T cell-dependent B cell hyperactivity in primary Sjögren's syndrome

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SERUM IMMUNOGLOBULIN FREE LIGHT CHAINS ARE SENSITIVE BIOMARKERS FOR MONITORING DISEASE ACTIVITY AND TREATMENT RESPONSE IN PRIMARY SJÖGREN'S SYNDROME

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ABSTRACT

Objective

Serum immunoglobulin free light chains (FLC) are frequently elevated in B cell-mediated autoimmune diseases, including primary Sjögren's syndrome (pSS). The objective of this study was to assess if serum FLC can contribute to classification, MALT-lymphoma detection, monitoring of disease activity and treatment response in pSS.

Methods

Serum samples of 100 consecutive patients suspected of pSS were included. Forty-five patients fulfilled ACR-EULAR criteria for pSS. Additionally, samples of 17 pSS patients with MALT-lymphoma and longitudinal samples of pSS patients treated with rituximab (n=20), placebo (n=10) or abatacept (n=15) were included. Serum FLCκ/FLCλ was measured by nephelometry.

Results

At diagnosis, FLCκ and FLCλ serum levels were significantly higher in pSS compared to non-SS sicca patients. The FLCκ/FLCλ ratio was abnormal in 11% of pSS patients. In established MALT-pSS patients, without recent rituximab treatment (n=12), 50% had abnormal FLCκ/FLCλ ratios. FLC measurement had no additional value for pSS classification, compared to IgG and anti-SSA. FLC levels correlated significantly with systemic disease activity, assessed by EULAR Sjögren's syndrome disease activity index (ESSDAI) and clinical ESSDAI, both cross-sectionally and longitudinally following treatment. Treatment with rituximab or abatacept significantly lowered FLC levels. FLC show a large sensitivity to change and relative changes induced by treatment were higher compared with IgG.

Conclusion

Serum FLC is elevated in pSS, and abnormal FLCκ/FLCλ ratios may be indicative for the presence of MALT-lymhoma. FLC levels can be used as biomarker for systemic disease activity and monitoring treatment responses. FLC is sensitive to change and has more favorable kinetics than IgG.

INTRODUCTION

Primary Sjögren's syndrome (pSS) is a chronic, systemic autoimmune disease which characteristically affects the salivary and lacrimal glands. B-cell hyperactivity is a major contributor to the pathology of pSS [1]. The involvement of B-cells in pSS pathogenesis is among others reflected by the presence of hypergammaglobulinemia and autoantibodies, such as anti-SSA/Ro, anti-SSB/La and rheumatoid factor (RF). The elevated intracellular levels of Bruton's tyrosine kinase in naive and memory B-cells further reflect their intrinsic, more activated state [2]. Also, pSS patients have an enhanced risk of developing mucosa-associated lymphoid tissue (MALT)-lymphoma, a subclass of malignant B-cell lymphoma [1,3]. In line with this role for B-cells in pSS pathogenesis, B-cell depletion therapy with rituximab shows favorable effects on salivary gland architecture and extraglandular manifestations of pSS [4]. However, not all patients benefit from this treatment, as shown by two large randomized controlled trials [5,6]. Validated biological markers for monitoring clinical response to therapy and/or disease activity in pSS are lacking. Promising markers are serological parameters of B-cell activity, including BAFF (B-cell Activation of the TNF Family, also named BLyS), CXCL13, β2-microglobulin and immunoglobulin free light chains (FLC). These markers are fairly associated with systemic disease activity in pSS patients [7,8].

FLC comprise kappa (κ) and lambda (λ) light chains, which are produced in excess compared to heavy chains during immunoglobulin synthesis by B-cells, plasmablasts and plasma cells. The surplus of light chains is secreted as FLC into serum [9]. The half-life of FLC in serum is very short (2-6 hours) compared to complete immunoglobulins and therefore the presence of FLC may reflect actual B-cell activity. FLC may have specific biological functions, which include binding to antigens on antigen-presenting cells and inhibition of apoptosis of neutrophils [10–12]. FLC serum levels correlate positively with other serological markers of B-cell activity and most strongly with serum IgG [13]. Levels of polyclonal FLC are not only elevated in pSS compared to healthy controls, but also in patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) [13–15]. In patients with SLE, FLC levels correlate with disease activity and are elevated in urine prior to relapse of symptoms [14,16]. However, the clinical relevance of serum FLC levels as a biomarker in pSS is unclear. The objectives of this study were to assess if serum FLC can contribute to classification, MALT-lymphoma detection, monitoring of disease activity and treatment response in pSS.

PATIENTS AND METHODS

Study population

Consecutive patients (n=129), referred to the Sjögren Expertise Center of the University Medical Center Groningen (UMCG) for suspicion of pSS, were screened for eligibility. Exclusion criteria were age<18 years, an incomplete diagnostic work-up, diagnosis of another systemic auto-immune disease, hepatitis C positivity and renal impairment (eGFR<60 mL/min, MDRD formula), which influences serum FLC levels [9]. Patients that fulfilled 2016 ACR-EULAR criteria for pSS were classified as pSS patients [17], and the remaining patients as non-SS sicca patients. Serum samples of 18 consecutive pSS patients with a current or pre-existing diagnosis of salivary gland MALT-lymphoma, who were enrolled as a subgroup in our Registry of Sjögren syndrome in UMCG-Longitudinal (RESULT) cohort, were also screened for eligibility. Of these 18 patients, one was excluded from FLC analysis because of renal impairment. The remaining 17 pSS-MALT patients were divided in a RTX-treatment group (rituximab treatment <1 year before inclusion) and a no-treatment group (untreated patients and patients who were treated with rituximab >1 year before inclusion). Informed consent was obtained from all patients according to the Declaration of Helsinki and the studies were approved by the Medical Research Ethics Committee of the UMCG (METc2013.066/METc2014.491).

