ultimate ROI.

Scintigraphy is a valuable tool to measure activity of the glands, and it can be performed in the same gland at different time periods to assess progression. Unfortunately, the diagnostic accuracy is low.

Computer tomography and magnetic resonance imaging

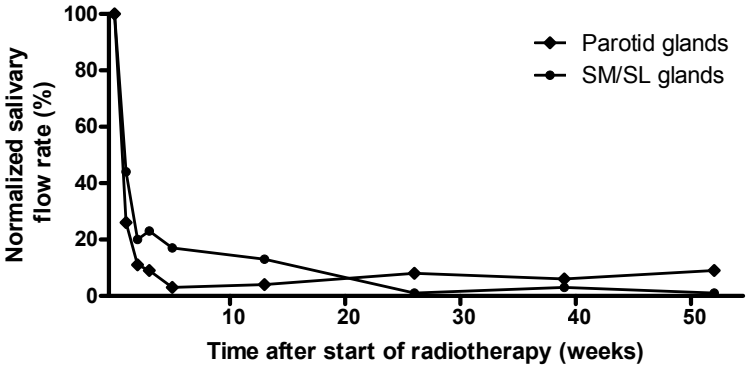
Magnetic resonance imaging (MRI) depicts more accurately because soft tissue contrast resolution is better in MRI than computer tomography (CT). Detailed knowledge of the anatomy of the parotid gland and surrounding structures is necessary for evaluating and diagnosing lesions. Bilateral imaging and comparison between right and left glands is essential. CT and MRI are of less value as diagnostic tools for salivary gland disorders as Sjögren's syndrome, sialadenosis and bacterial or viral sialadenitis.

Ultrasound

Ultrasound has no known contraindications and is a quick and well-accepted, non-invasive procedure. With color Doppler sonography, the complex vascular anatomy can be

Figure 2

Flow rate of parotid and submandibular/sublingual saliva (SM/SL) as a function of time after start of radiotherapy (conventional fractionation schedule, 2Gy per day, 5 days per week, total dose 60-70 GY). The parotid, submandibular and sublingual glands are located in the treatment portal. Initial flow rates were set to 100% (Adapted from Burlage et al. (12)).



accurately recorded.(5) Its potential in routine salivary diagnostics is restricted as tissue penetration depth is limited and proper interpretation of salivary sonograms requires a great deal of experience.

Histopathology

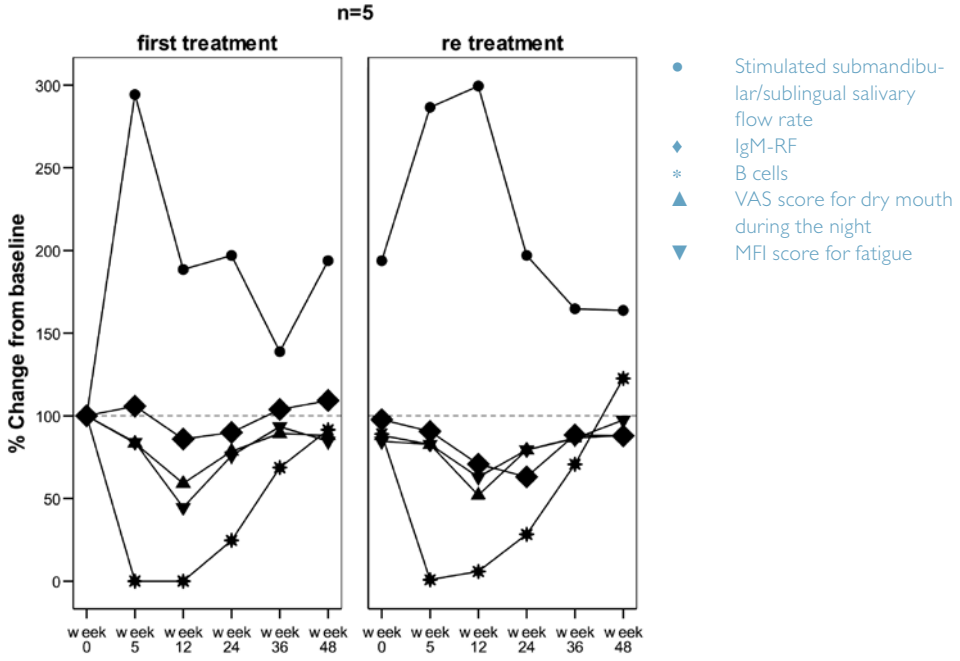
The labial and parotid glands are accessible for histopathological evaluation, and biopsies from these glands are often performed routinely. In SS, a disease affecting the salivary glands in which biopsies most often are taken as a routine procedure, the parotid and labial gland biopsies are diagnostically comparable. However, a parotid biopsy is preferred, due to lower morbidity than labial biopsies in which sensory loss may occur, easier access to larger tissue samples, and earlier detection of lymphomas.(6) In addition, repeated biopsies can be taken from the same parotid gland, making parotid biopsies an important tool in treatment evaluation (the outcomes can even be compared with saliva samples obtained from the same gland).

Cytology

A cytological puncture (ultrasound guided) can distinguish salivary gland disorders from lymph nodes disorders, and inflammation from malignancy.

Figure 3

Increase and decrease (mean values of 5 patients) in stimulated submandibular/sublingual flow rate, IgM-RF, B cells, VAS score for dry mouth during the night and multidimensional fatigue (MFI) score for fatigue following rituximab (re)treatment (baseline is 100%). Baseline values (week 0 first treatment) were stimulated submandibular/sublingual flow rate 0.09 ml/min (SD 0.07), IgM-RF 339 (SD 329), B cells 0.19 109/l (SD 0.09), VAS score for dry mouth during the night 85 (SD 12), MFI score for fatigue 16 (SD 3). (modified after Meijer et al.(13)).



Subjective evaluation

VAS

A Visual Analogue Scale (VAS) is a line of, for example, 100 mm on which the patient can mark the severity of the complaint. For SS, VAS scores are available for oral dryness, oral dryness during the day, oral dryness at night, difficulty swallowing *dry* food without any additional liquids, difficulty swallowing *any* food without any additional liquids, difficulty speaking without drinking liquids, dry eyes (sensation of sand or gravel in the eyes).

MFI

The Multidimensional Fatigue Index (MFI) is a 20-item self-report instrument designed to objectively measure fatigue, including the dimensions of general fatigue, physical fatigue, mental fatigue, reduced motivation and reduced activity. This validated questionnaire detects expected differences in fatigue between groups, within groups and between conditions. (7) A higher score (range 4-20) indicates a higher level of fatigue. Fatigue is a complaint not uncommon to patients suffering from salivary gland disorders, particularly patients with salivary gland disorders related to an autoimmune disease or as a result of cancer treatment.

SF-36

The 36-item short form (SF-36) is constructed to survey health status and was designed for use in clinical practice and research, health policy evaluations and, general population surveys. The SF-36 includes one multi-item scale that assesses eight health concepts. The questionnaire has been developed for self-administration by persons 14 years of age and older or for administration by a trained interviewer. A higher score indicates a higher level of well-being.(8) Health status can severely be impaired in patients suffering from salivary gland disorders particularly in patients with salivary gland disorders related to an autoimmune disease or as a result of cancer treatment.

Dry mouth questionnaires

Objective salivary gland function is not always consistent with the subjective perception. Whether the patient reports sipping liquids to aid in swallowing dry foods, dry mouth when eating a meal, or difficulties swallowing any foods is highly predictive of salivary gland function and, therefore, clinically useful in patients who report oral dryness.(9)

Serological parameters

In systemic diseases affecting the salivary glands, serological parameters can be useful in evaluating activity and progression of the disease and in evaluating treatment. For example, C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) are general parameters in peripheral blood for inflammation and are elevated in most autoimmune diseases. IgM-Rf (rheumatoid factor) and IgA correlate with B cell activity and are elevated in SS. IgM-Rf is also elevated in patients with rheumatoid arthritis and some other conditions. Antinuclear antibodies (ANA) and anti-Ro/SSA and anti-La/SSB can be detected in SS (anti-SSB is the most specific antibody).

Application of these tools in clinical research and clinical practice

The clinical application of the above-mentioned variables in treatment evaluation will be illustrated for patients with a reduced salivary flow due to head and neck radiotherapy and SS.

Radiotherapy

Xerostomia is a common and disturbing side effect of head and neck radiotherapy, leading to considerable morbidity, including severe oral discomfort, problems with speaking, dysphagia, and an increased incidence of caries and mucosal infections. Although new radiation techniques enabled significant sparing of the parotid glands, the amount of normal salivary gland tissue irradiated may still be substantial resulting in clinically relevant radiation-induced xerostomia. Therefore, protection against radiation-induced salivary gland damage may further improve the therapeutic gain.(10;11)

Although salivary gland tissue is a well-differentiated tissue and, theoretically, should be relatively radioresistant, studies have shown a rapid decline in parotid and submandibular/sublingual salivary flow, even after low doses of radiotherapy (figure 2). In humans, it has been reported that the TD_{50} (i.e., the dose to the whole organ leading to a complication probability of 50%) for parotid glands varies from 28.4 Gy to 31 Gy at 6 weeks increasing to 39 Gy at 1 year after completion of radiotherapy.

Sjögren's syndrome

SS is a chronic lymphoproliferative autoimmune disease with disturbances of T lymphocytes, B lymphocytes and exocrine glandular cells. SS can be primary (pSS) or secondary (sSS), the latter being associated with another autoimmune disease (e.g. rheumatoid arthritis and systemic lupus erythematosus). The main symptoms of SS are xerostomia, dry eyes (keratoconjunctivitis sicca), increased caries activity (exocrine glands) and fatigue and arthralgia (systemic features). The disease can have a great impact on the quality of life of the patients. There are no causal treatment options, and treatment used today is mainly symptomatic. Dry eyes are treated with eyedrops or gel, and sometimes anti-inflammatory or immunosuppressive medication is indicated. Dry mouth is treated with saliva-stimulating medication (pilocarpine) or with saliva substitutes. Currently, drug trials are evaluating biological agents with promising first results. (figure 3)

Sialometry and sialochemistry

Salivary flow rates have diagnostic and prognostic value in SS. Since the amount and composition of saliva reflects the effects of the autoimmune process in the salivary glands, analysis of saliva may also be valuable in diagnosis, prognosis and evaluation of treatment. SS is characterized by a high sodium and high chloride concentration and a low phosphate concentration in parotid gland saliva.

Sialometry and sialochemistry, easily performed and tolerated, are valuable in measuring disease progression (figure 1) and treatment outcome. For example, rituximab significantly increased salivary secretion (figure 3) and nearly normalized salivary sodium concentration.

A pilot study of ten SS patients and ten age- and sex-matched controls demonstrated that pSS patients' saliva contains proteomic and genomic diagnostic biomarker candidates. Proteonomics of saliva may also be useful in diagnosis, disease progression, and treatment evaluation, but further research is necessary to precisely assess its value.

Histopathology

In SS, widely accepted criteria for histologic confirmation is focal lymphocytic sialoadenitis in labial salivary glands and lymphoepithelial lesions in parotid salivary glands.

Moreover, repeated salivary gland biopsies might offer an objective method for evaluating treatment, in addition to serological and functional parameters. The parotid gland is the primary site to study changes after systemic therapy since SS lymphoproliferation occurs especially in these glands. Repeated parotid biopsies in SS patients treated with rituximab show redifferentation of lymphoepithelial lesions into regular ducts, which is in line with the sialochemical changes in parotid saliva.

Subjective evaluation

Fatigue is one of the most disabling complaints in SS, and it leads to a substantial decrease in health related quality of life. By using the MFI, patients with pSS reported more fatigue than healthy controls on all the dimensions of the MFI, and when controlling for depression significant differences remain on the dimensions of general fatigue, physical fatigue, and reduced activity. VAS scores have been used to assess subjective sicca complaints and have been validated for patients with xerostomia. After rituximab treatment, in patients with early pSS, assessment of mouth dryness, arthralgia, physical functioning, vitality and most domains of the MFI significantly improved.(3)

Serological parameters

Polyclonal expansion and secretory hyperactivity of B cells is an early event in pSS. This is demonstrated in the blood by increased amounts of different autoantibodies and by increased amounts of total Ig (primarily IgG). The more serious systemic complications occur mainly in patients with increased IgM-Rf levels, and levels of circulating IgM-Rf correlate positively with the number of extraglandular disease manifestations. Other researchers also reported an association between a high B cell autoreactivity (production of ANA, anti-Ro/SSA and anti-La/SSB) and the development of complications or more severe manifestations like neuropathy, kidney and pulmonary involvement. Rituximab treatment resulted in pSS patients in a rapid decrease in peripheral B cells, accompanied by a decrease in IgM-Rf levels. (figure 3)

Conclusion

Salivary research provides powerful tools to diagnose diseases affecting the salivary glands, to assess disease progression and to evaluate treatment. Important gland-specific parameters are sialometry, sialochemistry, and histopathology. More general tools are subjective questionnaires (e.g. VAS, MFI. SF-36) and serological parameters.

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Chapter 4b

Salivary proteomic and genomic biomarkers for primary Sjögren's syndrome

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Abstract

Objective To identify a panel of protein and messenger RNA (mRNA) biomarkers in human whole saliva (WS) that may be used in the detection of primary Sjögren's syndrome (pSS).

Methods Mass spectrometry and expression microarray profiling were used to identify candidate protein and mRNA biomarkers of pSS in WS samples. Validation of the discovered mRNA and protein biomarkers was also demonstrated using real-time quantitative polymerase chain reaction and immunoblotting techniques.

Results Sixteen WS proteins were found to be down-regulated and 25 WS proteins were found to be up-regulated in pSS patients compared with matched healthy control subjects. These proteins reflected the damage of glandular cells and inflammation of the oral cavity system in patients with pSS. In addition, 16 WS peptides (10 up-regulated and 6 downregulated in pSS) were found at significantly different levels ($p < 0.05$) in pSS patients and controls. Using stringent criteria (3-fold change; $p < 0.0005$), 27 mRNA in saliva samples were found to be significantly up-regulated in the pSS patients. Strikingly, 19 of 27 genes that were found to be overexpressed were interferon-inducible or were related to lymphocyte filtration and antigen presentation known to be involved in the pathogenesis of pSS.

Conclusion Our preliminary study has indicated that WS from patients with pSS contains molecular signatures that reflect damaged glandular cells and an activated immune response in this autoimmune disease. These candidate proteomic and genomic biomarkers may improve the clinical detection of pSS once they have been further validated. We also found that WS contains more informative proteins, peptides, and mRNA, as compared with gland-specific saliva, that can be used in generating candidate biomarkers for the detection of pSS.

Introduction

Sjögren's syndrome (SS), which was first described in 1933 by the Swedish physician Henrik Sjögren (1), is a chronic autoimmune disorder clinically characterized by a dry mouth (xerostomia) and dry eyes (keratoconjunctivitis sicca). The disease primarily affects women, with a ratio of 9:1 over the occurrence in men. While SS affects up to 4 million Americans, about half of the cases are primary SS (pSS). pSS occurs alone, whereas secondary SS presents in connection with another autoimmune disease, such as rheumatoid arthritis or systemic lupus erythematosus (SLE). Histologically, SS is characterized by infiltration of exocrine gland tissues by predominantly CD4 T lymphocytes. At the molecular level, glandular epithelial cells express high levels of HLA-DR, which has led to the speculation that these cells are presenting antigen (viral antigen or autoantigen) to the invading T cells. Cytokine production follows, with interferon (IFN) and interleukin-2 (IL-2) being especially important. There is also evidence of B cell activation with autoantibody production and an increase in B cell malignancy. SS patients exhibit a 40-fold increased risk of developing lymphoma.

SS is a complex disease that can go undiagnosed for several months to years. Although the underlying immune-mediated glandular destruction is thought to develop slowly over several years, a long delay from the start of symptoms to the final diagnosis has been frequently reported. SS presumably involves the interplay of genetic and environmental factors. To date, few of these factors are well understood. As a result, there is a lack of early diagnostic markers, and diagnosis usually lags symptom onset by years. A new international consensus for the diagnosis of SS requires objective signs and symptoms of dryness, including a characteristic appearance of a biopsy sample from a minor or major salivary gland and/or the presence of autoantibody such as anti-SSA.(2-4) However, establishing the diagnosis of pSS has been difficult in light of its nonspecific symptoms (dry eyes and mouth) and the lack of both sensitive and specific biomarkers, either body fluid- or tissue-based, for its detection. It is widely believed that developing molecular biomarkers for the early diagnosis of pSS will improve the application of systematic therapies and the setting of criteria with which to monitor therapies and assess prognosis (e.g., lymphoma development).

Saliva is the product of 3 pairs of major salivary glands (the parotid, submandibular, and sublingual glands) and multiple minor salivary glands that lie beneath the oral mucosa. Human saliva contains many informative proteins that can be used for the detection of diseases. Saliva is an attractive diagnostic fluid because testing of saliva provides several key advantages, including low cost, noninvasiveness, and easy sample collection and processing. This biologic fluid has been used for the survey of general health and for the diagnosis of diseases in humans, such as human immunodeficiency virus, periodontal diseases, and autoimmune diseases.(5-8) Our laboratory is active in the comprehensive analysis of the saliva proteome (for more information, see www.hspp.ucla.edu), thus providing the technologies and expertise to contrast proteomic constituents in pSS with those in control saliva.(9-11) Thus far, we have identified over 1,000 proteins in whole saliva (WS). In addition, we have recently identified and cataloged ~3,000 messenger RNAs (mRNA) in human WS.(12) These studies have provided a solid foundation for the discovery of biomarkers in the saliva of patients with pSS. We have previously demonstrated proteome- and genome-wide approaches to harnessing saliva protein and mRNA signatures for the detection of oral cancer in humans.(13,14)

There have been continuous efforts in the search for biomarkers in human serum or saliva for the diagnosis of pSS. Some gene products were found at elevated levels in SS

patient sera or saliva, including β_2-microglobulin (β_2m), soluble IL-2 receptor, IL-6, anti-Ro/SSA, anti-La/SSB, and anti-α-fodrin autoantibodies.(15-20) However, none of them individually is sensitive or specific enough to use for the confirmative diagnosis of SS.(15) Therefore, it is crucial to use emerging proteome- and genome-wide approaches to discover a wide spectrum of informative and discriminatory biomarkers that can be combined to improve the sensitivity and specificity for the detection of pSS.

Patients and methods

Patient cohort

Because sample quality is critical for clinical proteomics studies, a standardized procedure, in strict accordance with the American-European Consensus Group Criteria for SS (2), was used for the identification and recruitment of pSS patients for this study. A diagnostic evaluation of SS was performed in all patients and included assessments of subjective complaints of oral and ocular dryness, sialometry (unstimulated WS), sialography, histopathology of salivary gland tissue, serology (SSA and SSB antibodies), eye tests (rose bengal staining and Schirmer's test) according to the American-European classification criteria for SS (2), and screening for extraglandular manifestations. Three of the pSS patients were being treated with hydroxychloroquine, and 1 patient was being treated with prednisolone. Eight patients had a focus score of >1 on examination of parotid gland biopsy tissue.

The enrolled pSS patients and healthy control subjects were well matched for age, sex, and ethnicity. The mean ±SD age was 37.2±9.8 years in the pSS patients (n=10) and 37.0± 10.6 years in the healthy control subjects (n=10). All subjects enrolled in this study were Caucasian women, since pSS mainly affects women. All of the enrolled control subjects were negative for serum anti-SSA/SSB antibodies, and none of them reported any sicca symptoms, including oral and ocular dryness.

Saliva sample collection

Samples of WS and saliva from the parotid and submandibular/sublingual glands were collected from each pSS patient and control subject for comparative analysis. Saliva sample collection was performed at the University Medical Center Groningen, using our standardized saliva collection protocols. Subjects were asked to refrain from eating, drinking, smoking, or performing oral hygiene procedures for at least 1 hour prior to the collection. Samples were collected in the morning, at least 2 hours after eating and rinsing the mouth with water, according to established protocols.(21,22) WS was stimulated by chewing paraffin and was collected over a period of 15 minutes. Glandular saliva specimens from individual parotid glands and, simultaneously, from the submandibular/sublingual glands were collected into Lashley cups (placed over the orifices of the Stenson's duct) and by syringe aspiration (from the orifices of the Warton's duct, located anteriorly in the floor of the mouth), respectively.

After collection, the saliva samples were immediately mixed with protease inhibitors (Sigma, St. Louis, MO) to ensure preservation of the integrity of the proteins and then centrifuged at 2,600g for 15 minutes at 4°C. The supernatant was removed from the pellet, immediately aliquoted, and stored at –80°C. All samples were kept on ice during the process. Two patients who had very low submandibular/sublingual gland salivary flow rates (0.03 ml/ minute) did not produce enough submandibular/sublingual gland saliva for this study.

Sample preparation for proteomic analysis

The saliva samples were precipitated overnight at –20°C with cold ethanol. Following centrifugation at 14,000g for 20 minutes, the supernatants were collected and dried with a speed vacuum for use in the peptide biomarker study. The pellet was then washed once with cold ethanol and collected for assay of total protein using a 2-D Quant kit (Amersham, Piscataway, NJ). We pooled saliva samples according to the total protein content from all patients with pSS and those from all control subjects. However, both the patients and controls were analyzed individually for the peptide profiling experiment.

Matrix-assisted laser desorption ionization–time-of- flight mass spectrometry (MALDI-TOF-MS)

Profiling of saliva peptides in 10 pSS patients and 10 matched control subjects was performed using a MALDI-TOF-MS system (Applied Biosystems, Foster City, CA). The peptide fraction from each patient (n=10) and control (n=10) sample was dissolved in 10 µl of 50% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA). The sample was mixed with α-cyano-4-hydroxycinnamic acid (10 mg/ml in 50% ACN/0.1% TFA) at a ratio of 1:2, and 1 µl of the mixture was spotted on the MALDI plate for measurement. Three measurements were performed for each sample, and the signals were averaged for subsequent data analysis.

In order to achieve an accurate comparison of the MALDI-TOF-MS data between the patient and control groups, baseline correction and Gaussian smoothing were initially performed to eliminate broad artifacts and noise spikes. Afterward, peak alignment was undertaken to ensure accurate alignment of the mass/charge (m/z) values across the set of spectra, and peak normalization was performed against the total peak intensity. These steps ensured comparability of the MALDI-TOF-MS spectra among all subjects. Subsequent statistical analysis (t-test) was used to reveal peptides that were present at significantly different levels in the pSS patients as compared with the control subjects.

Two-dimensional gel electrophoresis

Saliva samples from the 10 pSS patients and from the 10 control subjects were equally pooled according to the total protein content and then precipitated using the same procedures described above. The pellet was washed once with cold ethanol and then resuspended in rehydration buffer. A total of 100 µg of proteins was loaded onto each gel for the 2-D gel separation procedure. Isoelectric focusing was performed using immobilized pH gradient strips (11 cm long, with an isoelectric point [pI] of 3-10 nonlinear) on a Protean isoelectric focusing cell (Bio-Rad, Hercules, CA), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed in 8-16% precast Criterion gels on a Criterion Dodeca Cell (Bio-Rad). Fluorescent SYPRO Ruby stain (Invitrogen, Carlsbad, CA) was used to visualize the protein spots.