Additionally, longitudinal serum samples of pSS patients that participated in our previously reported double-blind, randomized controlled trial with rituximab (1000 mg, days 1 and 15, n=30 with 2:1 randomization for rituximab and placebo) and open-label study with abatacept (~10 mg/kg of body weight, days 1, 15 and 29 and every 4 weeks thereafter, n=15) were included [18,19]. One patient in the placebo-arm of the rituximab study was lost to follow-up after week 12 and therefore no samples were available from this patient at week 24, 36 and 48. To minimize the risk of infusion reactions and serum sickness, all patients in the rituximab- and placebo-arm were treated with methylprednisolone (100 mg IV), acetaminophen (1000 mg orally) and clemastine (2 mg IV) prior to each infusion, and received oral prednisone (60 mg on days 1&2, 30 mg on days 3&4 and 15 mg on day 15). Except for these precautionary medications, treatment with concomitant immunosuppressants, including hydroxychloroquine and glucocorticoids, was discontinued during the study. All patients that participated in the rituximab and abatacept studies also fulfilled, retrospectively, the ACR-EULAR criteria. The EULAR Sjögren's syndrome disease activity index (ESSDAI) and clinESSDAI (ESSDAI without the biological domain) were completed in all pSS patients [20,21].

FLC measurement

Stored serum samples (-80°C) were thawed and FLC (κ and λ) were measured by quantitative turbidimetry using the Freelite assay and the Optilite analyzer (Binding

Site, UK). The Freelite assay estimates FLCκ and FLCλ by separate immunoassays based on affinity-purified polyclonal antibodies coated onto latex particles. Samples from the MALT-lymphoma group and the rituximab study were measured by quantitative nephelometry using the Freelite assay and the BNProSpec analyzer (Siemens AG, Germany), as part of the diagnostic work-up. Reference intervals were 3.3–19.4 mg/L for FLCκ, 5.7-26.3 mg/L for FLCλ, and 0.26-1.65 for the κ/λ ratio, according to the 95% percentile range provided by the manufacturer. An abnormal FLC level was defined as an abnormal kappa and/or lambda level and/or abnormal κ/λ ratio.

Statistical analysis

Continuous data are presented as medians and interquartile ranges. Mann-Whitney U test was used to compare FLC levels between different groups. Predictor analyses of pSS classification were performed using binary logistic regression. Multivariate analysis was performed using the forward Wald method for inclusion of predictors that had a P-value <0.05 in the univariate analysis. The explained variance is presented as Nagelkerke's R square (R2). Residual statistics were assessed and outlier cases were identified based on Cook's distance >1, or standard and normalized residual values outside the cut-off values of ±1.96 and ±2.58, respectively.

Cross-sectional correlations between serum FLC and clinical and biological parameters (i.e., ESSDAI, clinESSDAI, IgG) were evaluated with Spearman's correlation coefficient. Longitudinal correlations between FLC and clinical parameters were analyzed with generalized estimating equations (GEE). GEE were used to analyze changes in FLC levels over time, namely during B-cell depletion (week 0-24), B-cell repopulation (week 24-48), during abatacept treatment (week 0-24) and after cessation of abatacept treatment (week 24-38). For the placebo group, identical time points were analyzed as for the rituximab group. To evaluate sensitivity to change, the standardized response mean (SRM) was calculated as the mean change score divided by the SD of the change score, between baseline and each consecutive time point. SRM <0.5 were interpreted as small, 0.5–0.8 as moderate, and >0.8 as large [22]. P-values <0.05 were considered statistically significant. Statistical analyses were performed using IBM SPSS Statistics 23 (SPSS, USA).

RESULTS

From the diagnostic cohort, 100 out of 129 patients were included in the analyses. Reasons for exclusion were an incomplete diagnostic work-up (n=8), presence of another systemic auto-immune disease (n=12), missing serum samples (n=5), hepatitis C infection (n=2), or renal impairment (n=2). There were no patients with renal impairment in the treatment groups (rituximab, abatacept) at baseline or during

follow-up. Baseline patient's characteristics of the different cohorts are summarized in table 1. Detailed clinical characteristics of the MALT-lymphoma group are described in supplementary table 1.

Elevated FLC levels and κ/λ ratio in pSS patients at the time of diagnosis

Of the included patients from the diagnostic cohort, 45 (45%) patients were classified as having pSS. Twenty-nine (64%) of these pSS patients had abnormal FLC levels. Both FLCκ and FLCλ levels were significantly increased in pSS compared with non-SS sicca patients (figures 1A and 1B, P<0.001). In pSS, FLCκ and FLCλ levels were above the upper reference limits in 58% and 44% of patients, respectively. FLC levels were higher in anti-SSA positive, compared with anti-SSA negative patients (P=0.037 for FLCκ, P=0.064 for FLCλ). In addition to absolute levels of FLC, the κ/λ ratio was assessed. A small but significant increase in the κ/λ ratio was observed in pSS, compared with non-SS sicca patients (figure 1C, P=0.009), indicating a slightly higher production of FLCκ versus FLCλ. There was no significant difference in κ/λ ratio between pSS patients with or without anti-SSA antibodies.