The gel images were acquired and analyzed using PDQuest software (Bio-Rad). The images were initially processed through transformation, filtering, automated spot detection, normalization, and matching. The 2-D gel image was transformed to adjust the intensity of the protein spot and filtered to remove small noise features without affecting the protein spot. The images were then normalized based on the total density of the gel image. The 2-D gel images of the pSS patients (master gel) and the control subjects were used as a "match set" for automated detection of the protein spots on the gel. Within the match set, the detected spots were reviewed manually, and the relative protein levels in the patient sample compared with the control sample were summarized.

Table 1 Salivary proteins differentially expressed between pSS patients and healthy control subjects, as identified by LC-MS/MS and Mascot database searching*

Spot No.	Accession	Protein name	Mascot score	Peptide matched	PI	Mt	Ratio (pSS/ctrl)
1	IPI00295105	Carbonic anhydrase VI	163	4	6.65	35343	0.22
2	IPI00295105	Carbonic anhydrase VI	114	5	6.65	35343	0.35
3	IPI00295105	Carbonic anhydrase VI	78	2	6.65	35343	0.29
4	IPI00004573	Polymeric-immunoglobulin receptor	235	5	5.58	83262	0.48
5	IPI00004573	Polymeric-immunoglobulin receptor	293	7	5.58	83262	0.39
6	IPI00004573	Polymeric-immunoglobulin receptor	182	4	5.58	83262	0.56
7	IPI00019038	Lysozyme C	103	2	9.38	16526	0.21
8	IPI00022974	Prolactin-inducible protein	147	3	8.26	16562	0.52
9	IPI00009650	Von Ebner's gland protein	239	4	5.39	19238	0.32
10	IPI00032293	Cystatin C	153	3	9.0	15789	0.43
11	IPI00013382	Cystatin SN	152	3	6.82	16361	0.46
12	IPI00013382	Cystatin SN	130	3	6.82	16361	0.61
13	IPI00002851	Cystatin D	50	1	6.70	16070	0.56
14	IPI00032294 IPI00013382	Cystatin S Cystatin SA	166 208	3 4	4.95 4.85	16 214 16 445	0.65
15	IPI00007047	Calgranulin A	104	2	6.51	10828	0.53
16	IPI00007047	Calgranulin A	79	2	6.51	10828	Absent in pSS
17	IPI00027462	Calgranulin B	126	4	5.71	13234	1.05
18	IPI00219806	Psoriasin	133	4	6.28	11464	1.44
19	IPI00410714	Hemoglobin alpha-1 globin chain	157	5	7.96	15292	Absent in control
20	IPI00218816	Hemoglobin beta chain	48	1	6.75	15988	2.73
21	IPI00218816	Hemoglobin beta chain	51	1	6.75	15988	7.58
22	IPI00007797	Fatty acid-binding protein, epidermal	211	6	6.60	15155	3.21
23	IPI00472762 IPI00472610 IPI00430840	IGHG1 protein Hypothetical protein Ig gamma-1 chain C region	333 363 333	14 14 14	8.33 7.50 7.48	50822 52633 54866	22.64
24	IPI00472610 IPI00550718	IGHM protein Ig gamma-1 chain C region	260 257	11 11	7.50 8.46	53270 53331	Absent in control
25	IPI00465248	Alpha-enolase	409	12	6.99	47139	4.37
26	IPI00300786	Salivary alpha-amylase, fragment	241	5	5.73	57731	3.41
27	IPI00300786	Salivary alpha-amylase, fragment	230	4	5.73	57731	2.19
28	IPI00300786	Salivary alpha-amylase, fragment	375	7	5.73	57731	31.53
29	IPI00300786	Salivary alpha-amylase, fragment	260	5	5.73	57731	2.57
30	IPI00300786	Salivary alpha-amylase, fragment	171	5	5.73	57731	2.50

Table 1 continued

Spot No.	Accession	Protein name	Mascot score	Peptide matched	PI	Mt	Ratio (pSS/ctrl)
31	IPI00300786	Salivary alpha-amylase, fragment	194	4	5.73	57731	11.92
32	IPI00300786	Salivary alpha-amylase, fragment	149	4	5.73	57731	1.57
33	IPI00300786	Salivary alpha-amylase, fragment	148	4	5.73	57731	4.03
34	IPI00549682	Fructose-bisphosphate aldolase A	218	4	8.75	52306	2.59
35	IPI00332161	Ig gamma-1 chain C region	138	5	8.46	36083	2.54
36	IPI00215983	Carbonic anhydrase I	119	4	6.59	28852	7.4
37	IPI00218414	Carbonic anhydrase II	98	2	8.67	31337	2.11
38	IPI00013885	Caspase-14	172	5	5.44	27662	3.32
39	IPI00419424	Ig kappa chain C region	263	7	5.82	27313	1.79
40	IPI00004656	Beta-2-microglobulin	62	2	6.06	13706	2.21
41	IPI00021439	Actin	461	11	5.29	41710	3.18
42	IPI00022434	Serum albumin, fragment	492	10	5.41	69321	Absent in control

* Liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS) analysis and Mascot database searching were performed to identify the proteins. Shown are the theoretical isoelectric point and molecular mass of the protein precursors, as well as the ratio of protein levels in patients with primary Sjögren's syndrome (SS) and matched control subjects, as detected by 2-dimensional gel electrophoresis.

Liquid chromatography tandem mass spectrometry (LC-MS/MS) and database searching

Protein spots showing differential protein levels were excised by a spot-excision robot (Proteome Works; Bio-Rad) and deposited into 96-well plates. Proteins in each gel spot were reduced with dithiothreitol, alkylated with iodoacetamide, and then digested overnight at 37°C with 10 ng of trypsin. After digestion, the peptides were extracted and stored at -80°C prior to LC-MS/MS analysis.

LC-MS/MS analysis of peptides was performed using an LC Packings Nano-LC system (Dionex, Sunnyvale, CA) with a nanoelectrospray interface (Protana, Odense, Denmark) and a quadrupole time-of-flight (Q-TOF) mass spectrometer (QSTAR XL; Applied Biosystems). A New Objective PicoTip tip (internal diameter 8 mm; New Objective, Woburn, MA) was used for spraying, with the voltage set at 1,850V for online MS and MS/MS analyses. The samples were first loaded onto an LC Packings PepMap C18 precolumn (300 µm x 1 mm; particle size 5 µm) and then injected onto an LC Packings PepMap C18 column (75 µm x 150 mm; particle size 5 µm) (both from Dionex) for nano-LC separation at a flow rate of 250 nl/minute. The eluents used for LC were 1) 0.1% formic acid and 2) 95% ACN/0.1% formic acid, and a 1%/minute gradient was used for the separation.

The acquired MS/MS data were searched against the International Protein Index (IPI) human protein database (available at http://www.ebi.ac.uk/IPI/IPIhelp.html) using the Mascot (Matrix Science, Boston, MA) database search engine. Positive protein identification was based on standard Mascot criteria for statistical analysis of LC-MS/MS data.

Immunoblotting

Western blot analysis of α-enolase was performed on the same set of saliva samples (10 pSS and 10 control samples). Proteins were separated on 12% NuPAGE gels (Invitrogen) at 150V and then transferred to a polyvinylidene difluoride membrane (Bio-Rad) using an Invitrogen blot transfer cell. After saturating with 5% milk in Tris buffered saline-Tween buffer (overnight at 4°C), the blots were sequentially incubated for 2 hours at room temperature with polyclonal goat α-enolase primary antibody and horseradish peroxidase–conjugated anti-goat IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The bands were detected by enhanced chemiluminescence (Amersham) and quantified using Quantity One software (Bio-Rad).

Profiling of salivary mRNA by high-density oligonucleotide microarray analysis

Samples of stimulated parotid gland saliva or WS from 10 pSS patients and 8 matched controls were preserved in RNAlater reagent (Qiagen, Valencia, CA) at a 1:1 ratio and then frozen at –80°C. Total salivary RNA was isolated from 560 µl of RNAlater-preserved saliva (280 µl of parotid gland saliva/WS and 280 µl of RNAlater) using a viral RNA mini kit (Qiagen) as described previously (12). Isolated total RNA was treated with 2 rounds of recombinant DNase I (Ambion, Austin, TX) digestion, and the RNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The salivary RNA quality was examined by real-time reverse transcription–polymerase chain reaction (RT-PCR) analysis for expression of the salivary internal reference gene transcripts S100 calcium-binding protein A8 and annexin A2 (data not shown).

For microarray study, total salivary RNA was subjected to 2 rounds of T7-based RNA linear amplification (10). One microliter (200 ng/µl) of poly(dI-dC) (Amersham) was added to 11 µl of the salivary RNA sample, and 2 rounds of first-strand and second-strand complementary DNA (cDNA) synthesis were performed with a RiboAmp HS RNA amplification kit (Arcturus, Mountain View, CA) according to the manufacturer's instructions. After purification, the cDNA were in vitro transcribed to RNA and then biotinylated with GeneChip Expression 3'-Amplification Reagents for in vitro transcription labeling (Affymetrix, Santa Clara, CA). The labeled RNA was purified with the reagents provided with the RiboAmp HS RNA Amplification kit. The quality and quantity of amplified RNA were determined by spectrophotometry, with optical densities at 260/280 nm > 1.9 for all samples.

Biotinylated RNA samples (15 µg each) were subsequently fragmented, and the quality of the fragmented RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA). The Affymetrix human genome U133 Plus 2.0 array, which contains >54,000 probe sets representing >47,000 transcripts and variants, including ~38,500 well-characterized human genes, was applied to salivary mRNA profiling. Fragmented RNA were hybridized overnight to the microarrays. After a high-stringency wash to remove the unbound probes, the hybridized chips were stained and scanned according to the manufacturer's standard expression protocol. The scanned images were read with the Affymetrix microarray Robust Multiarray Average (RMA) software.(23) We deposited the microarray data we obtained into a Minimum Information About a Microarray Experiment (MIAME)–compliant database (available at http://.mged.org/workgroups/MIAME/miame.html); the accession number is GSE7451.

Statistical analysis for the mRNA study

The expression microarrays were scanned, and the fluorescence intensity was measured using Microarray Suite 5.0 software (Affymetrix). The arrays were then imported into the statistical software R (24). After quantile normalization and RMA background correction, the RMA expression index was computed in R using the Bioconductor routine.(25) Since most human RNAs are not present in saliva (12), we used the present/absent call generated by the Affymetrix Microarray Suite 5.0 software to exclude probe sets that were assigned an "absent" call in most (>75%) of the samples. Principal components analysis was performed to assess the information contained in the data to separate pSS and control cases. Student's 2-tailed t-test was used for comparison of the average gene expression signal intensity between samples from the SS patients (n=10) and controls (n=8). P values were adjusted with the Benjamini and Hochberg false discovery rate (FDR) criterion.(26) Fold ratios between SS and control samples were calculated for the transcripts. For the further validation study using real-time quantitative PCR, we applied stringent criteria: an alpha level of 0.001 for the t-test, which corresponded to a 5% FDR based on the data, and a fold ratio of 3. For functional analysis using MAPPFinder (27), we applied an alpha level of 0.01, which corresponded to an 8% FDR, and a fold ratio of 2, to obtain a larger list of genes.

Real-time quantitative RT-PCR

The biomarker candidates generated by microarray profiling were validated by real-time quantitative RT-PCR on the same set of samples used for the microarray analysis. All primers used for quantitative PCR were designed with the Primer3 program and synthesized by Sigma. Total RNA was reverse-transcribed using reverse transcriptase and gene-specific primers. One microliter of total RNA was used in a 20-µl volume of cDNA synthesis reaction and then subjected to the following thermal cycling conditions: 25°C for 10 minutes, 42°C for 45 minutes, and 95°C for 5 minutes. Three microliters of cDNA was used as template for each 20-µl PCR, which contained forward primer (200 nM), reverse primer (200 nM), and 10 µl of 2 x SYBR Green PCR Master Mix (Applied Biosystems). PCRs were performed in a 96-well plate on the Bio-Rad iCycler or IQ5 instrument (95°C for 3 minutes followed by 50 cycles of 95°C for 30 seconds, 62°C for 30 seconds, and 72°C for 30 seconds). All PCRs were performed in duplicate for all candidate mRNA.

The specificity of the PCR was confirmed according to the melting curve of each gene, and the average threshold cycle (C_t) was examined. The relative expression of the candidate genes was calculated according to the $2^{(-\Delta Ct)}$ method, where $\Delta C_t = C_t$ in pSS patients – C_t in controls. The expression ratio ([pSS patients/controls] = $2^{(-\Delta Ct)}$) is shown as the fold change.(28)

Pathway analysis

PathwayArchitect software, version 1.1.0 (Stratagene, La Jolla, CA) was used to investigate the functional pathways presented by the differentially expressed genes.

Results

Salivary flow rate and total salivary protein and mRNA contents in pSS patients

Patients with pSS who had been carefully diagnosed and monitored were enrolled in this study. All 10 patients were positive for anti-SSA/Ro antibodies, and 9 of them were also positive for anti-SSB/La antibodies. Their mean ±SD IgG level was 23.4±7.4 gm/liter, and

their mean ±SD IgM rheumatoid factor level was 136.3±99.6 kIU/liter. These patients exhibited significantly lower (~50%) salivary flow rates than did the age-, sex-, and ethnicity-matched healthy control subjects. The mean ±SD stimulated salivary flow rates in the 10 pSS patients were 0.13±0.12 ml/minute for the parotid glands (per gland), 0.32±0.38 ml/minute for the submandibular/sublingual glands, and 0.61±0.23 ml/ minute for WS. These rates in the 10 control subjects were 0.21±0.07 ml/minute for the parotid glands (per gland), 0.78±0.36 ml/minute for the submandibular/ sublingual glands, and 1.03±0.31 ml/minute for WS. Due to the low volume of saliva obtained from the pSS patients, the salivary proteins were equally pooled for the 10 pSS patients and separately for the 10 control subjects for the 2-DE analyses.

On average, the mean ±SD total protein concentrations in the controls were determined to be 1.26±0.40 mg/ml in submandibular/sublingual gland saliva (n=8 subjects), 0.93±0.38 mg/ml in parotid gland saliva (n=10 subjects), and 0.95±0.52 mg/ml in WS (n=10 subjects). The total protein concentrations in the pSS patients were 1.45±0.49 mg/ml in submandibular/sublingual gland saliva (n=8 patients), 1.40±0.56 mg/ml in parotid gland saliva (n=10 patients), and 1.38±0.37 mg/ml in WS (n=10 patients). There were consistently higher concentrations of proteins in the SS patients (WS, submandibular/sublingual gland saliva, and parotid gland saliva) than in the matched healthy control subjects. In addition, saliva from the pSS patients appeared to contain a higher concentration of total RNA than did that from the matched controls. In parotid gland saliva, the mean ±SD RNA concentration was determined to be 5.8±3.1 µg/ml in the pSS patients and 3.±.5 µg/ml in the controls (p=0.05). In WS, the average RNA concentration was 10.9±5.4 µg/ml for pSS patients and 6.6±3.6 µg/ml for matched controls (p=0.057).

Discovery of candidate peptide markers for pSS

The expression of 16 WS peptides was found to be significantly different (p=0.0046–0.0441) in pSS patients (n=10) and controls (n=10). Ten of the 16 peptides were overexpressed (m/z 1.107, 1.224, 1.333, 1.380, 1.451, 1.471, 1.680, 1.767, 1.818, and 2.039) and 6 were underexpressed (m/z 2.534, 2.915, 2.953, 3.311, 3.930, and 4.187) in the pSS patients. The peptide with an m/z of 1.451 exhibited the highest up-regulation (25.9-fold) in pSS patients (results not shown). We also compared the native peptide patterns in saliva from the parotid and submandibular/sublingual glands between pSS patients and control subjects (results not shown). WS was found to contain more informative peptides than did gland-specific (parotid or submandibular/sublingual) saliva. On average, 53 MALDI peaks were observed in WS from the 10 pSS patients, with only 24 peaks and 26 peaks detectable in saliva from their parotid and submandibular/sublingual glands, respectively.

Findings of 2-DE of WS proteins from pSS patients and matched control subjects

Figure 1 presents the 2-DE patterns of the proteins in pooled WS samples from 10 pSS patients and 10 control subjects. A number of proteins were found to be differentially expressed between the patient and control groups. By performing the PDQuest analysis and normalizing the protein spot signals, the relative levels of these proteins were quantified. The differentially expressed proteins (figure 1, spots 1-42) were excised and subsequently identified using in-gel tryptic digestion and LC-Q-TOF-MS. Pooled parotid and submandibular/sublingual gland saliva from pSS patients and control subjects was also analyzed by 2-DE (results not shown). WS was again found to be more informative than parotid or submandibular/sublingual gland saliva for generating candidate protein biomarkers

for the detection of pSS. A total of 325 protein spots were detected by 2-DE analysis of WS, whereas 232 and 267 spots were detected by 2-DE analysis of parotid and submandibular/sublingual gland saliva, respectively.

LC-Q-TOF-MS identification of proteins at altered expression levels

The differentially expressed WS proteins identified by LC-Q-TOF-MS and Mascot database searching, as well as their theoretical isoelectric point (pI), relative molecular mass (Mr), IPI accession number, the number of peptides matched, and ratios of expression levels between the pSS patient and matched control groups are shown in table 1.

Figure 2A depicts the tandem MS spectrum of a double-charged tryptic peptide (m/z 450.3). The precursor ion was well fragmented to yield sufficient structural information for confident identification of the peptide sequence TIAPALVSK, which originated from α-enolase. Mascot database searching indicated that 12 peptides were matched to this protein, resulting in a sequence coverage of 31%. Validation of α-enolase was also performed by Western blotting of the same set of samples used for the 2-DE study. (figure 2B) An equal amount of total proteins from each sample was used for immunoblotting of α-enolase and actin. Both α-enolase and actin were found to be up-regulated in WS from pSS patients, which is consistent with the 2-DE results. (table 1) P values were calculated to be 0.006 for α-enolase without actin normalization and 0.037 with actin normalization for comparisons between the pSS patient and healthy control groups.

Figure 1

Comparative analysis of proteins in whole saliva (WS) samples from patients with primary Sjögren's syndrome (pSS) and age-, sex-, and ethnicity-matched control subjects, as determined by 2-dimensional gel electrophoresis (2-DE) and liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-Q-TOF-MS). Shown are the 2-DE patterns of proteins in pooled WS from 10 control subjects and 10 pSS patients. A total of 100 µg of total proteins from each pooled sample was used for the 2-D gel separation. The differentially expressed proteins (spots 1-42; see Table 1 for the complete list) were identified using in-gel tryptic digestion and LC-Q-TOF-MS.

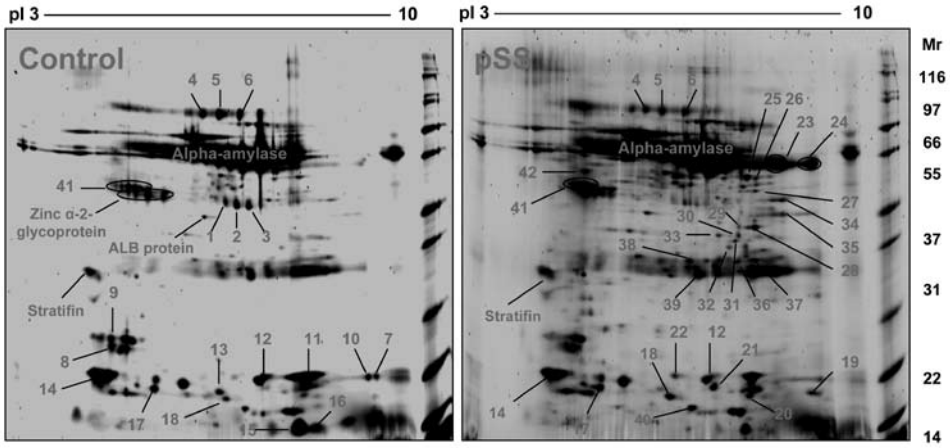


Table 2 Real-time quantitative RT-PCR validation of 13 genes selected from the top 27 genes found to be differentially expressed in pSS patients and healthy control subjects*

Gene	Average Ct Control	Average Ct pSS	Δ Ct (Control/ pSS)	quantitative RT-PCR, fold change $2^{(-\Delta Ct)}$	P value (t-test)	Microarray fold change
GIP2	44.5±1.9	35.5±2.1	9.0	495.5	<0.001	15.76
B2M	45.0±2.1	38.8±3.4	6.2	72.1	<0.001	8.67
IFIT2	41.1±2.0	35.9±2.6	5.1	35.5	<0.001	12.19
BTG2	38.5±5.3	33.5±2.0	5.0	32.4	0.01	3.22
IFIT3	43.8±0.5	39.1±2.4	4.7	25.3	<0.001	122.82
MNDA	37.3±1.2	33.7±2.1	3.7	12.7	<0.001	8.67
FCGR3B	40.6±1.5	36.9±2.2	3.6	12.5	<0.001	25.32
TXNIP	39.2±2.1	35.6±3.2	3.6	11.7	0.01	3.42
IL18	45.3±2.1	41.8±2.5	3.5	11.5	0.01	6.12
HLAB	36.4±2.7	32.9±2.0	3.5	11.2	0.01	4.34
EGR1	37.4±2.4	33.9±2.0	3.4	10.3	0.01	7.20
COPI	40.5±1.5	38.7±3.3	1.8	3.4	0.18	7.62
TNSF	39.6±0.4	38.9±2.9	0.7	1.6	0.95	8.03

* All real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) analyses were performed in duplicate. See Patients and Methods for calculations of the fold change (primary Sjögren's syndrome (SS) patients/healthy controls) and threshold cycle (C_t) data.

Identification of candidate genomic markers of pSS in saliva samples

For all the arrays, the mean ±SD percentage of genes present was 13.2±2.9%. This is similar to the finding in our previous study (12) and indicates consistency of the techniques used for sample preparation. Microarray profiling indicated that WS contains >10 times more informative mRNA than does parotid gland saliva. A total of 328 mRNA had a >2-fold change in WS from pSS patients, while only 21 mRNA had a >2-fold change in parotid gland saliva from these patients. Therefore, we focused on the discovery and validation of WS candidate mRNA biomarkers using microarray and real-time quantitative RT-PCR strategies.

Gene expression profiles of individual WS samples from 10 pSS patients and 8 controls were compared. After filtering the transcripts by the criteria of being "present" in >25% of the samples, a total of 6,413 transcripts were retained for further analysis. This number is consistent with our previous results, showing that only a small number of RNAs are present in saliva (12). Principal components analysis indicated that the information contained in the data could well segregate control subjects and pSS patients. (figure 3) We then performed statistical testing and fold change analysis to identify differentially expressed genes. Only a few mRNA were found at significantly lower levels in pSS patients as compared with the controls when using a threshold of >2-fold change and a significance level of P <0.01 (FDR 0.08). Yet, by the same criteria, 162 genes showed significant up-regulation in samples from patients with pSS.

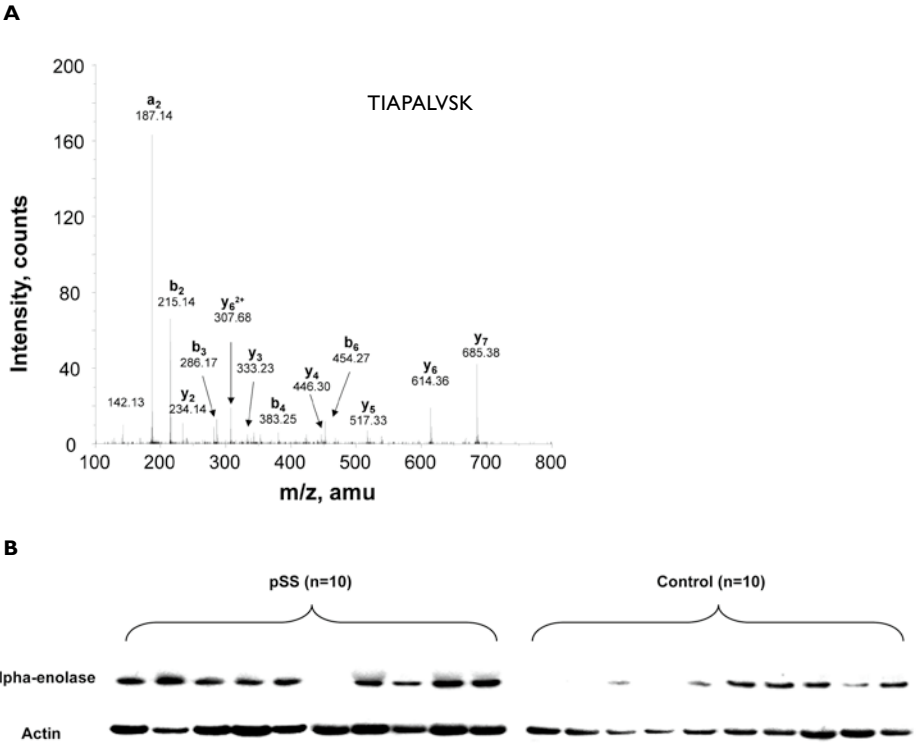
Pathway analysis indicated that 37 genes were involved in the IFN-α pathway, and most

of them have been reported to be IFN-α or IFN-β inducible.(29;30) These results suggest that activation of IFN pathways is involved in the pathogenesis of pSS and that the related information is reflected in the saliva. To facilitate biomarker discovery, we narrowed the number of candidate biomarkers by using more stringent threshold criteria of $P < 0.001$ (FDR 0.05) and 3-fold change. Based on these criteria, we found 27 genes that were highly overexpressed in samples from pSS patients. These genes are sufficiently informative for segregating the pSS patients from the control subjects. (figure 4)

Among the top 27 genes, 13 were validated by real-time quantitative RT-PCR. Eleven of the 13 genes were found to be significantly up-regulated in pSS patients (>10-fold change), including the IFN-inducible protein G1P2, which showed an ~500-fold change in pSS patients. Table 2 shows the average Ct values of these genes in pSS patients and control subjects, as well as the quantitative PCR fold change in comparison with that of microarray profiling.

Figure 2

Analysis of α-enolase by electrospray ionization tandem mass spectrometry (ESI-MS/MS) and immunoblotting. A, ESI-MS/MS spectrum of the tryptic peptide TIAPALVSK (mass/charge [m/z] 450.3 atomic mass units [amu]) from α-enolase. This protein was found to be overexpressed in whole saliva from patients with primary Sjögren's syndrome (pSS), as determined by 2-dimensional gel electrophoresis. B, Immunoblotting of whole saliva from 10 patients with pSS and 10 age-, sex-, and ethnicity-matched control subjects for α-enolase and actin. An equal amount of proteins from each sample was used for the immunoblots.

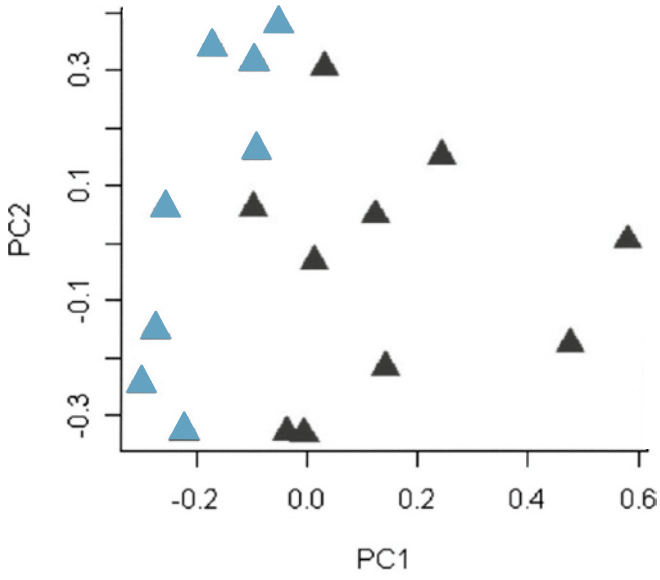


Discussion

Although saliva has been extensively explored as a source of information that can be used in the diagnosis of pSS, most of the previously published studies mainly examined individual components of the saliva. High-throughput profiling techniques, such as proteomics and expression microarray analysis, enable us to explore salivary proteins and mRNA in a global manner and may therefore provide new and deeper insights that may lead to the discovery of salivary biomarkers for pSS. Recently, surface-enhanced laser desorption ionization time-of-flight mass spectrometry and differential gel electrophoresis have been used to identify very promising candidate biomarkers of SS in tears and in parotid gland saliva.(31;32) It was found that the proteomic profile of parotid gland saliva from SS patients is a mixture of increased inflammatory proteins and decreased acinar proteins as compared with the profile in non-SS controls.(32)

In order to determine which oral fluid compartment is more informative for the discovery of biomarkers that can be used to detect pSS, we used both proteomic and microarray approaches to profile peptides, proteins, and mRNA in WS, parotid gland saliva, and submandibular/sublingual gland saliva from each study subject. WS as a fluid includes secretions from 3 major salivary glands, numerous minor salivary glands, and gingival fluid, as well as cell debris. There has therefore been concern about the complex background in WS for discovery of disease biomarkers, whereas parotid gland saliva, if collected carefully, may contain more specific biomarkers for pSS. Yet, there are no published reports of any advantage of using gland-specific saliva versus WS in terms of the diagnostic potential for

Figure 3
Principal components analysis of the gene expression data in patients with primary Sjögren's syndrome (SS) and in age-, sex-, and ethnicity-matched control subjects. Results of the principal components (PC1 and PC2) analysis suggest that the gene expression data we obtained segregated the 8 control subjects (green symbols) from the 10 pSS patients (black symbols).



pSS. The findings of our study allow us to conclude that WS is more informative than glandular saliva for generating biomarkers to be used for the detection of pSS.

Microarray profiling indicated that WS from pSS patients contained 328 mRNA with 2-fold change in expression, whereas the parotid gland saliva from pSS patients contained only 21 mRNA with a >2-fold change in expression. Similarly, findings of the MALDI-TOF-MS and 2-DE analyses suggested that WS from pSS patients has more informative proteomic components than does parotid or submandibular/sublingual gland saliva. Since the salivary flow rate varies from person to person, the peptide or protein composition among different individuals could be affected by the very low salivary flow rate of the parotid and submandibular/sublingual glands. With regard to the low flow rate of glandular saliva, as well as the additional skill set and clinical time necessary to collect gland-specific saliva, WS may be a more appropriate clinical diagnostic fluid for the discovery and detection of biomarkers of pSS.

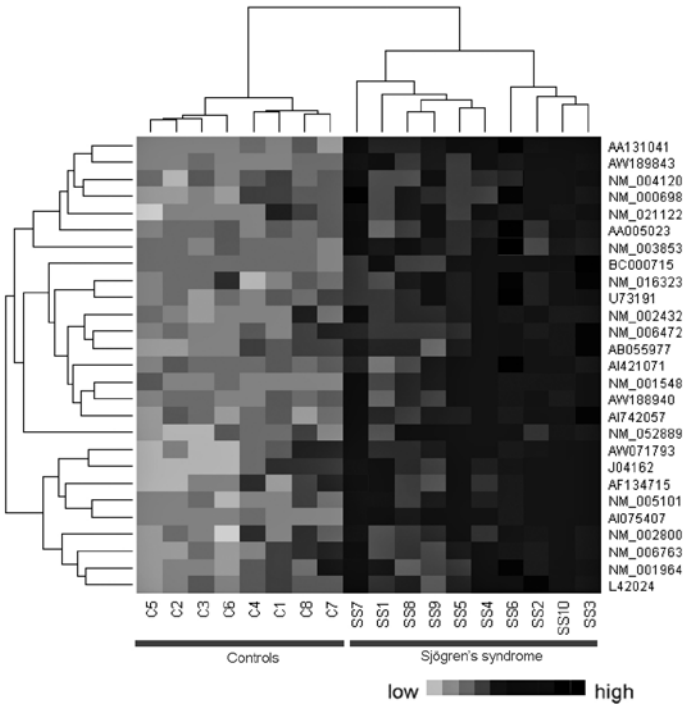
The panel of candidate peptide/protein markers for pSS is completely distinct from the panel we obtained for oral cancer.(13) This suggests that the panels of discriminatory salivary proteomic components are likely to be different for different diseases. The majority of underexpressed proteins found in WS from pSS patients are secretory proteins, including 3 glycoforms of carbonic anhydrase VI (figure 1, spots 1-3), cystatins, lysozyme C, polymeric immunoglobulin receptor (pIgR), calgranulin A, prolactin-inducible protein, and von Ebner gland protein. This suggests that the level of secretory proteins in WS from pSS patients may be directly affected by injury to salivary glandular cells. Several of these down-regulated proteins in the WS of pSS patients, including pIgR, lysozyme C, and cystatin C, were found up-regulated in the parotid gland saliva of pSS patients in a previously published study.(32) This may be factual, as evidenced by our comparative analysis of parotid gland salivary proteins in pSS patients and control subjects (results not shown). For example, in our 2-DE study, pIgR was also found to be up-regulated in the pooled parotid gland saliva of pSS patients as compared with the matched control subjects (results not shown). A future study of salivary proteins from the parotid gland versus WS in the same pSS patients would be of interest to the pSS research community.

Two glycolysis enzymes, fructose-bisphosphate aldolase A and α-enolase, were found at elevated levels in the WS of pSS patients. Fructose-bisphosphate aldolase A plays a central role in glucose metabolism, catalyzing either net cleavage or synthesis during glycolysis or gluconeogenesis. Alpha-enolase is a multifunctional glycosis enzyme involved in various processes, such as growth control, hypoxia tolerance, and allergic responses. Previously, α-enolase was identified as an autoantigen in Hashimoto encephalopathy, which is an autoimmune disease associated with Hashimoto thyroiditis.(33) Alpha-enolase was also found as an autoantigen in lymphocytic hypophysitis, and serum autoantibodies directed against α-enolase were detected in patients with lymphocytic hypophysitis as well as in patients with other autoimmune diseases. Excessive production of autoantibodies, which are generated as a consequence of uptake of enolase by antigen-presenting cells and subsequent B cell activation, can potentially initiate tissue injury as a result of immune complex deposition.(34;35) Overexpressed proteins in WS from patients with pSS also included psoriasin, fatty acid binding protein, carbonic anhydrases I and II, salivary amylase fragments, caspase 14, β_2m, hemoglobin (β and α1 global chains), and immunoglobulins. The elevated level of caspase 14 protein and caspases 1 and 4 RNA in pSS patients also suggested an interesting role of apoptosis in the pathogenesis of pSS.

Our study clearly demonstrates that pSS-related gene expression signatures are present

in saliva and they are able to differentiate pSS patients from control subjects. To the best of our knowledge, this is the first study on the discovery of candidate salivary mRNA markers for the detection of pSS. We identified 162 differentially expressed genes in the saliva of pSS patients, as compared with a reported 35 and 424, respectively, identified in 2 studies of microarray profiling of minor salivary gland biopsy tissues.(36;37) One of the important findings of this study is that the 37 up-regulated genes in the saliva of pSS patients were involved in the IFN pathway. This further confirmed the findings from previous tissue- based studies and demonstrated that the IFN-inducible gene signature associated with pSS is reflected in patients' saliva.(36-39) Beyond the IFN-inducible genes, the class I major histocompatibility complex is another major group of up-regulated genes found to be common to salivary gland and WS from patients with pSS.(36,37) Other genes reported to be of particular interest in the pathogenesis of pSS (37) that were found to be overexpressed in saliva are proteasome subunit β type 9, guanylate binding protein 2, IFN-induced protein 44, and IFN-inducible protein G1P2, and β_2m. These common genes found in saliva and minor salivary gland tissue from patients with pSS support our hypothesis that saliva harbors the biomarkers for pSS.

Figure 4
Heat map of 27 mRNA that were significantly up-regulated in patients with primary Sjögren's syndrome (SS) as compared with the age-, sex-, and ethnicity-matched control subjects, as determined by microarray profiling analysis. Control and SS patient numbers are shown at the bottom.



The mechanism of IFN pathway activation in the pathogenesis of pSS may be more complicated. Activation of IFN pathways (both type I and type II) in pSS suggests the involvement of viral infection in its pathogenesis. Immune complexes consisting of autoantibodies and DNA- or RNA-containing autoantigens derived from apoptotic or necrotic cells are also able to induce the production of type I IFN. However, IFN itself is not among the genes we found to be overexpressed in the saliva of the pSS patients. On the other hand, low-dose IFN-α has been reported to be effective in the treatment of some patients with pSS. A single-blind controlled trial showed that IFN-α therapy significantly improved salivary gland dysfunction in SS patients.(40) Serial labial salivary gland biopsy in 9 patients responding to IFN-α therapy showed a significant decrease ($p<0.02$) in lymphocytic infiltration and a significant increase ($p=0.004$) in the proportion of intact salivary gland tissue after IFN-α treatment.(41)

Type I IFN pathway dysregulation, however, has been reported in such distinct diseases as SLE, dermatomyositis, psoriasis, and SS (36), indicating that the consequences of activation of this pathway are likely to be tissue type-dependent and, from a therapeutic point of view, that local immune modulation (e.g., direct infusion into salivary glands) may be more efficient than systemic interference. An initial viral infection-induced type I IFN production in salivary glands, with prolonged activation triggered by autoantibodies from nucleic acid–containing immune complexes, has been proposed as the mechanism of pSS.(42) More importantly, activation of this IFN pathway may provide potential therapeutic targets for pSS, and saliva may be used to monitor the response to the IFN-related target modulation.

One of the up-regulated genes seen in the saliva of patients with pSS is β_2m, which is also regulated by IFN. Significantly elevated levels of β_2m have previously been detected in saliva from patients with pSS.(43) The concentration of salivary (but not serum) β_2m was highly related to the salivary gland biopsy focus score.(43) The value of salivary β_2m protein as a biomarker for pSS has been evaluated, and it has been suggested that determination of β_2m levels in the saliva could be used as a noninvasive measurement for confirmation of the diagnosis of SS.(44) Interestingly, but not surprisingly, we found that both the mRNA and protein levels of β_2m are concordantly overexpressed in the saliva of patients with pSS.

From the top 27 mRNA found to be overexpressed in WS from pSS patients, as revealed bymicroarray profiling, we were able to validate 11 of the genes; expression of the other 16 genes was too low for quantitative PCR assessment. The most overexpressed mRNA was found to be G1P2, which has a function in cell signaling and has been reported to be up-regulated at the mRNA level in minor salivary glands from patients with pSS.(37) There were discrepancies with regard to the fold change as determined by the quantitative PCR and the microarray studies.

There are many factors that may contribute to the observed discrepancies, including the procedures unique to the microarray analysis, such as nonspecific and/or cross-hybridization of labeled targets to array probes, as well as those unique to real-time quantitative RT-PCR, such as amplification biases.(45) Also, the increased distance between the location of the PCR primers and the microarray probes on a given gene was found to decrease the correlation between the 2 methods.(46) In our study, the amplified RNA used for microarray assay and the unamplified RNA used for the real-time quantitative RT-PCR validation studies can introduce variances in the fold change between the 2 methods. Furthermore, we do not expect there to be perfect correlation between the fold change as determined by quantitative PCR and by microarray analyses, since there is considerable variability in the fold change statistic, especially in the case of genes that are near the limit

of detection by quantitative PCR. For genes with expression levels that are too low for the quantitative PCR techniques in current use, it is still possible that they may be validated when the technology improves. Nevertheless, these 11 highly expressed genes, once they are further validated in a new and independent patient cohort, may be used in the clinical detection of pSS.

There was little correlation between the protein and mRNA markers identified. This has been observed for biologic systems when efforts were made to correlate the gene expression at both the protein and mRNA levels.(47;48) In a previous correlation analysis of the human saliva proteome and transcriptome, we demonstrated that complementary validation (e.g., Western blotting for protein or RT-PCR for mRNA) is required in the conduct of RNA-protein correlation studies of individual genes after initial mass spectrometry and expression microarray profiling.(49) If mutual validation is performed, there may be higher correlations between the protein and mRNA candidate markers in saliva identified in the present study. Nevertheless, the discrepancy we found may suggest that the combination of both mRNA and protein markers is important for improving the detection of pSS.

As a result of this preliminary study, a number of promising salivary protein and mRNA candidates that are characteristic of pSS have been identified. Many of these candidate biomarkers have not previously been associated with SS and, in combination, they may eventually be validated as specific biomarkers of pSS, thus improving the clinical diagnosis of pSS. Ideally, the biomarkers would be very specific for pSS and would discriminate pSS from other autoimmune diseases of a similar immunopathologic background. Future studies will include new pSS patients as well as patients with other autoimmune diseases as control groups, aiming to validate the candidate genes either through the use of real-time quantitative RT-PCR for mRNA or immunoassays for proteins. Absolute quantification will provide a cutoff value for each biomarker selected, and combination of the mRNA and protein markers will allow the eventual construction of a multimarker prediction model that can be used as an adjunct to the current diagnostic criteria for the clinical diagnosis of pSS.

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Chapter 5b

Clinical and histologic evidence of salivary gland restoration supports the efficacy of rituximab treatment in Sjögren's syndrome

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Abstract

Objective To assess the effect of rituximab (anti-CD20 antibody) therapy on the (immuno) histopathology of parotid tissue in patients with primary Sjögren's syndrome (pSS) and the correlation of histologic findings with the flow rate and composition of parotid saliva.

Methods In a phase II study, an incisional parotid biopsy specimen was obtained from 5 patients with pSS before and 12 weeks after rituximab treatment (4 infusions of 375 mg/m2). The relative amount of parotid parenchyma, lymphocytic infiltrate and fat, and the presence/quantity of germinal centers and lymphoepithelial duct lesions were evaluated. Immunohistochemical characterization was performed to analyze B:T cell ratio of the lymphocytic infiltrate (CD20, CD79a, CD3) and cellular proliferation in the acinar parenchyma (by double immunohistologic labeling for cytokeratin 14 and Ki-67). Histologic data were correlated to parotid flow rate and saliva composition.

Results Four patients showed an increased salivary flow rate and normalization of the initially increased salivary sodium concentration. Following rituximab treatment, the lymphocytic infiltrate was reduced, with a decreased B:T cell ratio and (partial) disappearance of germinal centers. The amount and extent of lymphoepithelial duct lesions decreased in 3 patients and was completely absent in 2 patients. The initially increased proliferation of acinar parenchyma in response to the inflammation was reduced in all patients.

Conclusion Sequential parotid biopsy specimens obtained from patients with pSS before and after rituximab treatment demonstrated histopathologic evidence of reduced glandular inflammation and redifferentiation of lymphoepithelial duct lesions to regular striated ducts as a putative morphologic correlate of increased parotid flow and normalization of salivary sodium content. These histopathologic findings in few patients underline the efficacy of B cell depletion and indicate the potential for glandular restoration in SS.

Introduction

Currently, there is no evidence-based intervention treatment for Sjögren's syndrome (SS), but biologic agents are promising.(1) Rituximab, a chimeric murine/human anti-CD20 monoclonal antibody that binds to the B-cell surface antigen CD20, is a well-established therapeutic agent in the treatment of B-cell non-Hodgkin lymphomas, and is a new promising therapeutic modality in different autoimmune disorders, such as rheumatoid arthritis (RA) and systemic lupus erythematosus.(2)

The salivary glands of patients with SS are histologically characterized by lymphocytic infiltration with progressive parenchymal atrophy and formation of the characteristic lymphoepithelial lesions in striated ducts, formerly called "epimyoepithelial lesions".(3) Our group has previously shown that lymphoepithelial lesions develop from basal cells of striated ducts, representing an aberrant metaplastic differentiation, triggered by the epitheliotropic autoimmune inflammation in SS.(3) In parallel, parenchymal acinar cells in SS demonstrate increased proliferation in an effort to partially compensate for enhanced apoptotic cell loss.(3-5) Our group previously reported clinical data from a phase II trial with rituximab treatment in 8 patients with primary SS, which showed significant improvement of subjective symptoms and increased salivary secretion with partial normalization of increased sodium concentration of saliva in patients with early-onset SS.(6) These findings might indicate partial recovery of salivary gland tissue.(7) In 5 of the 8 patients with pSS involved in the above-mentioned study, sequential parotid gland biopsy specimens were available for histologic analysis; these specimens were obtained before and 12 weeks after rituximab treatment. In the other 3 patients with pSS no second biopsy specimen was obtained, because these patients did not complete rituximab treatment due to the development of serum sickness.(6)

The biopsy material gave us the unique opportunity to correlate clinical findings, including the salivary flow rate and composition of saliva, with the findings of a detailed immunohistopathologic analysis of the parotid gland biopsy specimens obtained before and after rituximab treatment in order to histologically verify the effects of therapeutic B cell depletion in patients with SS.

Patients and methods

Study design

Five female patients (mean age 53 years, range 43-65 years), all of whom fulfilled the American-European consensus criteria for pSS, were treated with 4 infusions rituximab (Roche, Woerden, the Netherlands), given at a dosage of 375 mg/m2/week. No other immunosuppressive therapy was used. An incisional biopsy specimen of the parotid gland was obtained from the same gland before and 12 weeks after therapy.(8) These patients were part of an earlier reported phase II trial.(6)

Parotid gland function and salivary composition

Unstimulated and stimulated parotid saliva was collected in a standardized way at baseline and 12 weeks after treatment as described previously.(6) Flow rates were calculated and sialochemical analysis was performed, focusing on the concentration of sodium in parotid saliva, particularly because increased sodium in parotid saliva is indicative of SS and reflects damage to the ductal system. High levels of sodium in the saliva of patients with SS are associated with higher levels of disease activity and a more progressive course of the disease.(9)

Histopathologic analysis

Biopsy specimens were fixed in 4% neutral buffered formalin, embedded in paraffin, cut at a thickness of 3 µm, and stained with hematoxylin and eosin. The relative amount of glandular parenchyma, lymphocytic infiltrate, and fat was assessed semiquantitatively in steps of 10%, each in relation to the total amount of biopsied parotid tissue. The presence of secondary germinal centers was assessed as follows: 0 = no germinal centers, I = few (mostly small) germinal centers, and II = many (often large) germinal centers. The characteristic ductal alterations of SS (lymphoepithelial lesions) were evaluated by the following grading system: 0 = none, I = few and partially developed lymphoepithelial lesions (not circumferential, <50% of all striated ducts) and II = fully developed lymphoepithelial lesions (fully circumferential, >50% of all striated ducts). Biopsy specimens were independently scored as based on these criteria by 2 investigators (J.P. and S.I.) in a blinded manner. In case of discrepancy a definite score was determined by consensus.