In five (11%) pSS patients and three (5%) non-SS sicca patients, a κ/λ ratio above the upper cut-off value was found. One of these pSS patients was diagnosed with MALT-lymphoma. This patient also presented with abnormal levels of RF (>200 IU/mL), IgG (25 mg/L), and weak type III cryoglobulinemia (polyclonal), but without lymphopenia or low C4. A second pSS patient with an abnormal κ/λ ratio had an IgG level of 32 mg/L and developed urticarial vasculitis and unilateral parotid gland swelling a few months after inclusion. A third pSS patient with an abnormal κ/λ ratio developed progressive neuropathy with lymphopenia, low C4 level and weak type I cryoglobulinemia (monoclonal) one year later during follow-up. In the other two pSS patients and in the three non-SS sicca patients with an abnormal κ/λ ratio, risk factors for lymphoma such as cryoglobulinemia, lymphopenia or low complement levels were not detected. Together, these results indicate that an altered κ/λ ratio may indicate or precede severe clinical manifestations of pSS.

TABLE 1 | Baseline characteristics of different study cohorts.

Characteristic	Diagnostic cohort (n=100)		MALT- lymphoma cohort (n=17)	Rituximab trial (n=30)		Abatacept trial (n=15)
	pSS (n=45)	non-SS sicca (n=55)		RTX (n=20)	Placebo (n=10)	
Age, median (IQR), years	54 (46-63)	50 (40-56)	60 (50-64)	42 (32-53)	37 (32-60)	43 (32-51)
Female gender, n (%)	43 (96)	47 (86)	16 (94)	19 (95)	10 (100)	12 (80)
ESSDAI, median (IQR)	4 (1-9)	-	4 (1-15)	8 (6-11)	7 (5-9)	11 (8-14)
clinESSDAI, median (IQR)	4 (0-10)	-	4 (0-13)	7 (5-11)	5 (5-8)	11 (9-17)
IgG (g/L), median (IQR)	16 (12-20)	10 (9-12)	13 (9-17)	22 (19-26)	23 (16-27)	20 (15-27)
Anti-Ro/SSA positive, n (%)	34 (76)	3 (6)	16 (94)	20 (100)	10 (100)	15 (100)
Anti-La/SSB positive, n (%)	19 (42)	0 (0)	6 (35)	14 (70)	8 (80)	12 (80)
Biologic activity, n (%)	25 (56)	10 (18)	11 (65)	17 (85)	9 (90)	11 (73)
Treatment with corticosteroids, n (%)	2 (4)	2 (4)	2 (12)	0 (0)*	0 (0)*	0 (0)*
Treatment with DMARD, n (%)	7 (16)	3 (5)	7 (41)	0 (0)*	0 (0)*	0 (0)*
Kappa FLC mg/L, median (IQR)	22 (16-36)	15 (11-18)	24 (16-37)	23 (17-45)**	21 (15-27)**	38 (28-44)**
Lambda FLC mg/L, median (IQR)	21 (15-31)	15 (13-18)	15 (10-22)	26 (19-46)**	23 (19-24)**	30 (23-41)**
Ratio kappa/lambda	1.15 (0.89-1.51)	0.97 (0.78-1.20)	1.44 (1.15-1.95)	0.96 (0.75-1.24)**	0.98 (0.74-1.11)**	1.11 (0.86-1.52)**

*Treatment with prednisolone, hydroxychloroquine or other traditional disease-modifying anti-rheumatic drugs (DMARD) had to be discontinued at least 1 month before baseline. **Values at baseline, i.e. before start of treatment, are displayed. MALT: Mucosa-associated lymphoid tissue; IQR: Interquartile range; ESSDAI: EULAR Sjögren's Syndrome Disease Activity Index; clinESSDAI: ESSDAI without the biological domain.

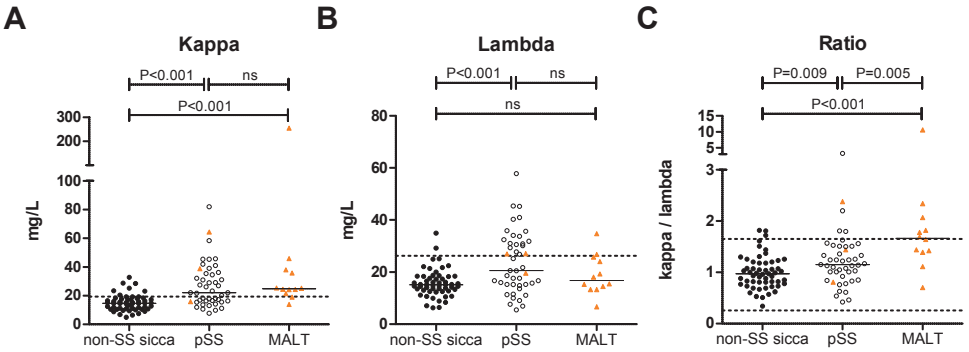

FIGURE 1 | Serum levels of FLCκ and FLCλ and the κ/λ ratio in pSS patients and sicca patients. FLC levels in a diagnostic cohort consisting of non-SS sicca patients (n=55) and pSS patients (n=45), classified according to the ACR-EULAR criteria, are displayed. In addition, results from established pSS patients with mucosa-associated lymphoid tissue (MALT)-lymphoma located in the salivary glands, without or with current/recent (<1 year) B-cell depletion therapy, are displayed (n=12 and n=5, respectively). Horizontal lines indicate the median. Dashed horizontal lines indicate the cut-off value(s). Orange triangles represent patients with salivary gland MALT-lymphoma without current/recent B cell depletion therapy, blue triangles represent patients with salivary gland MALT-lymphoma who have been treated with B cell depletion therapy <1 year before inclusion. Mann-Whitney U test was used for statistical comparisons between pSS and non-SS sicca patients. The one-way ANOVA (Kruskal Wallis) test was used to compare pSS, untreated pSS-MALT and treated pSS-MALT patients. P-values <0.05 were considered significant. Ns: Not significant.