Immunohistochemical analysis

In representative areas of lymphocytic infiltrates the numbers of B cells (staining for CD20) and T cells (CD3) were quantified in 1000 lymphocytes each, and consecutively calculated as B/T cell ratio. To evaluate a possible additional down-regulation of CD20 antigen presentation on persisting B cells due to anti-CD20 therapy, quantification of B cells was separately performed with antibodies to CD20 and CD79a. Due to technical limitations, it is not possible to quantify the absolute amount of B and T cells.

As described previously (4), a double immunohistochemical labeling technique for cytokeratin 14 (CK14) (labeling basal cells of striated ducts and myoepithelial cells) and Ki67 (labeling cellular proliferation) greatly enhances the exact identification and quantification of cellular proliferation of the various epithelial cells of the gland. In order to evaluate the regenerative potential of the glandular parenchyma, cellular proliferation in the CK14-negative acinar cells was calculated in representative areas of lymphocytic infiltration (nuclear positivity for Ki67 as a percentage of 400 acinar cells). For staining of

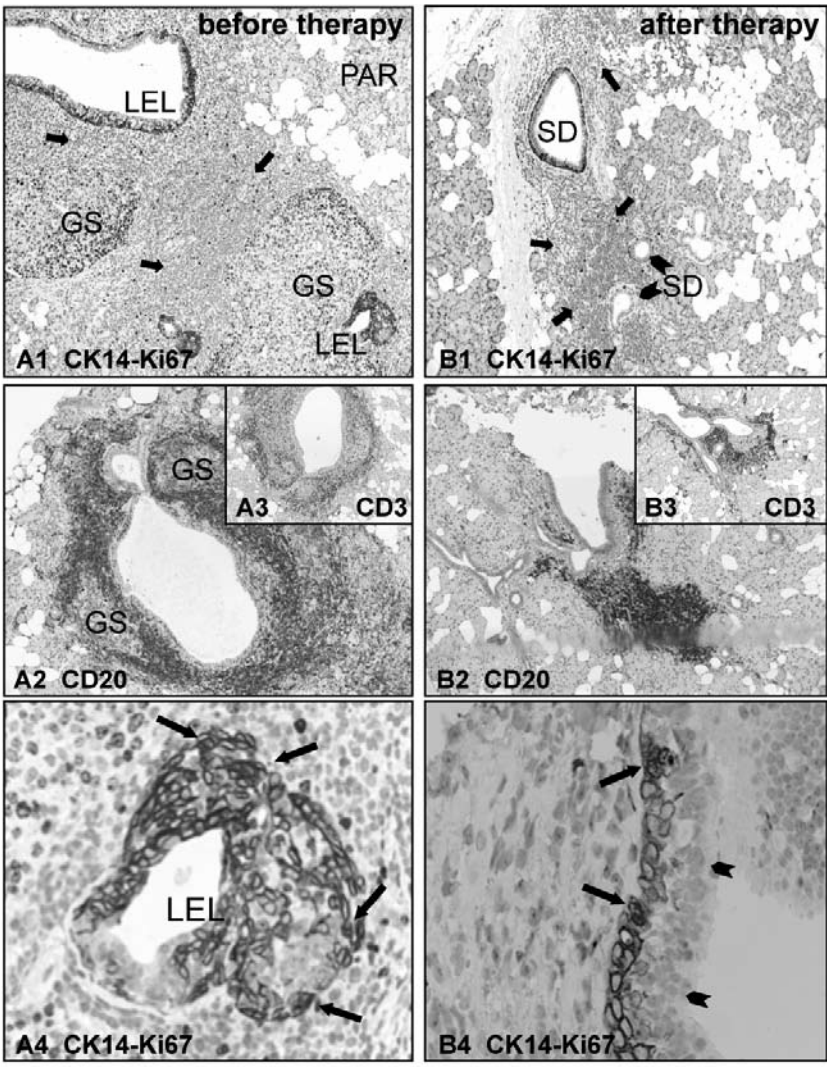


Figure 1

Comparison of parotid biopsy specimens obtained from patient 3 before therapy (left, figures 1A1-4) and 12 weeks after therapy (right, figure 1B1-4) Magnification 120x figures 1A1,1B1; 100x figures 1A2,1B2; 60x figures 1A3,1B3; 200x figures 1A4,1B4. Figure 1A1: Before treatment, double staining illustrates intense inflammation (arrows) with highly proliferating, large germinal centres (GS; Ki67 with nuclear staining), fully developed lymphoepithelial lesions (LEL; CK14 staining) and reduced glandular parenchyma (PAR). After therapy (figure 1B1), inflammation is reduced (arrows) with absence of germinal centres and presence of regular striated ducts (SD) devoid of lymphoepithelial lesions. Before therapy there was a dominance of B lymphocytes with germinal centres (GS; figure 1A2: CD20) in comparison to T lymphocytes (inset: figure 1A3: CD3). After therapy the overall reduced lymphoid infiltrate with slight dominance of T lymphocytes (inset: figure 1B3: CD3) in comparison to B lymphocytes (figure 1B2: CD20). In higher magnification (figure 1A4) fully developed lymphoepithelial lesions, many intraepithelial lymphocytes and increased basal cell proliferation (arrows), contrasting after therapy to regular striated duct with CK14-positive basal cells (arrows in figure 1B4) with regular differentiation into luminal ductal cells, devoid of intraepithelial lymphocytes (arrowheads).

Table 1 Clinical and (immuno-)histological data before and after rituximab therapy.

	Patient 1		Patient 2		Patient 3		Patient 4		Patient 5		Tendency
	Before	After	Before	After	Before	After	Before	After	Before	After	
Clinical findings											
Parotid flow	0.14	0.16	0.15	0.21	0.18	0.22	0.17	0.20	0.01	0.02	↑
Na+ in parotid saliva	39 (5)	27 (2)	12 (5)	7 (7)	19 (6)	8 (7)	4 (6)	3 (7)	N.A.	N.A.	↓
Histopathology											
Parenchyma (%)*	20-30	10-20	70-80	40-50	50-60	40-50	60-70	60-70	70-80	70-80	↓
Lymphocytic infiltrate (%)*	60-70	40-50	10-20	10-20	20-30	0-10	20-30	0-10	10-20	10-20	↓
Fat (%)*	10-20	20-30	10-20	50-60	20-30	40-50	20-30	20-30	0-10	0-10	↑
Germinal centres	II	II	I	No	II	No	I	No	No	No	↓
Lymphoepithelial duct lesions (LEL)	II	I	I	I	II	No	I	I	I	No	↓
Proliferation of acinar parenchyma in % (Ki67)	3.8	3.2	3.4	2.3	3.5	2.5	1.8	1.2	4.7	3.5	↓
B :T cell ratio (CD20/CD3)	76/24	67/33	59/41	28/72	58/42	35/65	54/45	52/48	43/57	35/65	↓

Parotid flow: stimulated parotid secretion (ml/min); Na: concentration of sodium in parotid saliva (mmol/l), value in brackets: sodium concentration to be expected in healthy subjects with the given parotid flow; N.A.: not available; * Percentages in steps of 10% represent assessment of the area of the biopsy specimen. Germinal centres, I = few germinal centres, II = many germinal centres; Lymphoepithelial duct lesions, I = partially developed lymphoepithelial lesions (not circumferential, <50% of all ducts), II = fully developed lymphoepithelial lesions (fully circumferential, >50% of all ducts); B:T cell ratio: ratio of B and T lymphocytes in the infiltrate; ↑: increase, ↓: decrease.

CK14 an aividin-biotin-peroxidase method was applied (ABC kit; Vector, Burlingame, CA), for staining of Ki67, the alkaline phosphatase-anti-alkaline phosphatase method was used (APAAP-ChemMate; Dako Cambridge, UK).

Results

The clinical and (immuno)histologic data for biopsy specimens obtained before and after rituximab treatment are summarized in table 1. All patients showed a clinical response as reflected, among other factors, by significant improvement of subjective symptoms.(6) Four of 5 patients showed a minor-to-moderate increase in the parotid flow rate (mean increase 24%). The baseline sodium concentration in parotid saliva was increased in the saliva samples from these 4 patients (patient 5 had no salivary parotid flow at baseline) (table 1). The sodium concentration decreased after treatment in all 4 of the above-mentioned patients, and values returned to near normal in 2 of these 4 patients.

The histologic data showed a tendency towards reduced lymphocytic infiltration after therapy with a decrease of the B:T cell ratio, indicating a major decrease especially in the number of B lymphocytes, in combination with a reduction of germinal centers (which were completely absent in 4 patients), (figures 1A2, 1B2, 1A3, 1B3). The number of B lymphocytes based on staining for CD20 and CD79a did not differ. The amount of acinar parenchyma did not change or was slightly decreased, and the amount of fat did not change or was increased. Parallel to the reduction in the number of intraepithelial lymphocytes, the amount and extent of lymphoepithelial lesions decreased in 3 of 5 patients, and these lesions were completely absent in 2 of 5 cases (figures 1A1, 1B1, 1A4, 1B4). Cellular proliferation of acinar parenchyma before therapy was higher (average 3.4% figure 2A) than that of normal acinar parenchyma of patients without SS (2.0%,(5)), and was found to be reduced in all patients after therapy (on average 2.5%). The most significant improvement of clinical and histological findings was observed in patient 3, (as shown in figures 1 and 2A). Statistical correlation of the different parameters could not be determined due to the small sample size.

Discussion

Rituximab is a promising treatment option for patients with pSS and systemic complications and/or active and progressive disease, but more data from randomized controlled trials are warranted before more accurate conclusions on the role of rituximab can be made.(10)

This study is the first to present histologic data demonstrating evidence of a reduction in glandular inflammation combined with signs of partial glandular restoration, parallel to increased parotid saliva flow and normalization of initially increased levels of salivary sodium. As expected, the reduction of inflammation was mainly attributable to a depletion of B lymphocytes, as has been previously described following rituximab therapy in RA.(11) Although quantification of the absolute amount of B and T cells was not possible for technical reasons, the overall decrease in the amount of infiltrate, combined with a decreased B:T cell ratio, suggests a relevant decrease in the amount of B cells. The preponderant absence of germinal centers and the reduction of intraepithelial lymphocytes in the salivary ducts after therapy underline the significant reduction of inflammatory activity. This correlates to complete depletion of B cells in the peripheral blood 12 weeks

after start of treatment (6), comparable to data from a recent study in patients with RA.(12) In addition, also T lymphocytes seemed to decrease slightly after therapy, although this could not be quantified.

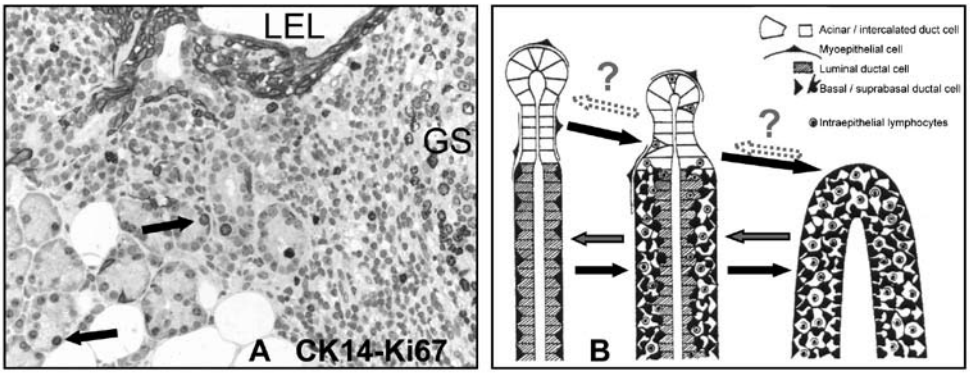
Although the number of B cells in parotid gland tissue was decreased, B cells were not completely depleted. The discrepancy with complete B cell depletion observed in peripheral blood might be explained by the expression of different protective factors in this tissue, such as BLyS (B lymphocyte stimulator) or BAFF (B cell activating factor). The same phenomenon has been observed in patients with RA treated with rituximab.(12) In contrast, another study of SS patients showed a complete depletion of B cells in labial salivary glands 4 months after rituximab treatment.(13) Possible explanations for this difference might be the increased inflammatory activity in parotid salivary glands (reflected by germinal centers) or a difference in the expression of BAFF or BLyS. Further studies are necessary to investigate the different B cell subsets before and after treatment and the expression of BAFF or BLyS.

The widespread presence of fully developed lymphoepithelial lesions before therapy and the reduction or complete disappearance of lymphoepithelial lesions after therapy offer histopathologic evidence that fully developed lymphoepithelial lesions can completely redifferentiate into regular striated ducts (see figure 2B). As shown previously by our group lymphoepithelial lesions in SS develop from enhanced proliferation of basal cells of striated ducts with an aberrant metaplastic lymphoepithelial differentiation, triggered by the epitheliotropic autoimmune inflammation.(3;14) Supposedly, this redifferentiation into regular striated ducts after therapy is recruited from surviving and proliferating basal cells in lymphoepithelial lesions with physiological differentiation into regular ductal cells (figure 1B4).

Figure 2

A. Minor increase of acinar cell proliferation (arrows) as demonstrated by double staining with CK14-Ki67, adjacent to lymphocytic infiltration with lymphoepithelial lesions (LELs) and germinal centre (GS; patient 3 prior to therapy; magnification 200x).

B. Schematic illustration of partially reversible glandular alterations in SS: Black arrows (bottom) indicate transformation of striated duct (left) into incomplete (middle) and fully developed lymphoepithelial lesions (right), in addition to progressive loss of acini and intercalated ducts (black arrows top). Grey arrows (bottom) illustrate evidence of complete redifferentiation of fully developed lymphoepithelial lesions to regular striated ducts after therapy. Effective regeneration of intercalated ducts and acini as an effect of successful Rituximab therapy is hypothetical (dotted grey arrows).



In healthy subjects, most of the high sodium content in primary saliva is actively reabsorbed during passage through striated ducts. The increased sodium content in saliva of patients with pSS has been attributed to severely impaired reabsorption in the structurally altered lymphoepithelial lesions.(9) Reduction or normalization, respectively, of the salivary sodium concentration after B cell depletion obviously is attributable to partial or complete redifferentiation of lymphoepithelial lesions to regular striated ducts, with reconstituted physiological function, including regular reabsorption of sodium.

Increased proliferation of acinar parenchyma in pSS in comparison with regular glands has been interpreted as a regenerative effort to compensate for increased apoptotic cell loss in the inflamed parenchyma.(5) Accordingly, the observed minor decrease of proliferation in acinar parenchyma after rituximab treatment of pSS presumably is attributable to a decrease of the inflammatory stimulus. There is no good explanation for the almost absent parotid salivary flow in patient 5, despite the amount of salivary parenchyma (table 1). It has been shown that many patients with SS have, within their salivary glands, large amounts of acinar tissue that is unable to function in vivo, possibly due to antimuscarinic antibodies.(9;15)

In summary, these findings are the first to provide histopathologic evidence that rituximab treatment in SS can induce reduction of glandular inflammation and structural redifferentiation of lymphoepithelial duct lesions, correlating to a gain in function of the glands, especially with respect to improved function of the structurally redifferentiated striated ducts. The decrease in lymphocytic infiltration, the number of germinal centers, intraepithelial lymphocytes, and acinar proliferation, combined with redifferentation of lymphoepithelial lesions in 3 patients, suggests efficacy of B cell depletion in salivary glands. A larger placebo-controlled randomized clinical trial investigating the immunohistologic correlation of sequential biopsy specimens obtained before and after therapy has been started by our group in order to prove the findings suggested in this uncontrolled study.

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Chapter 5a

Treatment of primary Sjögren's syndrome with rituximab: extended follow-up, safety and efficacy of retreatment

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Introduction

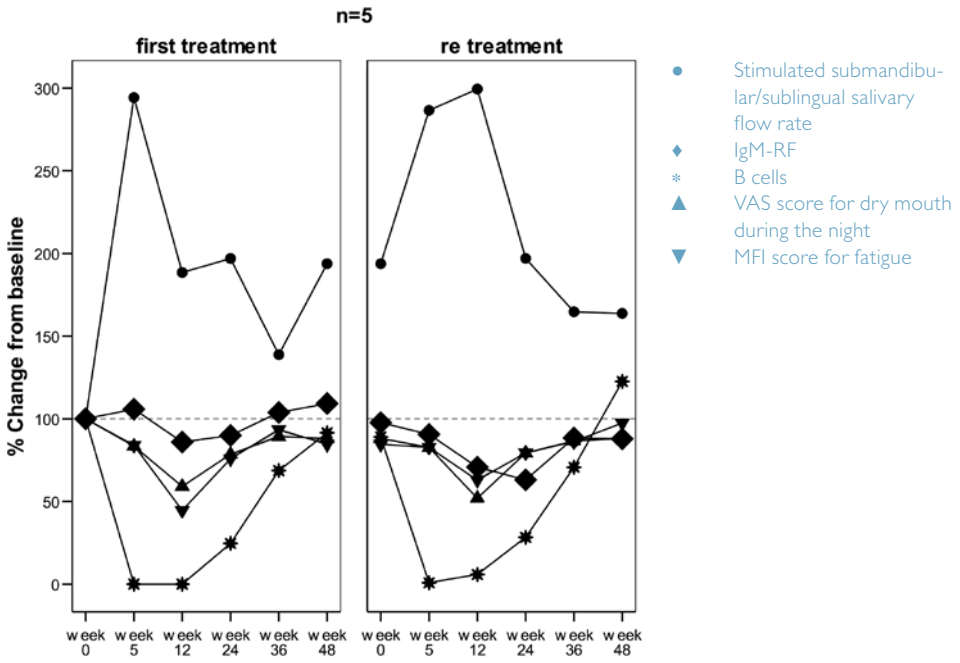
We previously reported that B cell depletion therapy with rituximab (4 weekly infusions of 375 mg/m2, premedication: 25 mg prednisolone intravenously) in eight patients with early primary Sjögren's syndrome (pSS) and 7 patients with mucosa-associated lymphatic tissue (MALT)/pSS was effective in reducing subjective and objective symptoms after 12 weeks of follow-up.(1) Three patients with early pSS developed serum sickness-like disease, of whom one patient declined to further participate. The MALT component of six of the 7 patients with MALT/pSS was initially effectively treated with rituximab, one of these six patients was successfully retreated 9 months after the first treatment and all six patients are still in remission of MALT > 2 years after treatment.

Patients and methods

We focused the present work on the extended follow-up and retreatment of the patients with early pSS. For seven of the eight patients with early pSS, 48 week follow-up data were available. In addition, five patients, who did not develop serum-sickness and in whom symptoms returned, were retreated with four infusions of rituximab and followed for another 48 weeks. Return of symptoms included decrease of salivary flow, increase of rheumatoid factor and return of B cells and subjective symptoms.

Figure 1

Increase and decrease (mean values of 5 patients with primary Sjögren syndrome (pSS)) in stimulated submandibular/sublingual flow rate, IgM-rheumatoid factor (RF), B cells, visual analogue scale (VAS) score for dry mouth during the night and Multidimensional Fatigue Inventory (MFI) score for fatigue following rituximab (re)treatment (baseline is 100%). Mean (SD) baseline values (week 0 first treatment) were: stimulated submandibular/sublingual flow rate 0.09 (0.07) ml/min, IgM-RF 339 (329) kIU/l, B cells 0.19 (0.09) 10^9/liter, VAS score for dry mouth during the night 85 (12), MFI score for fatigue 16 (3).



Results

First course of rituximab (n=7)

Depletion of peripheral B cells was complete 5 weeks after onset of therapy. By 36 weeks, median peripheral B cell numbers had returned, although levels were still low in some patients. Stimulated submandibular/sublingual salivary flow showed a significant increase at week 12, followed by a gradual decline to just above baseline at 48 weeks. Similarly, a significant improvement of most of the visual analogue scale (VAS) scores for dry mouth and most domains of the Multidimensional Fatigue Inventory (MFI) was observed, followed by a gradual decline to near baseline.

Retreatment with rituximab (n=5, figure 1)

Retreatment had a significant effect on B cells, levels of IgM-rheumatoid factor (RF) and stimulated submandibular/sublingual salivary flow similar to the effects of the first course. VAS scores for dry mouth, MFI scores for general fatigue and SF-36 questionnaire scores for physical functioning improved significantly too. For the other subjective symptoms a similar trend towards improvement was seen as after the first course. Again, almost all variables had returned to baseline 6-9 months after retreatment. One patient developed serum sickness-like disease (purpura, arthralgia, myalgia) after the second rituximab infusion during the retreatment course. Rituximab treatment was stopped, pain relief (non-steroidal anti-inflammatory drugs (NSAIDs)) and 120 mg methylprednisolone was given once. The patient recovered completely.

Discussion

Rituximab appeared to be effective for at least 6-9 months in patients with pSS with active disease, improving both subjective and objective symptoms. Development of serum sickness-like disorder in a substantial number of patients with pSS indicates that higher doses of corticosteroids might be needed during treatment. Retreatment resulted in a good clinical response in patients with pSS comparable to the response in patients with RA (2) and patients with systemic lupus erythematosus (SLE).(3) Based on these promising results, one might consider maintenance treatment. The best approach to and timing of maintenance treatment has, however, to be studied in future trials. Furthermore, attention has to be paid to among others the possibility of development of humoral immunodeficiency related to repeated treatment.(2)

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Chapter 5c

Effectiveness of rituximab treatment in primary Sjögren's syndrome: a randomized, double-blind, placebo-controlled trial

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Abstract

Objective To study the efficacy and safety of B cell depletion with rituximab, a chimeric murine/human anti-CD20 monoclonal antibody, in a double-blind, randomized, placebo-controlled trial of patients with primary Sjögren's syndrome (pSS).

Methods Patients with active pSS, as determined by the revised European-US criteria, and a stimulated whole saliva secretion ≥0.15 ml/min, were treated with either rituximab (1000 mg) or placebo infusions at days 1 and 15. Patients were assigned randomly in a 2:1 ratio (rituximab:placebo). Follow-up was conducted at 5, 12, 24, 36 and 48 weeks. The primary endpoint was stimulated whole salivary flow rate; secondary endpoints included functional, laboratory and subjective variables.

Results Thirty patients (29 female) were randomly allocated to treatment. Mean ages in the rituximab and placebo groups were 43±11 and 43±17 years, and disease duration was 63±50 and 67±63 months, respectively. In the rituximab group, significant improvements, in terms of the mean change from baseline compared with that in the placebo group were found for the primary endpoint of secretion of stimulated whole saliva ($p=0.038$), and for various laboratory parameters (B cells, rheumatoid factor), subjective parameters (multidimensional fatigue inventory (MFI) scores and visual analogue scale (VAS) scores for sicca complaints) and extraglandular manifestations. Moreover, rituximab treatment significantly improved stimulated whole saliva secretion ($p=0.004$) and several variables (e.g., B cells, rheumatoid factor, unstimulated and stimulated whole saliva, lissamine green test, MFI, short-form 36 (SF-36) and VAS scores), compared with baseline values. One patient developed mild serum sickness-like disease.

Conclusions This study indicated that rituximab is an effective and safe treatment modality for patients with pSS.

Introduction

Sjögren's syndrome (SS) is a systemic auto-immune disease characterized by chronic inflammation of the salivary and lachrymal glands, resulting in xerostomia and keratoconjunctivitis sicca in about 95% of patients.(1) These symptoms are frequently accompanied by extraglandular manifestations (EGM) such as Raynaud's phenomenon, arthritis, arthralgia and myalgia, and 85% of the patients suffer from severe fatigue. Moreover, B cell hyperactivity, reflected by increased serum levels of IgG and IgM-rheumatoid factor (IgM-RF) and the presence of anti-SS-A and anti-SS-B autoantibodies, is a common finding in SS. Furthermore, SS has a large impact on health-related quality of life, employment and disability as reflected by lower SF-36 scores and employment rates, and higher disability rates in SS patients relative to the general population.(1)

To date, no causal systemic treatment has been available for SS. In pilot trials, however, it has been shown that rituximab, a chimeric murine/human anti-CD20 monoclonal antibody which binds to the B cell surface antigen CD20, might improve subjective and objective symptoms related to primary SS (pSS) for at least 6-9 months.(2;3) Based on these promising results, a randomized, double-blind, placebo-controlled trial was performed to investigate the efficacy and safety of rituximab in the treatment of patients with pSS.

Patients and methods

Study design

This was a prospective, single-centre, randomized, double-blind, placebo-controlled study. The study protocol was approved by the institutional review board of the University Medical Center Groningen. All patients provided written informed consent.

Patients

All patients were ≥18 years and fulfilled the European–US criteria for pSS.(4) Eligibility criteria were a stimulated whole saliva secretion ≥0.15 ml/min and positivity for autoantibodies (IgM-RF ≥10 kIU/l and anti-SS-A and/or anti-SS-B autoantibodies). A recent salivary gland biopsy (≤12 months before inclusion) showing characteristic features of SS must be available.(5) During the study, patients were asked to use reliable methods of contraception. Secondary SS patients and pSS patients who had been treated previously with other monoclonal antibodies were excluded. Treatment with prednisone and hydroxychloroquine had to be discontinued at least one month before baseline, and treatment with methotrexate, cyclophosphamide, cyclosporin, azathioprine and other disease-modifying anti-rheumatic drugs at least 6 months before baseline. Patients were allowed to use artificial tears and artificial saliva, but the regimen had to remain identical during follow-up. The use of these substitutes had to be stopped one day prior to each assessment. All patients underwent a baseline electrocardiogram and chest radiography. Patients with a history of any malignancy, with underlying cardiac, pulmonary, metabolic, renal or gastrointestinal conditions, with chronic or latent infectious diseases, or with immune deficiency were excluded.

Drug administration

Twenty patients were treated with intravenous (i.v.) infusions of 1000 mg rituximab (Roche, Woerden, The Netherlands) and 10 patients were treated with i.v. placebo infusions on days 1 and 15. To minimize side effects (infusion reactions, serum sickness), all patients were pre-medicated with methylprednisolone (100 mg/i.v.), acetaminophen (1000 mg/p.o.) and clemastine (2 mg/i.v.), and received 60 mg oral prednisone on days 1 and 2, 30 mg on days 3 and 4, and 15 mg on day 5 after each infusion.

Outcome parameters

The primary endpoint was defined as a significant improvement of secretion of stimulated whole saliva (ml/min) in the rituximab group compared with the placebo group.
Secondary endpoints were salivary/lachrymal function, and immunological and subjective variables. All variables were assessed at baseline (within 4 weeks before treatment), and at weeks 5, 12, 24 and 48 after treatment.

Salivary gland function

Whole, parotid and submandibular/sublingual saliva were collected in a standardized manner and at a fixed time of the day (in this study between 1 and 4 p.m.) in order to minimize fluctuations related to a circadian rhythm of salivary secretion (6;7) and composition. Glandular saliva was collected from both individual parotid glands by use of Lashley cups and submandibular/sublingual saliva was collected simultaneously by syringe aspiration from the area with the orofices of the submandibular excretory ducts. Unstimulated saliva was

collected the first 5 minutes, followed by stimulated saliva for 10 minutes. The salivary glands were stimulated by citric acid solution (2%), applied with a cotton swab to the lateral borders of the tongue every 30 seconds. Flow rates were calculated and composition of saliva was analyzed according to the methods described by Burlage et al. and Kalk et al.(8-10)

Lachrymal gland function

Lachrymal gland function was evaluated by performing a Schirmer's test, a lissamine green (LG) test and measuring break-up time (BUT) according to the methods described in detail by Kalk et al.(11)

Laboratory assessments

Laboratory assessments included serum biochemical analysis and complete blood cell count. Levels of immunoglobulins (IgG, IgA, IgM) and IgM-RF were measured by nephelometry. Numbers of circulating CD19+, CD4+ and CD8+ T cells were quantified by FACSCalibur flow cytometer using TruCOUNTTM tubes (Becton Dickinson). The absolute number was determined by comparing cellular events to beads events using CellQuest software (Becton Dickinson).

Subjective assessments

Patients completed the Multidimensional Fatigue Inventory (MFI)(12) and the SF-36.(13) In addition, a 100-mm Visual Analogue Scale (VAS) was used for rating oral and ocular sicca complaints.

Extraglandular manifestations (EGM)

Arthralgia, arthritis, renal involvement, oesophageal involvement (confirmed by oesophageal scintigraphy), polyneuropathy, Raynaud's phenomenon, tendomyalgia and vasculitis were defined as EGM. At each visit, EGM were scored as present or not according to protocol.

Serum sickness

Serum sickness was defined as development of fever, lymph node swelling, purpura, myalgia, arthralgia, thrombocytopenia and proteinuria, and decrease in complement levels. Serum sickness-like disease was defined as development of some of the symptoms of serum sickness.

Sample size

Based on a formal sample size calculation, 30 patients were included, 20 assigned to rituximab and 10 to placebo. The patients were randomly assigned by the pharmacy department, using a random-number generator on a computer, to one of the two treatment arms in a 2:1 ratio (rituximab:placebo) in blocks of three. Investigators (who also provided care and assessed the outcome variables) and patients were blinded to the assigned study medication. The code was revealed to the investigators after follow-up of all patients was completed. Because of the double-blind design, we assumed a 5% rate of false-positive patients in the placebo group with clinical signs of serum sickness. This resulted in an obligation to terminate the trial if two patients developed clinical signs of serum sickness after the first or second infusion within the first nine patients, or if three patients developed clinical signs of serum sickness after the first or second infusion within the first 29 patients. If for any reason the protocol was terminated, patients were not replaced.

Statistical analyses

All data analyses were carried out according to a pre-established plan. To compare treatment effects in time between the two treatment groups, repeated measurements ANOVA was performed. To determine whether an improvement had occurred over time relative to baseline, repeated measurements ANCOVA was performed on change from baseline data. Statistical analyses performed on secondary endpoints are considered to be of explorative nature. Therefore no corrections were made for multiple comparisons. The assumptions on homogeneity were met. If data were not normally distributed, a log-transformation was performed on the data prior to statistical analysis or a distribution-free alternative was used.

Results

Patients

Between August 2006 and September 2007, 30 patients were randomly assigned to treatment (figure 1). Baseline characteristics are summarized in table 1. Six patients used medication which had to be discontinued before inclusion according to the inclusion criteria.

Efficacy (table 2)

Salivary gland function

Stimulated whole saliva (figure 2a; primary endpoint) significantly improved in the rituximab group ($p=0.018$ at week 5 and $p=0.004$ at week 12) while values in the placebo group significantly decreased in accordance with the natural progression of the disease. A significant difference in the mean change from baseline in the stimulated whole salivary flow between the groups ($p=0.038$) was found at week 12. Unstimulated whole salivary flow (figure 2b) and submandibular/sublingual flow also significantly increased in the rituximab group.

Lachrymal gland function

The LG test showed significant improvement in the rituximab group at weeks 5 to 48, whereas the Schirmer and BUT tests revealed no significant changes.

Laboratory assessments

B cells were completely depleted in patients treated with rituximab after the first infusion (figure 2c). No significant changes were found in B cell levels in the placebo group. In the patient with serum sickness (see safety section below), who received only one infusion of rituximab, B cells reappeared within 12 weeks after treatment. In the other 19 rituximab-treated patients, B cells returned within 24 to 48 weeks after treatment, although B cell levels still had not returned to baseline by week 48. Significant differences in the mean change in absolute B cell count from baseline between the groups were found at weeks 5, 12, 24, 36 and 48 ($p<0.05$). No significant changes were found in CD4+ and CD8+ T cell levels in either the rituximab or placebo groups. Rheumatoid factor (figure 2d) levels decreased significantly in the rituximab group over weeks 5 to 36, and in the placebo group at week 5. Significant differences in the mean change in rheumatoid factor levels from baseline between the groups were found at weeks 12, 24 and 36 ($p<0.05$). The same pattern of change was found for levels of IgG, IgM and IgA (results not shown).

Table 1 Patient characteristics. Baseline characteristics of the patients in the placebo and rituximab treatment groups. UWS=unstimulated whole saliva, SWS=stimulated whole saliva.

Variable	Placebo (n=10)	Rituximab (n=20)
Age (years)	43±17	43±11
Gender	10 females	1 male, 19 females
Disease duration (months)	67±63	63±50
IgG (g/l)	21±7	23±8
IgM-RF (kIU/l)	221±245	102±79
Anti-Ro/SSA positive	10 (100%)	20 (100%)
Anti-La/SSB positive	8 (80%)	14 (70%)
Parotid gland swelling	10 (100%)	17 (85%)
UWS, ml/minute*	0.06±0.09	0.17±0.19
SWS, ml/minute	0.42±0.26	0.70±0.57
Extraglandular manifestations		
Arthralgia	5 (50%)	15 (75%)
Arthritis	0 (0%)	6 (30%)
Renal involvement	0 (0%)	2 (10%)
Oesophageal involvement	1 (10%)	0 (0%)
Peripheral polyneuropathy	0 (0%)	1 (5%)
Raynaud's phenomenon	6 (60%)	11 (55%)
Tendomyalgia	8 (80%)	17 (85%)
Vasculitis	3 (30%)	6 (30%)
Thyroid dysfunction	0 (0%)	1 (5%)
Use of artificial tears	8 (80%)	14 (70%)
Use of artificial saliva	2 (20%)	2 (10%)

*Significant difference ($p<0.05$) between placebo and rituximab group.

Subjective assessments

MFI and SF-36 scores showed the strongest improvements in the rituximab group (figures 2e, 2f). Between the two treatment groups, a significant change was found for the MFI score for reduced activity ($p=0.023$) at week 36, the MFI score for reduced motivation ($p=0.039$) at week 12 and the SF-36 score for vitality ($p=0.013$) at week 36. Moreover, all VAS scores improved in the rituximab group (figures 2g, 2h; table 2), while scores in the placebo group only showed a significant improvement at week 5. Significant differences in VAS scores between the groups were seen for dry mouth during the night ($p<0.05$) at weeks 24, 36 and 48, and dry eyes ($p<0.05$) at weeks 36 and 48.

Extraglandular manifestations

At baseline there were no differences between the rituximab and placebo group (figure 2i). The number of reported EGM (absent or present) significantly decreased in the rituximab group compared to placebo for tendomyalgia at weeks 12 and 36 ($p=0.029$) and for vasculitis at week 24 ($p=0.030$). In addition, there was a strong tendency towards a decrease in the number of reported complaints of Raynaud's phenomenon ($p=0.057$), tendomyalgia ($p=0.074$) and arthralgia ($p=0.058$) at week 24. Six patients in the rituximab

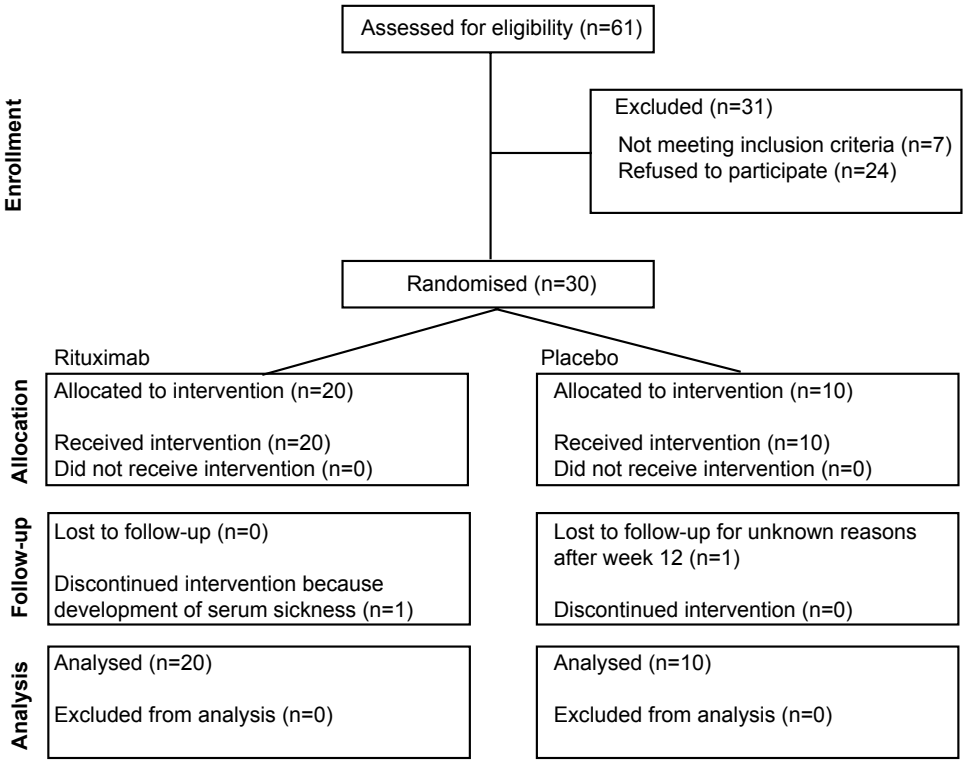
Table 2 Results of laboratory, functional and subjective assessments for the placebo and rituximab treatment groups (mean±SD (median)) at the assessed time-points. UWS=unstimulated whole saliva, SWS=stimulated whole saliva.

Variable	Baseline Placebo Rituximab	Week 5 Placebo Rituximab	Week 12 Placebo Rituximab	Week 24 Placebo Rituximab	Week 36 Placebo Rituximab	Week 48 Placebo Rituximab
UWS, ml/minute $ +	0.06±0.09 (0.03) 0.17±0.19 (0.08)	0.09±0.07 (0.08) 0.24±0.22 (0.20)*	0.05±0.05 (0.04) 0.23±0.22 (0.19)*	0.08±0.08 (0.09) 0.22±0.25 (0.14)	0.07±0.09 (0.02) 0.16±0.15 (0.11)	0.05±0.04 (0.04)* 0.18±0.18 (0.13)
SWS, ml/minute $	0.42±0.26 (0.36) 0.70±0.57 (0.47)	0.41±0.24 (0.37) 0.84±0.71 (0.48)*	**0.28±0.17 (0.25)*** **0.87±0.87 (0.56)***	0.36±0.28 (0.24) 0.74±0.60 (0.52)	0.29±0.18 (0.26)* 0.64±0.58 (0.44)	0.28±0.21 (0.22)* 0.66±0.71 (0.42)
Schirmer's, mm/5 minutes $	7±9 (3) 11±11 (7)	7±11 (4) 10±9 (10)	6±5 (5) 11±10 (11)	8±8 (6) 12±12 (5)	7 ±7 (5) 11 ±10 (7)	5±5 (6)* 10±11 (7)
Lysamine green $	4±1 (4) 3±2 (4)	5±1 (5) 3±2 (3)*	4±2 (4) 3±2 (3)*	4±2 (4) 2±2 (2)*	4 ±2 (4) 2 ±2 (2)*	4±2 (4) 2±3 (1)*
Tear break up time, seconds $ +	3±2 (3) 6±2 (6)	3±1 (3) 6±3 (6)	3±2 (3) 5±3 (5)	5±2 (6)* 6±3 (7)	5 ±3 (5)* 7 ±3 (8)*	4±3 (4) 6±3 (8)
B cells, 10^9/l $	0.27±0.12 (0.28) 0.21±0.17 (0.18)	**0.20±0.09 (0.17)*** **0.00±0.00 (0.00)***	**0.25±0.10 (0.27)** **0.01±0.03 (0.00)***	**0.28±0.11 (0.26)** **0.05±0.08 (0.03)***	**0.28 ±0.12 (0.31)** **0.10 ±0.08 (0.08)***	**0.33±0.15 (0.37)** **0.17±0.10 (0.15)***
IgM-R, kIU/l $	221±245 (108) 102±79 (83)	162±175 (96)* 55±36 (53)*	**156±138 (102)** **44±30(36)***	**258±260 (113)** **45±34 (32)***	**253 ±256 (119)** **71 ±68 (54)***	225±199 (126) 103±103 (72)
MFI, general fatigue	14±5 (17) 16±4 (18)	11±5 (12)* 15±4 (16)	13±5 (14) 13±4 (13)*	12±5 (12) 13±4 (12)*	*14 ±4 (14)* *14 ±4 (14)*	14±6 (17) 15±4 (16)
SF-36 total	64±17 (65) 52±20 (53)	70±17 (70) 56±18 (52)	67±15 (71) 63±15 (65)*	72±16 (82) 67±16 (70)*	63 ±16 (65) 60 ±17 (64)*	62±17 (62) 55±18 (55)
VAS oral dryness	59±28 (62) 55±28 (61)	50±28 (53) 47±27 (53)*	53±30 (60) 40±27 (40)*	*64±27 (74)* *34±27 (46)**	68 ±26 (79) 51 ±28 (61)*	*69±25 (76)* *50±28 (53)**
VAS dry eyes	65±27 (63) 59±29 (68)	55±28 (52) 49±28 (51)*	61±25 (54) 48±29 (47)*	*68±24 (74)* *41±28 (43)**	**70 ±27 (72)** **46 ±27 (52)***	**76±19 (80)** **46±28 (55)***

(mean±SD (median). $no normal distribution. *P<0.05 versus baseline in the same patient group, by ANCOVA analysis, + significant difference (p<0.05) between placebo and rituximab group at baseline. **Bold**: comparison of change from baseline between the placebo and rituximab group, by ANOVA analysis, results in a significant difference (p<0.05) *Italic*: comparison of change from baseline between the placebo and rituximab group, by ANOVA analysis, results in a difference (p>0.05 and p<0.10). Due to missing data, the differences between means in this table differ slightly from the means of differences as displayed in the figures.

Figure 1

Patient flow. Out of a cohort of 300 patients, a preselection was made of 61 patients based on last available sialometry, IgG, anti-SSA and anti SSB positivity and rheumatoid factor data.



group had complaints of arthritis at baseline; this resolved in four patients during follow-up. In the placebo group, no patients had symptoms of arthritis at baseline; however, three patients developed symptoms during follow-up. One patient with decreased thyroid function before rituximab treatment showed a normalization of thyroid function without additional thyrostatic supplementation. Renal function (two patients had renal tubular acidosis; both were treated with rituximab) remained stable during follow-up. Clinical symptoms of polyneuropathy (one patient; rituximab) improved after 12 weeks of follow-up.

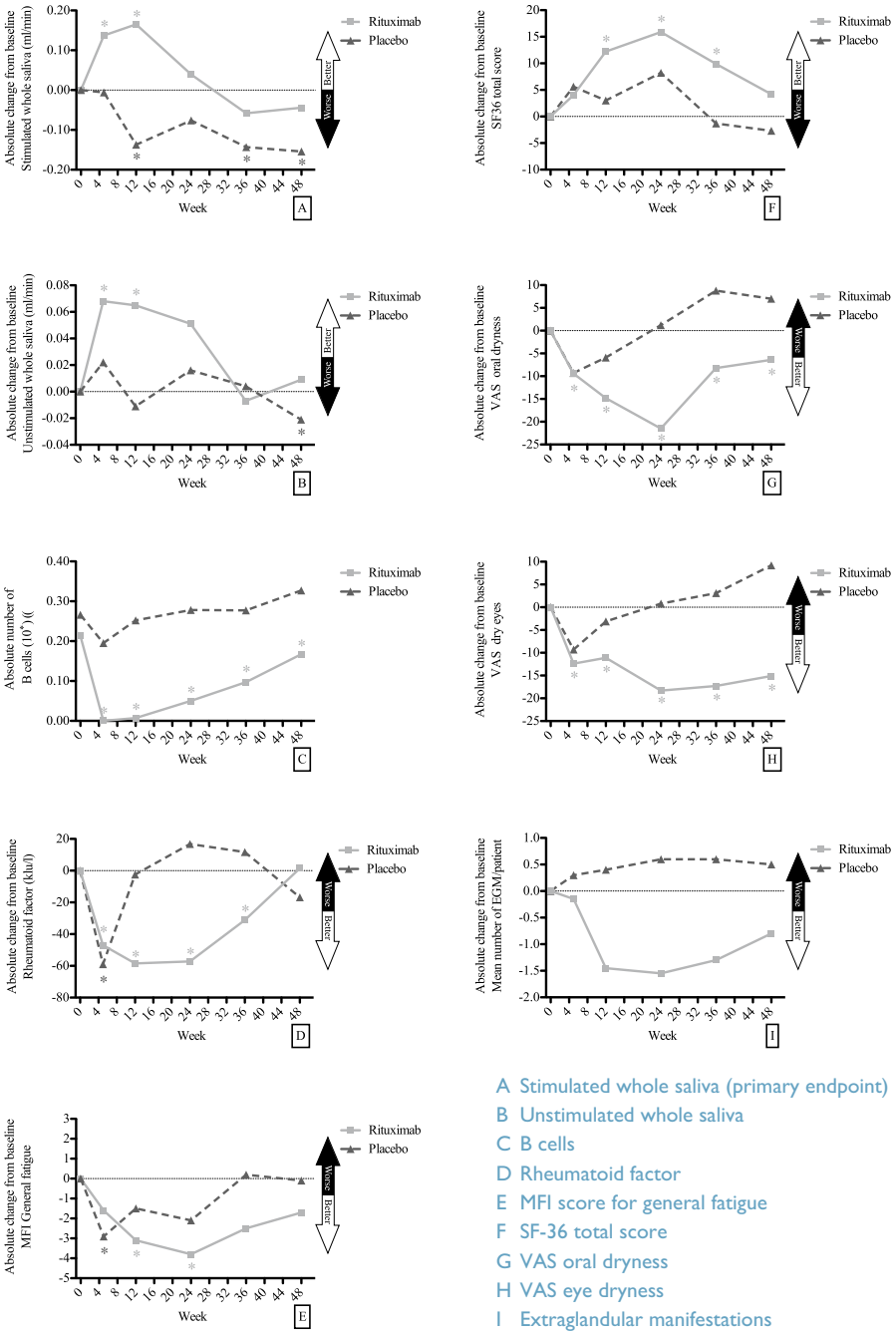
Safety (table 3)

Serum sickness

One diabetic patient developed a mild serum sickness-like disease, 14 days after the first infusion. She developed fever, purpura on both legs and arthralgia. She was admitted to hospital in order to control her serum glucose levels during administration of i.v. corticosteroids and non-steroidal anti-inflammatory drugs, and recovered completely in a few days without developing human anti-chimeric antibodies. The second infusion was not administered. This patient had not been treated with any immunosuppressive drug

Figure 2

Figures A, B, D, E, F, G, H and I are depicted as mean values of absolute change from baseline. Figure C is depicted as mean values of absolute numbers. (*) indicate significant ($p<0.05$) differences within the groups compared with baseline.