Abnormal κ/λ ratios in MALT-lymphoma patients

Because an abnormal κ/λ ratio was observed in a MALT-lymphoma patient in our diagnostic cohort, these ratios were also evaluated in established pSS patients with salivary gland MALT-lymphoma. Seventeen MALT-lymphoma patients were included, and five patients were analyzed separately because of recent or current B cell depletion therapy. Of the 12 patients who were not treated with rituximab, six (50%) patients had an abnormal κ/λ ratio (figure 1C). In only one of these six patients a weak monoclonal immunoglobulin (M-protein) was detected by immunofixation. Of the five patients who were recently treated with rituximab, only one had an abnormal κ/λ ratio (data not shown). The latter patient with a κ/λ ratio of 6.2 received maintenance treatment with rituximab every half year, because of recurrent symptoms of vasculitis and polyneuropathy in the last three years.

FLC versus IgG measurement for prediction of pSS

We next investigated if FLCκ and FLCλ could predict pSS classification by logistic regression analysis. Univariate logistic regression analysis showed that FLCκ and FLCλ are both significant predictors of fulfilling the ACR-EULAR criteria for pSS (R^2: 0.374 and 0.254, respectively). However, the explained variances for IgG and anti-SSA positivity were higher (R^2: 0.521 and 0.605, respectively). After inclusion of the variables FLCκ, FLCλ,

IgG and anti-SSA positivity in multivariate analysis, IgG and anti-SSA positivity were the only independent predictors of pSS classification (R^2: 0.696). When only FLCκ, FLCλ, and anti-SSA were included in the multivariate analysis, FLCκ contributed significantly to the model (supplementary table 2). Exclusion of 5 outliers (see Methods) improved the predictive value to 0.863, but did not change the independent variables included in the models (data not shown). Together, our results indicate that FLC levels are neither superior nor additional to IgG and anti-SSA positivity as a classification biomarker for pSS.

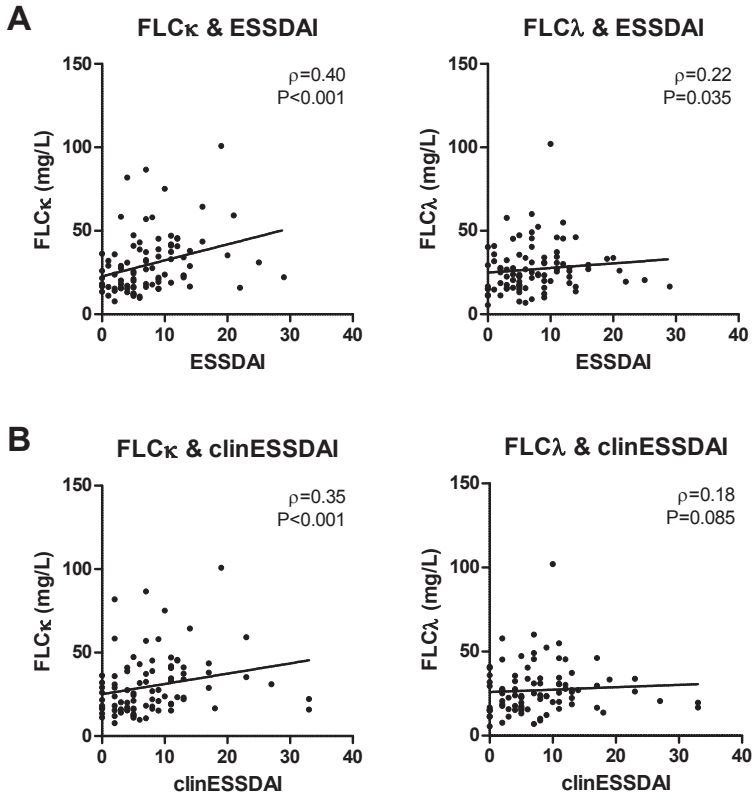

FIGURE 2 | Correlations between FLC and systemic disease activity. Correlations between FLC and systemic disease activity, as measured by ESSDAI and clinESSDAI, in 90 pSS patients classified according to ACR-EULAR criteria. Patients with pSS from the diagnostic cohort (n=45) and baseline data from the rituximab (n=30) and abatacept (n=15) cohorts were combined. Correlations were evaluated with Spearman's correlation coefficient (ρ).

Association between FLC levels and systemic disease activity at baseline

Previous studies have suggested that FLC levels correlate positively with extraglandular involvement in pSS patients [7,13]. Consistent with this finding, combined data from our diagnostic cohort and baseline data from the rituximab and abatacept trials, in which

ESSDAI scores were relatively high (table 1), indicated that FLCκ, and to a lesser extent also FLCλ, correlated significantly with ESSDAI scores (figure 2A). The ESSDAI domain that showed the highest correlation with FLC levels was the biological domain, which can be explained by the strong correlations between IgG and FLCκ and FLCλ (ρ=0.685 and ρ=0.621, respectively). Therefore, also correlations between FLC and clinESSDAI were assessed and only FLCκ was significantly correlated to clinESSDAI scores, although a trend was seen for FLCλ (figure 2B).