A Stimulated whole saliva (primary endpoint)
B Unstimulated whole saliva
C B cells
D Rheumatoid factor
E MFI score for general fatigue
F SF-36 total score
G VAS oral dryness
H VAS eye dryness
I Extraglandular manifestations

previously, while none of the 6 patients who had discontinued immunosuppressive drugs 1 to 6 months prior to rituximab treatment developed serum sickness-like disease.

Infections

Twelve of the infections were reported by eleven patients in the rituximab group; four patients in the placebo group reported a total of seven infections. The rates of infection were 76 and 65 events per 100 patient-years for the placebo and rituximab groups, respectively. None of the infections required hospitalization. No opportunistic infections were seen.

Discussion

This study showed that rituximab-induced B cell depletion can be considered an effective and safe treatment modality for patients with pSS. B cell depletion resulted in improvement of objective and subjective parameters of disease activity in pSS patients for at least 6 to 9 months. Amongst others, salivary function improved, fatigue diminished and the number of EGM was reduced.

Rituximab has already been shown to be a safe and effective treatment for rheumatoid arthritis (RA) resulting in a decrease in disease activity, diminished radiological progression and an improved quality of life.(14-16) Previously, the utility of rituximab for the treatment of SS had only been investigated in a few open-label, Phase II studies and one randomized, double-blind, placebo-controlled study. Results from open-label studies in terms of objective and subjective variables were promising(2;3) as was the improvement of systemic features. (17) Although the duration of treatment effect differed between the trials, in all trials a significant effect occurred 12 to 24 weeks after treatment. In the randomized, double-blind, placebo-controlled study of rituximab treatment of SS, a significant improvement in fatigue (primary endpoint) was noted compared with baseline data in the rituximab group, but there were no significant changes in secondary endpoints assessing glandular manifestations (unstimulated salivary flow, Schirmer test).(18) Moreover, this study by Dass et al.(18) used a less accurate objective eye test (Schirmer test); the Rose Bengal score and LG are considered to be more accurate.(11) This fact together with the small number of patients included in the trial (eight rituximab, nine placebo), might explain the lack of significant improvement in glandular manifestations following rituximab treatment.

In our trial, most significant improvements in endpoints associated with rituximab treatment were observed between 12 and 36 weeks following treatment. By contrast, improvement of most of the variables observed in patients in the placebo group occurred 5 weeks after the first infusion. We hypothesize that the improvements observed after placebo treatment are related to the prednisolone these patients had received before and during the days after the infusions, although data are inconclusive regarding the effect of prednisolone on SS symptoms. Although one study reported a significant increase in whole saliva during the use of low-dose prednisolone,(19) other studies noted no significant improvement in glandular function.(20;21)

Stimulated whole saliva provides a general indication of overall salivary glandular function, which is an important outcome in a disease that specifically affects salivary glands. Pijpe et al. (3) reported a significantly increase of stimulated whole saliva in rituximab treated pSS patients whose stimulated salivary flow rate was >0.10 ml/minute at baseline. These patients

Table 3 Adverse events observed in patients following treatment with rituximab or placebo.

	Events	Placebo (n=10)	Rituximab (n=20)
Early infusion reaction		0	2 (10%)
Late infusion reaction		0	2 (10%)
Serum sickness		0	1 (5%)
Infections within 2 weeks after infusion	Upper airway infection	0	1 (5%)
	Parvovirus	0	1 (5%)
Infections during 48 weeks of follow-up	Otitis media	0	2 (10%)
	Upper airway infection	4 (40%)	4 (20%)
	Recurrence of ocular toxoplasmosis	0	1 (5%)
	Parotid gland infection	0	3 (15%)
	Recurrence of herpes zoster	1 (10%)	0
	Epstein-Barr virus	1 (10%)	0
	Rubella	1 (10%)	0

also showed significant improvement of subjective parameters as mouth dryness, arthralgia, physical functioning, vitality and most domains of the MFI. In other words, patients with some residual secretory potential may benefit the most from rituximab treatment. The secretory potential at baseline might even discriminate between patients that are considered to be good responder to rituximab treatment or not. Therefore, stimulated whole saliva was chosen as the primary endpoint of our study. As cut off value, a stimulated whole saliva flow rate ≥0.15 ml/min was chosen as this is a flow rate that discriminates between patients showing disease activity (e.g., progressive loss of secretory function) and patients with an end stage pSS.(21)

We observed an increase in salivary flow in the rituximab group that exceeded the intra-patient variability observed for repeated collections of saliva.(8) This increase is also reflected by the improvements of subjective scores for dry mouth and indicates that these changes are clinically meaningful for the patients. The, non-significant, baseline difference between the groups for the flow rate of stimulated whole saliva was caused by high salivary flow rates before inclusion in a few patients. All patients in the study were required to have a stimulated whole saliva flow ≥0.15 ml/min. This meant that all patients had a clinically relevant functional secretory salivary gland capacity. Our pilot study revealed that no relevant improvement in salivary gland function can be expected in patients with little or no secretory potential at baseline.

In RA clinical trials of rituximab, the number of reported (serious) infections and infusion reactions is within the range expected for patients with RA treated with biological agents. Therefore, the risk:benefit ratio is considered to be good regarding rituximab treatment of RA.(22) In clinical trials of rituximab treatment of other autoimmune diseases (including SS), reported numbers of infusion reactions and infections vary widely; this is possibly due to variability in how these adverse events are defined or to small patient numbers. The incidence of infusion reactions and infections reported for the rituximab group in this trial was largely comparable to that of the placebo group and was lower or within the same range

as that reported previously.(23) Also, the rate of infections per 100 patient-years is lower compared with the rate in RA patients treated with rituximab. This might be explained by the fact that our patients did not use any other immunosuppressive therapy.(24)

When compared to lymphoma patients, RA patients and systemic lupus erythematosus (SLE) patients treated with rituximab, patients with pSS develop serum sickness(-like) disease more frequently (6% to 27%).(25) A therapy-related explanation for this phenomenon might be that usually higher doses of steroids and/or other immunosuppressive drugs besides rituximab have been or are given to RA and SLE patients, whereas our pSS patients received no other co-medication than a 5 days period of steroids after i.v. administration of rituximab. Another therapy-related explanation is that RA and SLE patients often have been treated with intensive immunosuppressive regiments including biological agents before they were subjected to rituximab treatment, whereas our pSS patients are far more medication-naïve at the time of rituximab treatment. The higher susceptibility for serum sickness could also be inherent to the disease itself. The pSS patients in this trial, as well as in our pilot trial, (3) who developed serum sickness were more likely to have an active, early and progressive form of SS. It is possible that such pSS patients are more prone to develop serum sickness. Furthermore, hypergammaglobulinemia is common in pSS patients, which could make these patients prone to the development and deposition of immune complexes and thus to serum sickness(-like) disease.(18)

Because of the higher risk of developing serum sickness(-like) disease in SS patients, we decided to increase the steroid dose. Only one patient in the current study developed serum sickness-like disease (5%), which is considerably lower than the incidence reported in our open-label study (27%).(3) Based on these findings, we would recommend administering 100 mg methylprednisolone immediately prior to each infusion of rituximab. The oral regimen of prednisolone in the days following each infusion is a point of interest and should be explored in future trials. The administration of higher doses of prednisolone in the days following infusion, such as is performed during lymphoma treatment, should also be considered.

This study indicates that rituximab treatment could be effective for pSS patients with active disease and remaining salivary gland secretory potential as well as for pSS patients with EGM. Future trials with rituximab in pSS are warranted with inclusion of larger groups of patients and with defining less strict inclusion criteria (e.g., no restriction to salivary gland function ≥ 0.15 ml/min and auto-antibody positivity) in order to be able to extrapolate the results to a larger group of pSS patients. Besides inclusion criteria, attention should be given to defined criteria for response to treatment. Activity scores for pSS have now been developed and wait for validation. These scores should be included in response criteria to be used in future trials.

Based on the promising results of this study and on our study on retreatment with rituximab, which resulted in a beneficial effect comparable to that of the first treatment with this biological (26), a maintenance therapy with rituximab infusions every 6 to 9 months may be a reasonable approach. Advantages of maintenance therapy might be a reduction or even arrest of disease progression and improvement of quality of life for a long period. This improvement will be a great achievement in SS patients, as SS has a large impact on health-related quality of life, employment and disability.(1) A threat might be the, so far unknown, long-term side effects of repeated B cell depletion. The timing of retreatment could be based on return of symptoms, however, retreatment just before return of symptoms would even be better.

In conclusion, this study indicates that rituximab could be an effective and safe treatment

modality for patients with pSS. B cell depletion resulted in improvement of the primary endpoint stimulated whole saliva. Explorative analyses also showed improvements for at least 6 to 9 months' duration of objective and subjective secondary endpoints of disease activity. As pSS has a great impact on health-related quality of life, employment and disability(1), it is worthwhile to further explore the role of rituximab in a large size randomized controlled trial.

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Chapter 6

Sjögren's syndrome and localized nodular cutaneous amyloidosis: coincidence or a distinct clinical entity?

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Abstract

Objective To report 8 patients with Sjögren's syndrome (SS) and localized nodular cutaneous amyloidosis and to examine serologic and immunohistologic findings that may link the 2 diseases.

Methods The databases of 3 amyloidosis centers were searched for patients with localized nodular cutaneous amyloidosis and SS. Eight patients with this combination were identified, and clinical, serologic and histologic parameters were retrospectively evaluated.

Results Among the 8 patients with a clinical diagnosis of SS, 6 fulfilled the American-european Consensus Group criteria for SS. All of the patients were women in whom SS had been diagnosed at a median age of 47 years (range 30-61 years) and amyloid had been diagnosed at a median age 60 years (range 42-79 years). The presence of the immunoglobulin light chain type of amyloid (AL) was confirmed in 4 patients. In 3 of these 4 patients as well as 2 other patients, a light chain-restricted plasma cell population was observed near the amyloid deposits. Progression to systemic amyloidosis was not observed in any patient during a median follow-up of 3.5 years.

Conclusions SS should be considered in patients with cutaneous amyloidosis. The combination of cutaneous amyloid and SS appears to be a distinct disease entity reflecting a particular and benign part of the polymorphic spectrum of lymphoproliferative diseases related to SS.

Introduction

Sjögren's syndrome (SS) is a chronic lymphoproliferative autoimmune disease characterized by disturbances in T lymphocytes, B lymphocytes and exocrine glandular cells.(1) SS can be primary or secondary, with the latter entity being associated with another autoimmune disease such as rheumatoid arthritis or systemic lupus erythematosus. Lymphocytic infiltrates, consisting of T lymphocytes and B lymphocytes, are a characteristic histopathologic finding in SS. The presence of autoantibodies and hypergammaglobulinemia is considered to reflect polyclonal B lymphocyte hyperactivity. Systemic complications of SS are associated with this polyclonal B lymphocyte hyperactivity and with the development of clonal B lymphocyte proliferation. As a reflection of the latter, a malignant B cell lymphoma develops in ~5% of patients with SS.(2)

Amyloidosis refers to a variety of protein-folding diseases caused by extracellular deposition of amyloid fibrils. The peptide subunit of the protein fibrils varies among the different types of amyloidosis and is the basis for the current chemical classification.(3) AL amyloid (formerly called primary amyloid) refers to the immunoglobulin light chain-associated amyloid, and AA amyloid (formerly called secondary amyloid) refers to the inflammation-associated amyloid. The diagnosis of amyloidosis is based on the characteristic apple-green birefringence under polarized light of a biopsy specimen stained with Congo red.(4) Amyloidosis can be divided in systemic and localized forms. In the systemic form, there is widespread deposition of amyloid in organs and tissues and in the localized form, amyloid deposition is restricted to one single organ, tissue, or site of the body.(4;5)

Although it is very rare, systemic amyloidosis has been observed in patients with SS; systemic AA amyloidosis may occur because of longstanding inflammation(6), and systemic AL amyloidosis may occur because of the development of an immunoglobulin light chain-producing lymphoproliferative disease.(7) Systemic AL amyloidosis itself can affect the lacrimal and salivary glands and is therefore one of the causes of sicca syndrome.(7) SS has also been associated with the presence of localized amyloid in sites such as the lungs(8), the breast(9), the tongue(10), and the skin.(11) Three types of localized cutaneous amyloidosis can be recognized: macular, lichen and nodular types, of which the nodular type is the rarest.(12) Nodular cutaneous amyloidosis has been related frequently to deposition of immunoglobulin light chains that have been produced by a clonal plasma cell population. The typical clinical presentation involves single or multiple nodules or plaques on the trunk or limbs.(11) Deposition of amyloid usually takes place in the dermis, subcutis and associated blood vessels.(12;13)

The estimated prevalence of SS in the general population is ~0,5-2%.(14;15) Cutaneous nodular amyloidosis is extremely rare, with ~60 cases reported in the literature.(16) Despite the rare occurrence of cutaneous amyloidosis, 16 cases have been reported in patients with SS, and these 16 cases represent ~25% of the reported cases.(11-13) The link between cutaneous amyloidosis and SS is, however, still unresolved. Here we will discuss the possible relationship between the 2 entities after reporting clinical, serologic, and histopathologic data for 8 new cases.

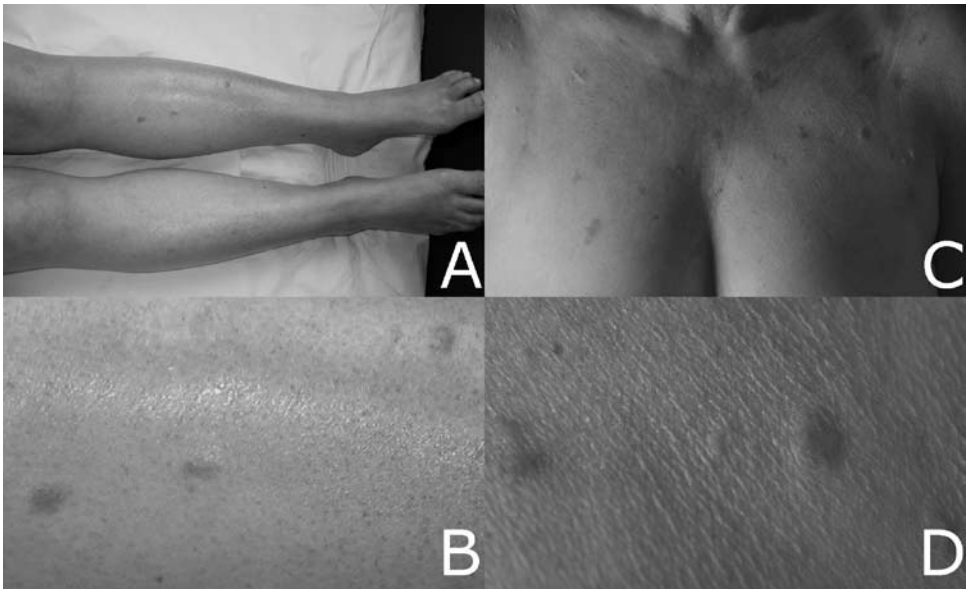
Patients and methods

We retrospectively searched the patient registries for 3 amyloidosis referral centres (a total of 2306 patients (1421 Italian, 520 Dutch and 365 German patients), to identify the combination of cutaneous localized amyloid and SS. Eight patients (0.3%) with this combination of diseases were identified. The current American-European Concensus Group criteria were used to determine how many cases adhered to the current definition of SS.(17) Extraglandular manifestations of SS were defined as the presence or confirmed records of purpura, lung or neurological involvement, synovitis, myositis, vasculitis, lymphadenopathy, enlarged spleen or previous lymphoma during the evolution of the disease. Histopathologic data and information regarding recently determined serologic parameters were collected. The local ethics committees approved the study, and all patients gave informed consent.

The serologic parameters comprised antinuclear antibodies, extractable nuclear antigens, SSA antigens, SSB antigens, rheumatoid factor, cryoglobulins, anti-double-stranded DNA, total protein, gamma globulin, serum amyloid A protein, serum M (monoclonal) protein, serum κ free light chain, serum λ free light chain, serum κ:serum λ free light chain ratio, alkaline phosphatase, and creatinine. Kidney function was evaluated by measuring the amount of proteinuria and the creatinine clearance.

Histopathology reports for biopy specimens obtained from the parotid gland, other salivary glands, skin and abdominal fat were retrieved; if the biopsy specimens were judged inadequate for the current purpose, they were reexamined if the original tissue blocks were still available.

Figure 1
A. Cutaneous amyloid papules and nodules located on the lower legs of patient 2
B. Higher-magnification view of papules and nodules shown in A.
C. Cutaneous amyloid papules and nodules located on the upper front side of the thorax of patient 7
D. Higher-magnification view of papules and nodules shown in C.



Histologic examination focused on whether amyloid was present or absent (as determined by Congo red staining) in the investigated tissues and whether signs of SS (such as lymphocytic infiltrates, myoepithelial islands, focus score ≥ 1) were present in the parotid gland or salivary gland. The skin biopsy specimen was evaluated specifically for the type of amyloid involved, by using a panel of antibodies directed to amyloid A protein, λ and κ light chains, and transthyretin, and also for the presence of a light chain-restricted plasma cell population located nearby the amyloid deposits.

Results

The characteristics of the patient are shown in table 1. All of the patients were women. One patient (patient 6) was of Indonesian descent and the other 7 patients were white. SS had been diagnosed at a median age of 47 years (range 30-61 years). Amyloid had been detected at median age 60 years (range 42-79 years): in 3 patients amyloid was detected 18 years, 5 years, and 2 years, respectively, before the diagnosis of SS; in 1 patient, amyloid was detected simultaneously with the diagnosis of SS; and in 4 patients amyloid was detected 10 years, 29 years, 34 years, and 34 years after the diagnosis of SS. In all 8 patients the determination that SS had been present for many years was made on a clinical basis. Six of these eight patients (75%) fulfilled the current strict American-European concensus Group criteria for SS (see table 2). In the remaining 2 patients all of the criteria could not be applied, because data about salivary gland involvement (flow and biopsy) were not available. In the latter 2 cases, however, the diagnosis of SS was very likely on clinical grounds. All patients had sicca symptoms; 6 patients had xerostomia, and 7 patients had xerophtalmia. No amyloid deposits were observed in the salivary gland biopsy specimens that were investigated. Six patients had extraglandular manifestations of SS (see table 1).

The amyloid deposits were generally asymptomatic, sparse, erythematous yellowish or orange papules and small nodules on the limbs, or, less frequently, the chest or abdomen. (figure 1) One patient (patient 4) also presented with large, brownish, hyperkeratotic dorsal patches.

Figure 2
Photomicrographs of a skin biopsy specimen obtained from patient 5, showing Congo red-stained amyloid deposits in the skin.
A. Normal light shows amyloid staining (arrows).
B. Polarized light shows apple-green birefringence of positively stained amyloid deposits.
(Original magnification 200 x.)

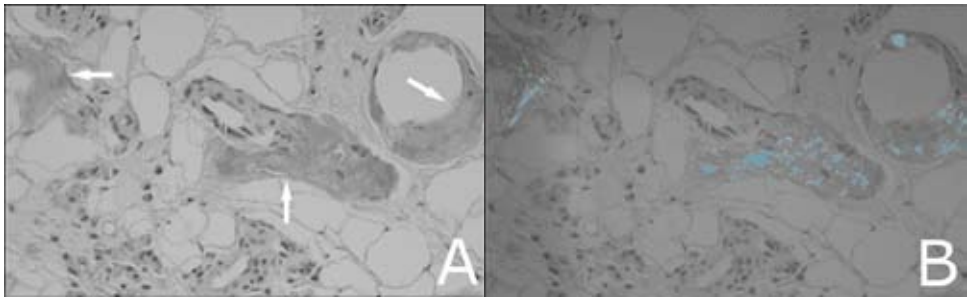


Table 1 Characteristics of the patients*.

Characteristics	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8
Age, years, at diagnosis of Sjögren (year of diagnosis)	57 (1994)	47 (2005)	61 (2000)	47 (1994)	45 (1965)	44 (1970)	30 (1975)	61 (2006)
Age, years, at detection of amyloid (year of detection)	67 (2004)	44 (2003)	43 (1982)	42 (1989)	79 (1999)	79 (2004)	59 (2004)	61 (2006)
Sicca symptoms	Xerophthalmia	Xerophthalmia, xerostomia	Xerophthalmia, xerostomia	Xerostomia	Xerostomia, xerophthalmia	Xerostomia, xerophthalmia	Xerostomia, xerophthalmia	Xerophthalmia
Extraglandular manifestations	Fatigue, arthralgia, Raynaud's		Fatigue, arthralgia, Raynaud's	Fatigue, arthralgia	CREST syndrome, fatigue	Fatigue		Fatigue, arthralgia, Raynaud's
Serology:								
SAA, mg/liter (normal <4.3)	7.3	8.3	3.7	15.7	1.0	5.0	1.0	ND
Antinuclear antibodies:	pos	pos	pos	neg	pos	pos	pos	pos
IgM-Rf, IU/ml (normal < 15)	ND	ND	pos	ND	76	75	188	Pos
total protein, mg/dl (normal 6.5-7.9)	6.3	10.2	7.2	7.4	7.0	7.9	9.3	8.8
Gamma globulin, mg/dl (normal 0.7-3.0)	0.9	4.17	1.39	1.07	1.49	1.54	3.97	3.31
κ FLC level, mg/liter (normal 3.3-19.4) †	12.4	64.7‡	ND	20.9	19.4	18.5	70.3	43.1
λ FLC level, mg/liter (normal 5.7-26.3) †	12.3	16.6	ND	8.9	45.9‡	23.2	41.1	52.7
FLC ratio (normal 0.26-1.65) †	1.01	3.90‡	ND	2.34‡	0.42	0.80	1.71§	0.82
Pathology:								
Salivary gland biopsy	ND	SS	ND	SS	SS	ND	SS (parotid)	ND
Skin								
Congo red staining	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
Immunohistology	ND	AL κ ¶	AL λ ¶	ND	AA neg, AL λ pos	AA neg	AA neg	AL κ
Predominant plasma cells	ND	λ	No plasma cells	ND	λ	λ	κ	κ
BMPCs, (%)	ND	5	ND	ND	3	ND	1	9
Location of amyloid	Left leg	Arm, back, legs	abdomen	Back	Legs	Legs	Arms, legs, trunk	abdomen
Disease progression:								
Local cutaneous progression / new cutaneous sites	Yes / yes	No / yes	No / no	No / no	Yes / yes	Yes / no	Yes / yes #	
Systemic amyloidosis	No	No	No	No	No	No	No	No
Treatment and medication	Methylprednisolone 4 mg/day	No	No	No	Surgical excision, electrocoagulation	No	No	Surgical excision

* All patients were women. SS= Sjögren's syndrome (for salivary gland biopsy, SS=histologic changes specific for SS); Raynaud's = Raynaud's phenomenon; CREST syndrome = calcinosis, Raynaud, esophagial dysmotility, sclerodactily, telangiectasis; SAA = serum amyloid A protein; ND = Not Done; IgM Rf = IgM Rheumatoid factor; FLC = free light chain; AL = amyloid light chain, BMPCs = bone marrow plasma cells. † reference intervals according to Katzmann et al.(21) ‡ indication of clonal light chain overproduction. § levels of both λ and κ are increased, but the level of κ is more increased than that of λ, resulting in an increased ratio. ¶ Investigated using immunoelectronmicroscopy. # Still slowly progressive in terms of the number and size of the lesions.

In 6 patients the amyloid was thought to be of type AL: in 4 of these patients, amyloid was positive for a particular light chain by immunoelectron microscopy, and in 3 of these 4 patients as well as 2 other patients, a light chain-restricted plasma cell population was observed in close proximity to the amyloid deposits in the skin. (figures 2 and 3) In the remaining 2 patients immunohistologic analysis was not possible, because material had not been obtained for that purpose. Biopsy samples of subcutaneous fat did not show any amyloid in 7 patients and were not obtained in 1 patient (patient 8).

Symptoms of systemic AL amyloidosis were absent in all patients. In particular, echocardiograms were normal, there was no significant proteinuria, and results of serum creatinine and liver function tests were within reference limits in all patients. None of the patients had Bence Jones proteinuria or a serum M protein, and results of immunofixation studies of serum and urine were negative in all patients. Serum immunoglobulin free light chain concentrations were measured in 7 patients (table 1). The κ: λ ratio was above the reference range in 3 patients (patients 2, 4 and 7), and 1 patient (patient 5) had an increase in the level of λ free light chain despite a normal κ: λ ratio. The type of amyloid (in patients 2 and 5) and the type of predominant plasma cells in the skin biopsy (in patients 2, 5 and 7) corresponded to the type of circulating free light chains with concentrations above the upper reference limit (figure 4). In patient 4, the type of amyloid was not characterized.

The median followup was 3.5 years (range 1-25 years) after the diagnosis of amyloidosis. Localized amyloid remained stable for years or showed only minor cutaneous progression. Progression to systemic AL amyloidosis was not seen in any of the patients. No clinical relevant comorbidity was observed in any of the patients.

Discussion

The current results provide support for the hypothesis that cutaneous nodular amyloidosis in SS is a distinct clinical entity. Amyloid derived from immunoglobulin light chains (type AL) is locally produced by a light chain-restricted plasma cell population in the skin. This

Figure 3
Immunoperoxidase-stained skin biopsy specimen obtained from patient 5, showing amyloid deposits in the skin with plasma cells nearby.
A. Many plasma cells immunoreactive for λ light chain, located in close proximity to λ-positive amyloid deposits.
B. Plasma cells immunoreactive for κ light chain and κ-negative amyloid deposits.
(Original magnification 200 x)

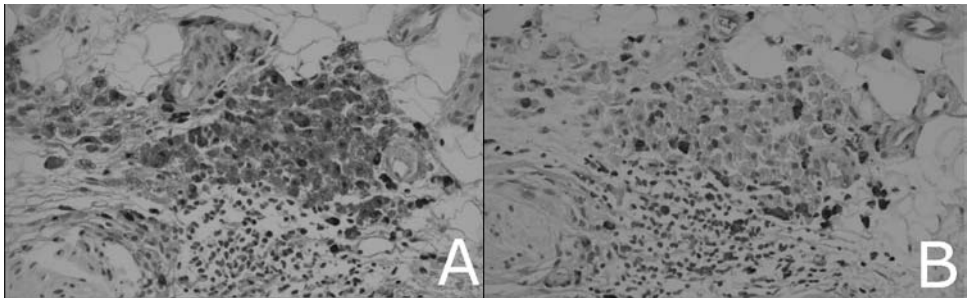


Table 2 Characteristics of the patients based on the American-European Concensus group revised criteria for Sjögren's Syndrome. *

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8
1. Ocular symptoms	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
2. Oral symptoms	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
3. Ocular signs (Schirmer and Rose Bengal score)	Yes	Yes	ND	Yes	Yes	Yes	Yes	Yes
4. Histopathology	ND	Yes	Yes	Yes	Yes	ND	Yes	ND
5. Salivary gland involvement	ND	Yes	Yes	ND	Yes	ND	Yes	ND
6. Autoantibodies to SSA or SSB (SSA/SSB)	No (-/-)	Yes (+/+)	Yes (+/ND)	No (-/-)	Yes (+/-)	No (-/-)	Yes (+/+)	Yes (+/+)
Total score	3	6	5	4	6	3	6	5
Diagnosis of Sjögren's Syndrome according to the criteria	No	Yes	Yes	Yes	Yes	No	Yes	Yes

* According to the American-European Concensus Group criteria (17), primary Sjögren's syndrome may be defined as the presence of any 4 out of the 6 following items (including 4 or 6), or any 3 of item 3,4,5 or 6.
Ocular symptoms: a positive response tot at least one question; Have you had daily, persistent, troublesome eyes for more than 3 months? Do you have a recurrent sensation of sand or gravel in the eyes? Do you use tear substitutes more than 3 times a day?
Oral symptoms: a positive respons to at least one question; Have you had daily feeling of dry mouth for more than 3 months? Have you had recurrently or persistent swollen salivary glands as an adult? Do you frequently drink liquids to aid in swallowing dry food?
Ocular signs: posititve results for at least one test; Schirmer's test, without anesthesia (≤ 5 mm in 5 minutes) or Rose Bengal score (≥ 4 van Bijsterveld's scoring system)
Histopathology: Labial salivary gland: focusscore ≥ 1 or Parotid gland: MESA, myoepithelial islands
Salivary gland involvement: at least one positive test; Unstimulated whole saliva flow (≤ 1.5 ml in 15 minutes) or Sialectasia on parotid sialography or Abnormal salivary scintigraphy
Autoantibodies to SSA/Ro and/or SSB/La

hypothesis concerning a distinct disease entity has been based on 4 related issues, as follows: localized deposition of AL amyloid, the type of AL amyloid involved, the presence of light chain-restricted plasma cells near the amyloid deposits, and the relationship with SS.

Systemic amyloidosis was not detected in any of our patients nor in the patients described in the literature, and no evidence of systemic amyloidosis was observed in any patient during followup. Therefore, localized deposition of amyloid is thought to be present in all these patients.

In 6 patients, the amyloid was characterised to be type AL, by immunohistology of amyloid itself or by the presence of a light chain-restricted plasma cell population found near the amyloid deposits. This finding strongly supports the light chain origin of this amyloid. It should be noted that detection of AL amyloid by immunohistology is frequently (~32-35% of patients) negative because of lack of reactivity with the antibodies used.(18;19) In the patients with cutaneous nodular amyloidosis and SS described in the literature, only type AL has been detected, when typing of amyloid was possible.(11) Therefore, it is likely that AL amyloid was the actual type all 8 patients.

In 5 of our patients, the presence of light chain-restricted plasma cells near the amyloid was actually detected in the skin biopsy specimen, suggesting a possible relationship between local production of a single free light chain by plasma cells followed by amyloid deposition. This finding is consistent with the literature.(11;13) In the remaining 3 patients (patients 1, 3 and 4) a search for plasma cells was not performed in the original skin biopsy specimen, and a specimens were unavailable to allow performance of this search at the time of our retrospective study.

The situation of plasma cells being in close proximity to the cutaneous epithelium may be explained by subclinical homing of these cells (or their precursor B lymphocytes) to the skin as a result of SS itself, which is an autoimmune epithelitis.(15) This explanation is highly speculative because of the large differences between glandular epithelium and cutaneous epithelium.

Serum free light chain concentrations are increased in patients with primary SS, especially those with extraglandular involvement, as compared with healthy control subjects.(20) However, Gottenberg et al. reported only 3% of patients in whom serum free light chains were elevated in the same high range as that in 4 patients in the current study who had a single increased free light chain; i.e., >45 mg/l for λ light chain and >50 mg/l for κ light chain. (20) In the 4 patients in the current study, the increased serum concentration of a free light chain might reflect overflow of local intracutaneous production into the systemic circulation. No M protein in serum, Bence Jones protein in urine, or plasma cell clonality in salivary glands and bone marrow was observed in the specimens that were studied, and systemic AL amyloidosis did not develop in any of the patients. Therefore, no cause of the increased light chain concentration in the blood than the skin seems to be likely. Symptoms of SS usually develop very gradually, and therefore this syndrome often has not been diagnosed until years after onset of the first symptoms. In patients 2 and 4, we know that the symptoms started before amyloidosis was diagnosed; therefore, SS was probably already present before the development of amyloid. Why amyloid was detected 18 years before SS in the third patient (patient 3) cannot be explained and this has not been reported previously in the literature.

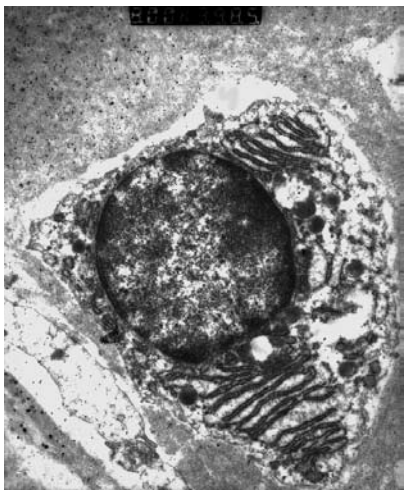


Figure 4
Immunoelectron microscopy of a skin biopsy specimen obtained from patient 2, showing immunogold labeling with anti-κ antibodies of amyloid fibrils located around the plasma cell and of vacuoles inside the plasma cell.

The 4 issues mentioned above may lead to the following hypothesis of a distinct disease entity: although SS is considered to be a T lymphocyte-mediated disease, its extraglandular manifestations are associated with an increase in B lymphocyte activity. Cutaneous nodular amyloidosis in SS seems to be the result of a benign clonal proliferation of plasma cells in the skin that is part of the spectrum of lymphoproliferative diseases associated with SS. This disease entity can be distinguished from the other lymphoproliferative diseases of this type by the differentiation of B lymphocytes to plasma cells and the homing of plasma cells to the skin, with local production of a single immunoglobulin light chain that is used to be deposited locally as AL amyloid fibrils.

It is remarkable and puzzling that most lesions develop on the extremities and especially on the legs; a plausible explanation is lacking. The course of the disease has proven to be benign in the current cases as well as the cases described in the literature. Treatment options for nodular localized amyloidosis are therefore limited to local removal of amyloid, such as surgical excision, cryotherapy, electrodessication and carbon dioxide laser treatment.(12) In our opinion, treatment is recommended only if there is any discomfort for the patient or for esthetic reasons.

Apart from the skin, localized nodular AL amyloidosis in SS has also been described sporadically in the lung(8) and in the breast(9). If these other 2 sites are also consistently connected to SS, then these 3 different amyloid sites may be grouped together in an even larger disease entity, i.e., SS-associated localized nodular amyloidosis (for which the acronym SALNA can be used).

In conclusion, localized nodular cutaneous amyloidosis is very rare and many of the reported cases are related to SS. Therefore, it is useful to look for signs of SS in patients with cutaneous amyloidosis. This type of amyloid appears to be related to local production of one of the free light chains by light chain-restricted plasma cells that had infiltrated the skin, possibly as part of the autoimmune epithelitis. We hypothesize that this combination of amyloid and SS is a distinct disease entity reflecting a particular and benign part of the polymorphic spectrum of lymphoproliferative diseases related to SS.

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Chapter 7

Summary and general discussion

Summary

Sjögren's syndrome (SS) is a systemic autoimmune disease characterised by chronic inflammation of the salivary and lacrimal glands, resulting in complaints of xerostomia and keratoconjunctivitis sicca in about 95% of the patients. These symptoms are frequently accompanied by extraglandular manifestations, and 85% of the patients suffer from severe fatigue.(1) Furthermore, the presence of SS has a large impact on health related quality of life (HR-QoL), employment and disability.

Yet, no causal systemic treatment is available in SS and therefore only symptomatic treatment can be given. Currently, biological agents have been introduced in various systemic autoimmune diseases including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). However, no biological agent has been approved thus far for the treatment of SS, but several phase II and III studies have recently been completed or are currently being conducted. The effect of treatment with biological agents is aimed at reducing disease activity and to slow down progression of SS.

In the research described in this thesis the impact of SS on quality of life has been evaluated, the different approved and experimental treatment options have been reviewed, existing and new tools to evaluate treatment were assessed and treatment results with anti-CD20 monoclonal antibodies (rituximab) are presented.

Chapter 2 describes HR-QoL, employment and disability in patients with primary (pSS) and secondary (sSS) SS, compared to data available from the general Dutch population. A questionnaire was sent to the total cohort of SS patients within the University Medical Center Groningen that is seen for scheduled follow-up. 195 out of 235 patients (83%) responded. The results revealed that SS has a large impact on HR-QOL, employment and disability as reflected by lower Short Form-36 (SF-36) scores (measuring subjective well-being), lower employment rates and higher disability rates in SS patients when compared to the general Dutch population. In addition, physical functioning, bodily pain and general health were worse in sSS than in pSS patients. The results of this trial underscore the necessity for the development of causal treatment for SS.

Therefore, in chapter 3 an overview is given of the trials performed in SS with biological agents up to 2006 and future perspectives are presented. The gain in knowledge regarding the cellular mechanisms of T and B lymphocyte activity in the pathogenesis of SS and the current availability of various biological agents (anti-TNF-α, IFN-α, anti-CD20, and anti-CD22) have resulted in new possibilities for therapeutic intervention. In SS, various phase I and II studies have been performed to evaluate these biologicals. Currently, B cell directed therapies, and especially the use of anti-CD20 monoclonal antibodies, have been shown to be more promising than T cell related therapies. In the near future a large role for treatment with biologicals for SS is expected. Larger phase II and III trials are necessary to confirm these first promising results.

In general, evaluation of a new treatment modality requires well defined and usable tools to evaluate the effect of treatment. Chapter 4a gives a general overview of existing tools for evaluation of treatment for diseases affecting salivary glands. Assessments of salivary gland function (sialometry, sialochemistry) and histopathological examination of salivary gland biopsies provide powerful tools to diagnose diseases affecting the salivary glands, to

assess disease progression and to evaluate treatment. More general tools are subjective questionnaires (e.g., visual analogue scale (VAS) scores, Multidimensional Fatigue Inventory (MFI) score and SF-36) and serological parameters.

Chapter 4b describes the development of a new evaluation tool, the genomic and proteonomic profile of whole saliva. In the study described in this chapter, the profiles for SS patients were compared to healthy age and sex matched controls. This preliminary study indicated that both glandular and whole saliva from pSS patients contain molecular signatures that reflect damaged glandular cells and an activated immune response. Whole saliva was shown to be more useful in SS diagnostics than parotid and submandibular/sublingual saliva. The candidate proteonomic and genomic biomarkers found in whole saliva may improve the clinical detection of pSS once they have been further validated in a larger group of patients.

The evaluation tools described in chapter 4 were used in evaluating treatment with rituximab, described in chapter 5. In chapter 5a a study is described assessing the efficacy and safety of (re)treatment of SS patients with rituximab after extended follow-up (mean follow-up 57 weeks) of B cell depletion therapy. Included were 8 early pSS patients and 7 pSS patients with a mucosa-associated lymphoid tissue (MALT)-type lymphoma (MALT/pSS). Rituximab was effective for 6-9 months in pSS patients and, probably, even longer in MALT/pSS patients. Retreatment of 5 pSS patients resulted in a comparable beneficial effect as observed after the first course. Development of serum sickness-like disorder in 27% of pSS patients indicated that higher doses of corticosteroids might be needed during rituximab treatment.

In chapter 5b the results of histopathological evaluation of parotid tissue after rituximab treatment were correlated with clinical results of parotid function in order to evaluate rituximab treatment on a more fundamental level. Sequential parotid biopsies before and 12 weeks after rituximab treatment in pSS patients demonstrated histopathological evidence of reduced glandular inflammation and redifferentiation of lymphoepithelial duct lesions to regular striated ducts as a putative morphological correlate of increased parotid flow and normalization of salivary sodium content. These histopathological findings underline the efficacy of B cell depletion and prove the potential for glandular restoration in SS. This study was performed as a pilot in the 5 pSS patients that received retreatment described in chapter 5b. Analysis of larger groups of patients biopsied before and after rituximab treatment are necessary to confirm these first results.

Based on these promising results, a randomized double-blind placebo-controlled trial was performed (chapter 5c). In this trial 30 pSS patients were included, of which 20 were treated with rituximab, while 10 patients received placebo. All 30 patients received an additional dose of corticosteroids in order to prevent the development of side effects. In this trial, B cell depletion led to improvement of objective and subjective parameters of disease activity. Salivary function improved, fatigue diminished, extraglandular manifestations improved. Most improvements were seen 12 to 36 weeks after treatment. These promising results suggest that a larger phase III trial should be performed in order to receive approval for rituximab treatment of SS.

Although SS is considered to be a T lymphocyte mediated disease, there are more and more signs that the role of the B cells should not be underestimated.(2) The description of the cases described in chapter 6 has deepened our insight into the B cell component of SS.

In this chapter, we retrospectively evaluated 8 patients with the combination of SS and

localized cutaneous amyloidosis. The databases of 3 amyloidosis centres (Italy; University of Pavia, Germany; University of Heidelberg and the Netherlands; Medical Center Groningen) were searched in order to find this rare combination. It was likely that AL amyloid was the actual type in all 8 patients, which is an immunoglobulin light chain associated amyloid, locally produced by a light chain-restricted plasma cell population in the skin. The combination of cutaneous amyloid and SS appeared to be a distinct disease entity reflecting a particular and benign part of the polymorphic spectre of lymphoproliferative diseases related to SS.

General discussion

Sjögren syndrome: is there a need for treatment and which treatment is available?

SS is known to affect patients' physical, psychological and social functioning (3), but the impact of SS on health-related quality of life (HR-QOL), and especially on employment and disability, has not been studied extensively before. However, this information is necessary to interpret the burden of the disease and also to gain insight into the necessity for treatment. Therefore, the analysis described in chapter 2 was performed. Comparable to other autoimmune diseases, SS has a large impact on HR-QOL, employment and disability as reflected by lower SF-36 scores and employment rates, and higher disability rates when compared with the general Dutch population. The impact on socioeconomic status described in chapter 2 justifies further research on biologicals in the treatment of SS, even though these treatments are expensive and intensive. In addition, the overview of the reports on biological treatment for SS (chapter 3) revealed that anti-CD20 (rituximab) is the most promising biological agent so far.(4-6) The results of some of therapies targeting TNF-α (infliximab, etanercept and adalimumab) and IFN-α were also promising in phase I and II studies, but in larger placebo controlled randomized trials results were disappointing. So, although the first results with rituximab seem promising, also regarding this biological larger placebo controlled trials are needed to confirm these promising results (see section on rituximab treatment). Moreover, as rituximab is a chimeric anti-CD20 agent that has the inherent hazard of inducing serum sickness, humanized anti-CD20 (ocrelizumab) that more recently has become available might, in potential, be an even more promising B cell therapy. Another promising B cell directed therapy is anti-CD22 (epratuzumab). This agent seemed to be effective in a small open-label trial, although to a lesser extent than rituximab as it only partially depletes B cells.(7). Other potential targets for biological therapy include cytokines such as IL-6 and BlyS (BAFF), interferons, adhesion molecules and chemokines. No trials in SS have yet been performed with these biological therapies, however.

Which evaluation tools are useful?

With the increasing number of trials performed aiming to treat SS, there is a growing need for more specific assessment parameters to monitor treatment effects, both subjectively and objectively. For studies on intervention in SS, especially evaluation of the parotid gland might be of use. Assessment of parotid secretory function (sialometry), composition of parotid saliva (sialochemistry) and histological examination of parotid gland tissue (repeated incisional biopsies) are routinely used in our setting to evaluate the effect of an intervention therapy as a function of time. Also scintigraphy, functional MRI, PET scans and ultrasound can be used repeatedly in evaluating the parotid gland. The diagnostic accuracy of the latter tools is lower and these are therefore less often used in our setting for treatment

evaluation. More general tools, but very valuable in evaluating intervention in SS, are subjective questionnaires (e.g. VAS scores, MFI scores and SF-36) and serological parameters such as rheumatoid factor and immunoglobulin levels, and B cell counts in the case of B cell depletion therapy.

Furthermore, both glandular and whole saliva are easy to obtain and the first results from studies on genomics and proteonomics (chapter 4b) showed valuable results. As a continuation of this study, a validation paper reported on the discovery of highly specific autoantibody biomarkers for pSS using protein microarray technology.(8) If the genomics and proteonomics can be used in the future as diagnostic tools for SS and as tools for monitoring the effect of treatment, for example rituximab treatment, in depth saliva analysis might even replace more invasive diagnostic tools such as parotid biopsies, PET and scintigraphy.

What about rituximab treatment?

Based on the promising results described in the review (chapter 3) and in the open label phase II study (chapter 5a and 5b), a randomised, placebo-controlled trial with rituximab was performed (chapter 5c). The results of the latter trial confirmed the promising results of the phase II trials, but, also some criticism was raised related to the treatment of early pSS patients without extraglandular manifestations with this biological. Because the long term (side-)effects of treatment with biological agents in SS are not known yet, some SS experts suggest to use treatment only for those SS patients with severe extraglandular manifestations(9;10). However, we observed that patients with remaining glandular function at the time of diagnosis benefit more from rituximab treatment than patients without any function left. Thus, in our opinion patients with active disease, as reflected by high levels of IgG and rheumatoid factor, increasing complaints of fatigue, and/or sicca complaints and/or swelling of the parotid gland (but still having glandular function), are the preferred patients to be treated with rituximab. Besides this group of early patients, also patients with severe extraglandular manifestations may benefit significantly from treatment. Of course, the long-term side effects of rituximab treatment have to be thoroughly investigated in larger phase III trials before implementation of this biological as therapy for SS.

In contrast to patients with lymphoma or RA treated with rituximab, serum sickness or serum sickness-like adverse events are more frequently reported in SS patients, with a rate between 6% and 27%. (chapter 3) This initially unexpected finding may be due to the use of different co-medication. Patients with RA and systemic lupus erythematosus (SLE) usually receive higher doses of steroids or concomitantly immunosuppressive drugs as compared with SS patients, which may prevent certain adverse events. In addition, RA and SLE patients often have been treated with a wide range of medication (including biological agents) before receiving treatment with rituximab, whereas SS patients are far more medication-naïve at the time of rituximab treatment. We also observed in the trial described in chapter 5c, as well as in our pilot trial, that patients who developed serum sickness were more likely to have an active, early and progressive form of the disease.(6) It is possible that such patients are more prone to develop serum sickness; however, such patients might also be the ones that most likely benefit from rituximab therapy. Another possibility is that SS patients may be more prone to develop and deposit immune complexes because of hypergammaglobulinaemia and/or cryoglobulinemia.(4) Consequently, because of the inherent risk of developing serum sickness (like) disease, we decided to increase the steroid dose in the trial described in chapter 5c. Of the 30 included patients, only

Table 1 Number of patients who actually received placebo or rituximab and the estimation of the patients and the physicians.

	Patient **True**	Patient **False**	Physician 1 **True**	Physician 1 **False**	Physician 2 **True**	Physician 2 **False**
Rituximab (20)	16	4	18	2	17	3
Placebo (9)	7	2	8	1	8	1
Total (29)	23 (79%)	6 (21%)	26 (90%)	3 (10%)	25 (86%)	4 (14%)

one patient developed serum sickness-like disease (5%), which is considerably lower than the incidence reported in our open-label study (27%).(6) Furthermore, HACA (human antichimeric antibodies) development, which occurred in 27% of patients in our open-label trial, was not found in the only patient who developed serum sickness-like disease. Based on these findings, we would recommend administering 100 mg methylprednisolone immediately prior to each infusion of rituximab. The oral regimen of prednisolone in the days following each infusion differ between different trials and should be explored in future trials. The administration of higher doses of prednisolone in the days following infusion, such as is performed during lymphoma treatment, should also be considered, because most lymphoma patients are, as SS patients, medication-naïve at time of rituximab treatment, and no serum sickness has been reported in these patients.