FLC levels are affected by immunomodulatory treatment

Rituximab

In the group of rituximab-treated and placebo-treated patients, 19 (63%) patients had abnormal FLC levels at baseline, which is comparable to the diagnostic cohort. None of the patients had an abnormal κ/λ ratio. Median values are displayed in table 1. No patients had monoclonal gammapathy, and no MALT-lymphoma patients were included in this treatment study. Rituximab reduced both FLCκ and FLCλ levels significantly over time (figure 3A). In placebo-treated patients, FLC also decreased significantly over time (figure 3B), which could be attributed to a drop only at week 5. In line with this notion, the decrease in FLC in the rituximab group over time was stronger, of longer duration, and significantly different from the placebo group ($P<0.001$ for both FLCκ and FLCλ). Furthermore, during B cell repopulation in the rituximab group, FLCκ and FLCλ levels increased to baseline values (figure 3A), whereas no changes were seen in the placebo group between weeks 24 and 48 (figure 3B). Relative changes in FLCκ and FLCλ after 24 weeks of treatment with rituximab were higher compared to IgG (median Δ -28%, -24% and -21%, respectively). SRM values for responsiveness were large (>0.8) for both FLC in the rituximab group, persisting until week 36 for FLCκ and week 24 for FLCλ (figure 4A). In the placebo group, a high SRM was found only at week 5 (figure 4B).

Abatacept

At baseline, 14 (93%) patients had abnormal FLC levels, which is higher compared to the diagnostic cohort and the rituximab group. Three (15%) patients had an abnormal κ/λ ratio. Serum FLC levels were reduced by abatacept treatment (figure 3C). Levels of FLCκ decreased to a larger extent than FLCλ (figure 3C). Median relative changes between baseline and week 24 for FLCκ, FLCλ and IgG were -18%, -10% and -12%, respectively, indicating that FLCκ was most strongly reduced by abatacept treatment. SRM values were larger for FLCκ than FLCλ (figure 4C).