Retreatment with rituximab resulted in a positive effect comparable to that of the first treatment with this biological (chapter 5a). Therefore, offering patients maintenance therapy with rituximab infusions every 6 to 9 months may be a reasonable approach. Advantages of maintenance therapy might be a reduction or even arrest of disease progression and better quality of life for a long period. A threat might be the, so far unknown, long term side effects of repeated B cell depletion. The timing of retreatment could be based on return of symptoms, however, retreatment just before return of symptoms would even be better. A prediction model based on the results of our placebo controlled trial, showed that levels of rheumatoid factor could be a good predictor for return of subjective symptoms such as dry mouth and fatigue (unpublished results). However, these preliminary results were based on 20 pSS patients and, therefore, in future trials, attention should be paid to the correlation between objective and subjective symptoms. We even like to pose that such a correlation might provide a base for selecting the most optimal retreatment schedule. Probably, for each patient an individual time scheme has to be made because we observed that the time period in which rituximab reduced SS related symptoms/complaints differed considerably between patients.

The dose of rituximab that patients should receive during maintenance treatment should also be investigated. Based on the positive results after 2 infusions of 375mg/m² (which is in total about 1000 mg) as reported by Devauchelle et al.(5), probably even only one infusion of 1000 mg could be sufficient. Another issue concerns the question which group of patients should be offered retreatment. In RA patients, results of trials on retreatment of non-responders to first treatment are not conclusive. Thurlings et al. (11) reported that only responders to the first treatment benefit from retreatment, while Vital et al. reports that retreatment of non-responders before circulating plasma cells return to baseline levels enhances B cell depletion and results in a better clinical response.(12) With respect to SS, criteria for defining responders versus non-responders should first be formulated

and validated and results of retreatment of both responders and non-responders should be evaluated in future trials.

As a general rule, a placebo effect should not be underestimated in clinical trials with a long follow up period. In order to obtain some insight into a placebo effect in a clinical trial with only 30 patients (chapter 5c) all patients were asked after 24 weeks by mail if they thought they received placebo or rituximab and the reason why they thought to have received the active drug or placebo. One patient did not respond and was therefore excluded from this analysis. Both study coordinators (physicians of the departments of rheumatology and oral and maxillofacial surgery), who regularly assessed the patients and who were blinded for the study medication, also guessed whether the patient had used rituximab or placebo. In 23 out of 29 patients estimation of treatment was correct for both physicians. The physicians correctly scored treatment modality of 25 and 26 patients out of the 29 patients, respectively (Tables 1 and 2).

In conclusion, both the blinded patients and doctors could quite accurately estimate if a patient received placebo or rituximab. Therefore, the placebo effect in this particular study is small which gives us an additional hint that rituximab is an effective treatment for SS.

Role of B cells

The classical view on the role of B cells in immunity is focused on the production of antibodies and autoantibodies in the case of autoimmune diseases. However, over the past years the role of B cells seems to have acquired much more dimensions such as regulating T cell subsets and dendritic cells through cytokine production, activation of T cells and antigen presentation to T cells.(13;14) As other autoimmune diseases, SS is long considered to be a T-lymphocyte mediated disease, however, in the light of these new developments the role of B cells might be more prominent than thought in the past. The promising results of B cell depletion therapy in SS also support the theory that there is a role for B cells in the pathogenesis of SS. E.g., cutaneous nodular amyloidosis in SS seems to be the result of a benign clonal proliferation of plasma cells in the skin that is part of the spectre of lymphoproliferative diseases associated with SS. Despite its rare occurrence, 16 cases of cutaneous amyloidosis have been reported in patients with SS, which is about 25% of the reported cases of cutaneous amyloidosis These cases and the description of the cases described in chapter 6 support the role of the B cell in SS.

Future perspectives

Today, SS is diagnosed more and more in an early stage of the disease. Screening might become much easier if, in the future, e.g., the proteonomic profile can be used for diagnosis. Only one drop of saliva might be sufficient for diagnostics and/or treatment evaluation.

Today no causal treatment is available, however, so far, the performed trials revealed that B cell depletion with rituximab is probably the most effective therapy available to date. Also our randomized double-blind placebo-controlled trial (chapter 5c) with rituximab treatment showed promising results. A trial investigating retreatment of all patients involved in that trial is in progress. Focus of that study will be a longer follow up period (64 weeks), the effect of retreatment and the effect of treatment in patients who have received initially a placebo. A histopathological study of parotid gland biopsies before and after rituximab treatment of the patients described in chapter 5c has also been initiated and hopefully confirms our clinical findings and the results of our pilot study on histopathological effects of rituximab treatment (chapter 5b).

Table 2 Number of correct estimations. Maximum score is 3: patient and both physicians scored correct.

Number of correct estimations	0	1	2	3
Number of patients	1 (3%)	2 (7%)	4 (14%)	22 (76%)

Besides the already performed phase II trials, larger phase III trials are needed before approval can be obtained for rituximab treatment in SS patients. In these larger phase III studies, additional attention should be paid to the long term side effects, possibility of retreatment, and the oral dose of prednisolone during the days after each infusion. We also like to pose that rituximab treatment is especially effective for patients with active disease, extraglandular manifestations and/or remaining salivary gland secretory potential. To confirm these hypotheses, in future larger trials less strict inclusion criteria related to baseline salivary gland function and a larger number of patients are needed. In order to define treatment protocols, criteria regarding responders/non-responders have to be implemented. Studies regarding disease activity scores are currently being performed and are also important for future treatment protocols.

In addition to phase III rituximab trials, also other types of B cell depletion therapies should be investigated including completely humanized anti-CD20, anti-CD22 and anti-BAFF. To our opinion, there is a large role in the future for biologicals in the treatment of SS which could add substantially to a good quality if life of SS patients.

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Chapter 8

Samenvatting

Samenvatting

Het syndroom van Sjögren (SS) is een auto-immuunziekte die wordt gekarakteriseerd door een chronische ontsteking van onder andere de speeksel- en traanklieren. Deze ontsteking leidt bij 95% van de patiënten tot klachten van een droge mond (xerostomie) en droge ogen (keratoconjunctivitis sicca). Dit beeld wordt het primair syndroom van Sjögren (pSS) genoemd. Wanneer de aandoening gepaard gaat met een andere auto-immuunziekte zoals reumatoïde artritis (RA) of systemische lupus erythematosus (SLE) spreken we van een secundair syndroom van Sjögren (sSS). De mond- en oogproblemen worden vaak vergezeld door klachten buiten de klieren (extraglandulair) zoals nier- en long problemen, ontsteking van gewrichten (artritis), pijnlijke gewrichten (artralgie) en ontsteking van bloedvaten (vasculitis). Daarnaast ondervindt 85% van de patiënten klachten van ernstige vermoeidheid. Tenslotte heeft SS een grote impact op de ziekte gerelateerde kwaliteit van leven.

De huidige behandelingen voor SS onderdrukken alleen, in wisselende mate, de symptomen van de ziekte. Sinds een aantal jaren wordt therapie met biologicals toegepast voor auto-immuunziekten, zoals RA. Bij therapie met biologicals wordt gepoogd op celniveau in te grijpen in de ontstaanswijze (pathofysiologie) van de ziekte. Voor SS zijn echter nog geen goedgekeurde therapieën met biologicals beschikbaar voor klinische toepassing. Inmiddels zijn wel een aantal fase I en II studies uitgevoerd of in uitvoering waarin het nut van een dergelijke therapie voor SS wordt onderzocht.

In het onderzoek beschreven in dit proefschrift wordt de invloed van SS op de kwaliteit van leven geëvalueerd, worden de tot op heden toegepaste goedgekeurde en experimentele behandelingen voor SS beschreven, worden bestaande en nieuwe instrumenten voor het evalueren van de behandeling van SS besproken en worden de effecten van behandeling met rituximab, een antilichaam gericht tegen bepaalde witte bloedcellen (B cellen), in SS patiënten geëvalueerd.

In hoofdstuk 2 wordt de aan de ziekte gerelateerde kwaliteit van leven en de invloed op werk en arbeids(on)geschiktheid van pSS en sSS patiënten beschreven. De gegevens van SS patiënten werden vergeleken met data afkomstig uit de gemiddelde Nederlandse populatie. Aan het gehele cohort van SS patiënten, die regelmatig voor controle in het Universitair Medisch Centrum Groningen werd gezien, werd een vragenlijst toegestuurd. 195 van de 235 patiënten (83%) bleken bereid te zijn aan dit onderzoek deel te nemen en stuurden de ingevulde vragenlijst terug. Analyse van de resultaten toonde aan dat SS een grote invloed had op de aan de ziekte gerelateerde kwaliteit van leven en op werk en arbeids(on)geschiktheid. In vergelijking met de gemiddelde scores van de Nederlandse populatie scoorden SS patiënten lager op het Short Form-36 (SF-36; deze vragenlijst scoort het subjectieve welbevinden), bleek het percentage SS patiënten dat werkte lager te zijn en was het percentage arbeidsongeschiktheid onder SS patiënten beduidend hoger dan in de Nederlandse populatie. Patiënten met sSS scoorden slechter dan patiënten met pSS op de gebieden fysiek functioneren, lichamelijke pijn en algemene gezondheid van de SF-36 vragenlijst. De resultaten van dit onderzoek benadrukken de noodzaak tot het ontwikkelen van een meer causale behandeling voor SS.

In hoofdstuk 3 wordt een overzicht gepresenteerd van de tot en met 2006 gepubliceerde resultaten van onderzoek verricht naar de effecten van therapie met biologicals in de behandeling van SS. Tevens worden in dit hoofdstuk de toekomstperspectieven betreffende de behandeling van SS met deze therapieën geschetst. Toegenomen inzicht in de werking op

celniveau van witte bloedcellen (cellulaire mechanismen van T en B cel activiteit), toegenomen kennis van de pathofysiologie van SS en het beschikbaar zijn van een aantal therapieën met biologicals (anti-TNF-α, anti-CD20, anti-CD22) hebben geresulteerd in nieuwe mogelijkheden voor therapeutische interventie. Inmiddels zijn een aantal fase I en II onderzoeken uitgevoerd om de effectiviteit en veiligheid van therapieën met biologicals voor SS te evalueren. Momenteel lijken B cel gerichte therapieën, in het bijzonder de therapie waarbij anti-CD20 antilichamen worden toegepast, veelbelovend te zijn. Aangezien de uitkomsten van de tot op heden gerapporteerde inventariserende studies met B cel gerichte therapieën hoopvol zijn, is het de inschatting dat in de nabije toekomst therapieën met biologicals een belangrijke rol zullen gaan spelen bij de behandeling van SS. Alvorens deze therapieën algemeen kunnen worden toegepast, moeten eerst grotere fase II en III studies worden verricht om de gerapporteerde resultaten te bevestigen.

Evaluatie van een nieuwe therapie vereist goed gedefinieerde en gebruiksvriendelijke instrumenten om het effect van de behandeling te beoordelen. In hoofdstuk 4a wordt een overzicht gegeven van de bestaande instrumenten waarmee het effect van een therapie gericht op speekselklier gerelateerde ziekten kan worden geëvalueerd. Evaluatie van de speekselklierfunctie (hoeveelheid speeksel (sialometrie) en samenstelling (sialochemie)) en onderzoek van de biopten op weefselniveau (histopathologie) van de speekselklieren lijken zeer geschikte instrumenten om zowel speekselklier gerelateerde ziekten te diagnosticeren als de progressie en behandeling van de onderliggende aandoening te evalueren. Daarnaast is het zinvol om meer algemene instrumenten, zoals subjectieve vragenlijsten (bijvoorbeeld visual analogue scale (VAS) scores, Multidimensional Fatigue Inventory (MFI) score en SF-36) en serologische parameters, zoals gehaltes van autoantilichamen in het bloed, bij de evaluatie van het effect van een bepaalde therapie op SS te betrekken.

In hoofdstuk 4b wordt de ontwikkeling van een nieuw evaluatie instrument beschreven: analyse van het genetische en eiwitprofiel van totaal speeksel. In het in dit hoofdstuk beschreven onderzoek werden de genoemde speeksel profielen van SS patiënten vergeleken met die van gezonde mensen van dezelfde leeftijd en hetzelfde geslacht. Uit het onderzoek kwam naar voren dat zowel het speeksel van specifieke klieren als totaal speeksel van pSS patiënten moleculaire profielen bevat die weergeven dat de speekselklier beschadigd is en het immuunsysteem geactiveerd is. De eiwit en genetische biomarkers die werden gevonden in het totaal speeksel kunnen mogelijk van belang zijn voor de vroegdiagnostiek van pSS. Hiervoor moeten de gevonden markers worden gevalideerd in grotere groepen patiënten en worden afgezet tegen het profiel van patiënten met andere auto-immuunziekten, zoals reumatoïde artritis, SLE en sSS.

De in hoofdstuk 4 beschreven evaluatie instrumenten zijn gebruikt om de behandeling met rituximab (hoofdstuk 5) te evalueren.

In hoofdstuk 5a wordt een studie beschreven waarin de effectiviteit en de veiligheid van (her)behandeling met rituximab van patiënten met SS wordt geëvalueerd. De follow up bedroeg gemiddeld 57 weken. Acht patiënten met vroege pSS en 7 patiënten met een mucosa-associated lymphoid tissue (MALT)-type lymfoom en pSS (MALT/pSS) werden geïncludeerd. De behandeling met rituximab bleek ongeveer 6-9 maanden effectief voor vroege pSS patiënten en langer voor de MALT/pSS patiënten. Herbehandeling van de 5 pSS patiënten die geen serumziekte hadden ontwikkeld, resulteerde in een vergelijkbaar positief effect zoals werd gezien na de eerste behandeling. Het ontwikkelen van een serumziekte-

achtig beeld, veroorzaakt door de ontwikkeling van antilichamen tegen rituximab, bij 3 van de 8 behandelde patiënten zou kunnen beteken dat er een hogere dosis corticosteroïden nodig is tijdens de behandeling met rituximab.

In hoofdstuk 5b wordt een studie beschreven waarin de resultaten van de histopathologische evaluatie van weefsel van de oorspeekselklier (parotis) na rituximab behandeling werden gecorreleerd aan de parotisfunctie met als doel om een structuur-functie analyse van de behandeling met rituximab te kunnen verrichten. Sequentiële parotisbiopten, vóór en 12 weken ná behandeling met rituximab van vroege pSS patiënten, toonden een afname van de ontsteking en herstel van de in het ontstekingsproces veranderde klierbuisjes. De toegenomen speekselvloed van de parotisklier en de normalisatie van de natrium concentratie in het parotisspeeksel zijn in overeenstemming met de op histopathologisch niveau waargenomen veranderingen. Deze bevindingen onderstrepen de effectiviteit van B cel depleterende therapie en duiden er op dat regeneratie van speekselklierweefsel mogelijk is bij SS. Het in dit hoofdstuk beschreven onderzoek werd verricht bij de 5 patiënten die werden herbehandeld met rituximab (zie hoofdstuk 5a). Analyse van grotere groepen patiënten waarbij een biopt is genomen voor en na behandeling met rituximab is nodig om deze eerste resultaten te bevestigen.

Gebaseerd op deze veelbelovende resultaten werd een dubbel-blinde placebo-gecontroleerde studie verricht (hoofdstuk 5c). In dit onderzoek werden 30 patiënten met een vroege vorm van pSS geïncludeerd, waarvan 20 patiënten werden behandeld met rituximab en 10 patiënten met een placebo. Alle 30 pSS patiënten kregen een hogere dosering corticosteroïden dan de pSS patiënten in de eerdere inventariserende studie (hoofdstuk 5a) om het ontwikkelen van bijwerkingen, in het bijzonder een op serumziekte gelijkend klachtenpatroon, te voorkomen. In dit onderzoek leidde B cel depletie tot verbetering van zowel de objectieve als subjectieve parameters van de aan pSS gerelateerde ziekteactiviteit. De speekselklierfunctie verbeterde, de vermoeidheid verminderde en de extraglandulaire manifestaties namen af. De meeste verbeteringen werden 12 tot 36 weken na de start van de behandeling met rituximab gezien. Deze veelbelovende resultaten suggereren dat het zinvol is om een grotere fase III studie uit te voeren met als doel het verkrijgen van goedkeuring voor behandeling met rituximab bij SS.

Hoewel SS wordt beschouwd als een ziekte waarbij met name T cellen betrokken zijn bij het ontstaan van de afwijkingen, bestaan er steeds meer aanwijzingen dat de rol van de B cellen niet moet worden onderschat. De beschrijving van de casus in hoofdstuk 6 vergroot het inzicht in de betrokkenheid van een B cel component bij SS. In dit hoofdstuk wordt een retrospectief onderzoek beschreven naar 8 patiënten met de combinatie van SS en een lokale huidaandoening waarbij er neerslag plaatsvindt van eiwitten (gelokaliseerde cutane amyloidose). In databases van 3 amyloidose centra (Italië: Universiteit van Pavia; Duitsland: Universiteit van Heidelberg; Nederland: Universitair Medisch Centrum Groningen) werd gezocht naar deze zeldzame combinatie. Meest waarschijnlijk was er sprake van AL type amyloidose bij alle 8 SS patiënten. Dit is een lichte keten immunoglobuline geassocieerde amyloidose waarbij deze ketens lokaal worden geproduceerd door plasma cellen in de huid die uitsluitend lichte ketens produceren. Cutane AL amyloidose lijkt samen te hangen met SS. Hiermee wordt een nieuw element toegevoegd aan het spectrum van lymfoproliferatieve ziekten dat gerelateerd is aan SS.

In hoofdstuk 7 worden de algemene conclusies uit de verschillende onderzoeken gecombineerd,

besproken en in een breder kader geplaatst. Tevens worden toekomstperspectieven geschetst ten aanzien van de causale behandelmogelijkheden van SS.

Tegenwoordig wordt SS steeds vaker in een vroeg stadium van het ziekteproces gediagnosticeerd. Screening op SS zou in de toekomst kunnen worden vereenvoudigd wanneer hiervoor bijvoorbeeld het eiwitprofiel van speeksel kan worden gebruikt.

Tot op heden is geen causale behandeling beschikbaar voor SS. Wel is aangetoond dat het hebben van SS een grote invloed heeft op de kwaliteit van leven, op werk en arbeidsgeschiktheid. Daarom is het van belang dat er onderzoek wordt gedaan naar nieuwe behandelingen voor SS, ook al zijn deze behandelingen duur en intensief.

Uit literatuuronderzoek is gebleken dat behandelingen die gericht zijn op depletie van B cellen het meest succesvol zijn. Ook de resultaten van een placebo gecontroleerde studie (hoofdstuk 5c) lieten positieve effecten zien van behandeling met rituximab, een B cel depleterende behandeling. Een probleem van een behandeling met rituximab is dat bij SS patiënten veel vaker een serumziekte-achtig beeld wordt gezien dan bij patiënten met andere auto-immuun aandoeningen, bijvoorbeeld RA en SLE. Een aantal hypotheses die dit verschil zouden kunnen verklaren, worden uiteengezet in hoofdstuk 7. Toediening van prednisolon lijkt de kans op het ontwikkelen van dit serumziekte-achtige beeld te verkleinen.

Herbehandeling van SS met rituximab lijkt even effectief te zijn als een eerste behandeling. Momenteel loopt een studie waarbij alle 30 in hoofdstuk 5c beschreven pSS patiënten worden herbehandeld met rituximab en waarbij een langere follow up periode (ruim 1 jaar) in acht wordt genomen. In deze studie krijgen alle patiënten, dus zowel de patiënten die aanvankelijk rituximab kregen als ook de patiënten die een placebo hebben gekregen, rituximab toegediend. Naast een beoordeling van het klachtenpatroon, en serologisch- en speekselklierfunctieonderzoek, worden bij deze 30 pSS patiënten opnieuw parotisbiopten genomen (vóór en/of 12 weken ná behandeling met rituximab). Deze biopten zullen histopathologisch worden geanalyseerd. Met deze studie hopen we de in hoofdstuk 5b beschreven resultaten te bevestigen.

Naast de al uitgevoerde fase II studies moeten grotere fase III studies worden verricht om toestemming te krijgen voor routine behandeling van SS patiënten met rituximab. In deze grote fase III studies zal aandacht moeten worden geschonken aan de lange termijn effecten van rituximab, aan de mogelijkheid tot herbehandeling en aan het optimale doseringsschema van prednisolon in de dagen van en na het toedienen van rituximab.

Op basis van de resultaten van het in dit proefschrift beschreven onderzoek kan gesteld worden dat behandeling met rituximab effectief is bij pSS patiënten met een actief ziektebeeld en/of met een restfunctie van de speekselklieren. Daarnaast is rituximab effectief voor de behandeling van extraglandulaire manifestaties. Om deze stelling te bevestigen zullen bij toekomstige grote(re) studies minder strikte inclusiecriteria moeten worden gehanteerd, dat wil zeggen dat ook patiënten met een langere ziekteduur en/of een lagere speekselsecretie bij aanvang van de studie moeten worden geïncludeerd. Voorts moeten, om behandelprotocollen te kunnen opstellen, eerst algemeen geaccepteerde responder/non-responder criteria worden opgesteld. Binnen dit kader worden momenteel studies uitgevoerd waarbij wordt gekeken naar scores die ziekteactiviteit meten.

Naast de fase III studies met rituximab zouden ook andere op B cel gerichte therapieën moeten worden onderzocht, zoals gehumaniseerd anti-CD20, anti-CD22 en anti-BAFF. In de toekomst lijkt een grote rol weggelegd voor de therapie met biologicals in de behandeling van SS. Dergelijke therapieën zouden substantieel kunnen bijdragen aan het verbeteren van de kwaliteit van leven van de patiënten met SS.

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Curriculum vitae

Jiska Meijer werd 6 maart 1979 geboren te Vlaardingen. In 1997 deed ze eindexamen VWO aan het Carolus Clusius College te Zwolle. Van september 1997 tot september 1998 studeerde zij Industrieel Ontwerpen aan de Technische Universiteit Delft. In september 1998 startte zij met de studie geneeskunde aan de Rijksuniversiteit Groningen. Haar artsenbul behaalde zij cum laude in augustus 2004. Tijdens haar studie was Jiska actief in diverse commissies, zij vervulde onder andere een tweejarige functie als studentlid van de faculteitsraad. In september 2004 startte zij als arts-onderzoeker op de afdeling Kaakchirurgie van het Universitair Medisch Centrum Groningen. Van september 2005 tot en met april 2009 combineerde zij haar promotieonderzoek met de studie tandheelkunde aan de Rijksuniversiteit Groningen. In april 2009 behaalde zij haar tandartsenbul cum laude. Sinds september 2009 is Jiska in opleiding tot kaakchirurg.
Jiska woont samen met Janwillem Kocks en samen hebben zij twee zoons, Nander geboren op 20 februari 2008 en Borrit geboren op 14 september 2009.

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