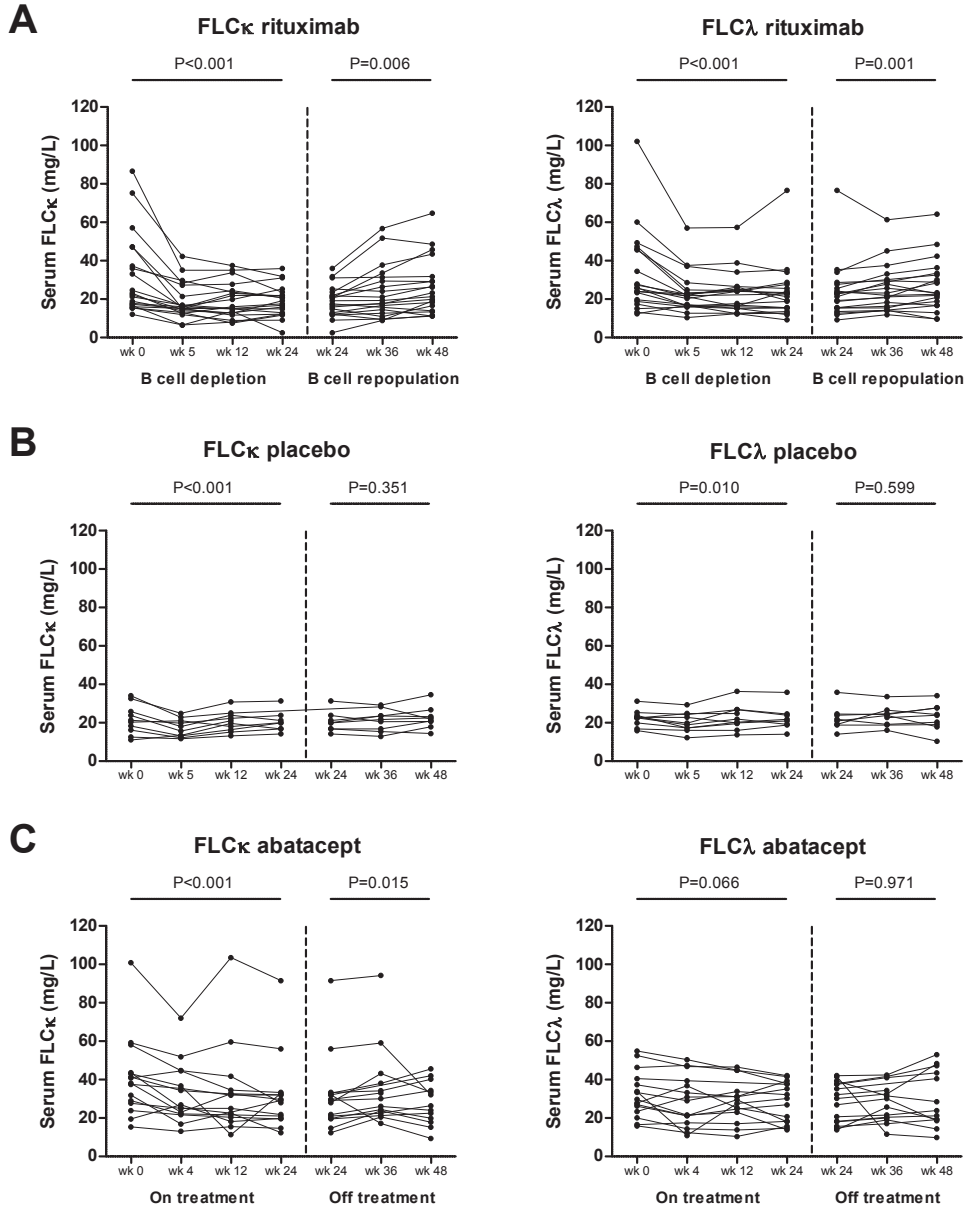

FIGURE 3 | FLC levels are lowered by immunomodulatory treatment. (A) FLCκ and FLCλ serum levels during B cell depletion (week 0-24) and B cell repopulation (week 24-48). (B) FLCκ and FLCλ serum levels in placebo-treated patients at the same time points as rituximab-treated patients. (C) FLCκ and FLCλ serum levels during abatacept treatment (week 0-24) and after cessation of treatment (week-24-48). P-values were calculated using generalized estimating equations with log-transformation of dependent variables.

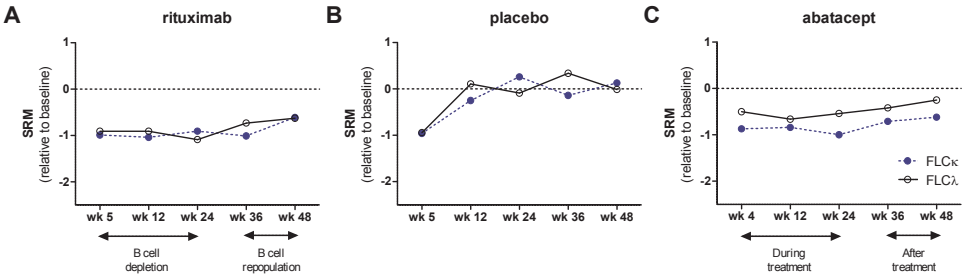

FIGURE 4 | Responsiveness of FLCκ and FLCλ in pSS patients treated with rituximab, placebo or abatacept. Standardized response means (SRM) are displayed for each treatment group at different time points during treatment (week 4/5, week 12, week 24) or after treatment (week 36 and 48). A negative SRM indicates lowering of FLC. SRM <0.5 is interpreted as small, 0.5–0.8 as moderate, and >0.8 as large.

Associations between changes in FLC levels and systemic disease activity during treatment

Finally, we assessed if the observed changes in FLC levels correlated with clinical and biological parameters over time during treatment, namely the period of B cell depletion by rituximab, and the period of treatment with abatacept (both week 0-24). We performed the same analyses for the period after treatment (week 24-48). The decrease in FLCκ and FLCλ in the rituximab group was significantly associated with lowering of ESSDAI and clinESSDAI scores from baseline to week 24 (table 2). Weaker correlations were observed in the placebo group (table 2). In the abatacept group, only FLCκ was significantly associated with ESSDAI and clinESSDAI scores over time during treatment (week 0-24).

TABLE 2 | Associations between FLC and systemic disease activity scores during treatment.

	ESSDAI		**ClinESSDAI**		**ESSDAI**		**ClinESSDAI**	
	During treatment (week 0-24)				**After treatment (week 24-48)**			
	Beta	***P-value***	***Beta***	***P-value***	***Beta***	***P-value***	***Beta***	***P-value***
FLCκ rituximab	0.030	**<0.001**	0.024	**<0.001**	0.015	**<0.001**	0.011	**<0.001**
FLCκ placebo	0.015	**<0.001**	0.009	0.079	0.009	0.138	0.004	0.082
FLCκ abatacept	0.008	**0.003**	0.007	**0.002**	-0.004	0.425	-0.003	0.324
FLCλ rituximab	0.018	**<0.001**	0.015	**<0.001**	0.007	**0.020**	0.005	**0.037**
FLCλ placebo	0.005	0.052	0.005	**0.011**	0.013	**0.007**	0.007	**0.012**
FLCλ abatacept	0.002	0.188	0.002	0.235	-0.006	0.236	-0.006	0.141

Longitudinal analysis were conducted for the period during treatment (week 0-24) and after treatment (week 24-48). Logarithmic transformation of FLC values was performed, and transformed values were entered into the Generalized Estimating Equation (GEE) models. Beta represents the regression coefficient (log-transformed). ESSDAI: European League Against Rheumatism (EULAR) Sjögren's syndrome Disease Activity Index; clinESSDAI: ESSDAI without inclusion of the biological domain. Significant P-values are displayed in bold.

Also after treatment, in the rituximab group, FLCκ and FLCλ levels correlated significantly with ESSDAI/clinESSDAI scores (table 2), suggesting that the increase in both parameters after treatment occurs in a similar manner.

DISCUSSION

Serum FLCs are biomarkers for actual B-cell activity and are frequently elevated in systemic autoimmune diseases [13,15]. This study shows that serum FLCκ and FLCλ levels are frequently elevated in pSS patients, compared to non-SS sicca patients, at the time of diagnosis. Importantly, we found that an abnormal FLC κ/λ ratio can be indicative for the presence of MALT-lymphoma. This study further shows that FLC levels rapidly decrease following treatment with rituximab or abatacept, and that these levels are associated with systemic disease activity at baseline and longitudinally in response to treatment.

In our diagnostic cohort, FLCκ and FLCλ levels were abnormal in 58% and 44% of patients, respectively. This percentage is considerably higher than the previously reported percentages of 22-24% by Gottenberg et al. [7,13]. This difference may be explained by higher B-cell activity in our cohort, reflected by higher IgG levels, and/or by the lower frequency of immunosuppressive drug use in our cohort. In line with a previous study by Sudzius et al. [23], we observed that increased FLC levels were more pronounced in anti-SSA-positive, compared with anti-SSA-negative pSS patients, suggesting that patients with anti-SSA antibodies exhibit higher B-cell activity and hence produce more FLC. We also found a subtle, but significant increase in the κ/λ ratio in pSS patients, though only 11% of the pSS patients had an abnormal ratio according to the 95-percentile range [7]. The reason for this skewing is not clear, but one possibility is that it is due to the autoantibody status, indicating that autoreactive B-cells might express relatively more kappa than lambda chains. However, no difference in the κ/λ ratio was observed between anti-SSA-positive and -negative patients, in line with a previous report [23]. In addition to anti-SSA/-SSB antibodies, pSS patients frequently produce RF. RF-positivity in pSS is associated with a higher risk of lymphoma development [24]. Bende et al. showed that 41% of salivary gland MALT-lymphomas expressed B-cell receptors with strong RF-homology [25]. Interestingly, the vast majority of these lymphomas was typed as κ-predominant by immunohistochemistry. These data suggest that RF-producing B cells may contribute to the skewed κ/λ ratio in a subgroup of pSS patients.

Because one of the pSS patients with an abnormal κ/λ ratio in our diagnostic cohort was diagnosed with MALT-lymphoma, we evaluated FLC levels in a cohort of established pSS patients with MALT-lymphoma located in the salivary gland. A previous study by Witzig et al. revealed elevated FLC levels in 31% of MALT-lymphoma patients,

but lymphoma localization was not specified [26]. Whether also salivary gland MALT-lymphomas in pSS patients present with abnormal FLC levels and/or ratios, is to the best of our knowledge not known. Our study shows that the κ/λ ratio was increased in 50% of patients with salivary gland MALT-lymphoma. In only one of the 12 (8%) pSS patients with MALT lymphoma a weak M-protein was detectable, indicating that secretion of complete immunoglobulins occurs only infrequently. A similar low frequency (9%) of M-protein in pSS-MALT patients was revealed by Wöhrer et al. [27], whereas we showed previously that M-proteins were present in 8 out 35 (23%) pSS-MALT patients [28]. Recent studies showed that the frequencies of monoclonal gammopathy in larger cohort of pSS patients were 7.4%-22% [29,30]. However, only few of the patients with M-proteins (0-6%) developed salivary gland MALT-lymphoma (follow-up time 6.3-10 years). These data strongly argue that the κ/λ ratio in serum may be more useful as indicator for the presence of MALT-lymphoma than finding a M-protein. This situation is analogous to patients with a monoclonal gammopathy of unknown significance (MGUS) in which the risk of progression to a malignant monoclonal gammopathy is higher in patients with an abnormal κ/λ ratio than in patients with a normal ratio [31]. In contrast to other pSS-lymphoma cohorts [8,32], the median ESSDAI score in our pSS-MALT cohort was relatively low. This may be explained by the fact that in our clinical work-up parotid gland biopsies are taken for diagnostic purposes. This setting results in unexpected detection of MALT-lymphoma in patients with low disease activity, as shown previously [28]. A second, not-mutually exclusive, explanation could be that both patients with a current or pre-existing MALT-lymphoma diagnosis were included. The finding that patients with a pre-existing MALT-lymphoma diagnosis frequently present with abnormal κ/λ ratios suggests that this biomarker may also be useful as indicator of MALT-lymphoma persistence and/or recurrence. Recurrence appears in 29-35% of the parotid gland MALT-lymphoma cases [28,33].

In addition to the findings in pSS-MALT patients, our study confirmed that abnormal FLC levels in pSS are associated with higher systemic disease activity, as measured by ESSDAI scores [7]. The biological domain of the ESSDAI score also includes elevated serum IgG levels, which obviously may bias the correlation between FLC levels and ESSDAI. For this reason we correlated FLC levels not only to ESSDAI, but also to clinESSDAI scores [20]. Interestingly, FLCκ was significantly and more strongly correlated to both ESSDAI and clinESSDAI scores than FLCλ. Thus, increased disease activity of pSS is not only associated with increased polyclonal B-cell activity, but remarkably also with preferential expansion of the kappa subtype, which is confirmed in other studies in pSS [7,8,13]. The reason for this preferential expansion remains obscure.

Besides a role as biomarker for systemic disease activity, FLC are potentially useful in monitoring the effect of immunomodulatory treatment on B-cell activity in pSS patients. Therefore, the effect of treatment with rituximab and abatacept on serum FLC

levels was evaluated. Rituximab treatment significantly reduced FLCκ and FLCλ levels, reflecting the decreased number of antibody-secreting cells (i.e., B-cells, plasmablasts, plasma cells). A reduction in FLC was also seen in the placebo group at week 5, likely due to corticosteroid treatment around the rituximab infusions, but a significantly stronger decrease was observed in the rituximab group. The decrease in FLC correlated significantly with lowering of ESSDAI and clinESSDAI during B-cell depletion. These findings are consistent with studies in RA and SLE, showing a correlation between normalization of FLC levels and disease activity upon rituximab treatment [34,35]. We found that abatacept treatment also significantly reduced FLCκ and, to a smaller extent, FLCλ levels. FLCκ levels were significantly associated with ESSDAI and clinESSDAI scores over time during treatment. As B-cells are not directly targeted by abatacept, the observed decrease in FLC is likely a result of reduced T-cell help to B-cells. This is further substantiated by the previously observed decrease in serum levels of IgG, anti-SSA antibodies and RF, as well as circulating plasmablasts [19,36]. Additionally, our study reveals that FLC have a large sensitivity to change, assessed by SRM, and that relative changes in FLCκ were higher compared with IgG. An advantage of monitoring FLC is that changes in serum levels are more rapidly induced after initiation of treatment, compared with IgG. FLC have a half-life of several hours, while IgG has a half-life of several weeks [9]. Therefore, B-cell activity can be monitored by FLC levels without the delay that is seen for IgG.

In summary, this study shows for the first time that the κ/λ ratio in serum is abnormal in 50% of salivary gland MALT-lymphoma patients. Abnormal ratios should be taken seriously and might be indicative for the presence, persistence and/or recurrence of MALT lymphoma. Prospective analysis of the κ/λ ratio in pSS patients is necessary to confirm its potential role as predictor of MALT-lymphoma development, and to compare sensitivity with M-protein analysis. Moreover, our data underline the pathogenic role of B-cell hyperactivity, and particularly kappa-expressing B-cells, in pSS. Finally, we suggest that FLC are useful biomarkers to monitor the effect of immunomodulatory treatment on B-cell activity.

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SUPPLEMENTARY MATERIALS

SUPPLEMENTARY TABLE 1 | Characteristics of the pSS-MALT patients at inclusion.

	Age	Sex	FLCκ (mg/L)	FLCλ (mg/L)	FLC κ/λ ratio	Anti-SSA positive	IgG (g/L)	RF (IU/mL)	M-protein	Cryo-glubulins	Low complement (C3/C4)	Parotid gland swelling	ESSDAI*	ClinESSDAI*	Time since pSS diagnosis (years)	Time since MALT diagnosis (years)	Location MALT	Current RTX treatment*	Previous MALT treatment
1	50	M	24.5	14.5	**1.69**	Yes	14.2	6.2	Neg	Neg	Low C3	No	15	14	1	1	Parotid right	No	None
2	70	F	25.4	15.5	1.64	Yes	13.2	10	Neg	Neg	Low C3	Yes*	15	14	7	5	Parotid right + left	No	Radiotherapy
3	68	F	35.8	15.3	**2.34**	Yes	18.5	45	Oligo	type III (weak)	No	No	7	5	27	12	Parotid right	No	None
4	50	F	24.2	13.3	**1.82**	Yes	11.2	14	Neg	type III (weak)	No	No	2	0	8	3	Parotid left+stomach	No	RCP
5	60	F	45.8	25.8	**1.78**	Yes	19.8	100	Neg	Neg	No	No	1	0	12	11	Parotid left	No	RCP
6	29	F	19.1	13.3	1.44	Yes	10.1	54	Neg	Neg	Low C3	No	1	0	3	2	Parotid right	No	RCP
7	61	F	25.2	18.1	1.39	Yes	13.4	38	Neg	Neg	No	Yes	10	12	4	3	Parotid right	No	None
8	61	F	38.1	26.8	1.42	Yes	14.2	6.3	Neg	type II poly-Ig	No	No	2	2	25	11	Parotid right	No	RCP
9	60	F	254.0	24.0	**10.58**	Yes	30.7	>200	Neg	type II	No	Yes	30	29	0	0	Parotid right	No	None
10	49	F	21.5	19.3	1.11	Yes	17.9	0	Neg	Neg	No	No	1	0	4	3	Parotid right	No	None
11	65	F	13.9	6.7	**2.07**	Yes	11.5	39	Neg	Neg	No	No	0	0	6	6	Parotid left	No	None
12	61	F	24.3	34.8	0.7	Yes	16.3	>200	IgG-λ (weak)	type II	No	Yes	24	23	2	0	Parotid right	No	None
13	49	F	10.9	9.3	1.17	No	8.6	0	Neg	Neg	No	No	0	0	8	2	Parotid right	Yes	RCP, radiotherapy
14	75	F	7.7	7.2	1.07	Yes	6.2	2.7	Neg	Neg	No	No	4	4	7	3	Parotid right	Yes**	RCP
15	49	F	18.0	14.2	1.27	Yes	11	4.3	Neg	type I (weak)	No	No	14	12	4	2	Parotid left	Yes**	RCP
16	62	F	12.5	11.2	1.12	Yes	6.6	8.1	Neg	Neg	No	No	2	2	22	12	Parotid left	Yes**	RCP
17	54	F	39.6	6.4	**6.2**	Yes	6.7	17	Neg	type II	Low C4	No	14	12	3	3	Parotid left	Yes	RCP

Abnormal FLC ratios are displayed in bold. *Because of gland enlargement due to lymphoma, lymphoma was scored in the lymphadenopathy and lymphoma domain and parotid gland swelling was not scored in the glandular domain in this patient. **Rituximab was continued for treatment of extraglandular manifestations, after finishing induction treatment with RCP. The treated MALT-lymphoma in these patients was considered in remission and therefore lymphoma was not scored positively in the ESSDAI. FLC: Serum immunoglobulin free light chains; RF: rheumatoid factor (IgM); M-protein: monoclonal protein; ESSDAI: European League Against Rheumatism Sjögren's Syndrome Disease Activity Index. ClinESSDAI: Clinical ESSDAI (ESSDAI without inclusion of the biological domain); MALT: mucosa-associated lymphoid tissue (lymphoma); RTX: rituximab; RCP: rituximab, cyclophosphamide, prednisone.

SUPPLEMENTARY TABLE 2 | Biomarkers associated with fulfillment of ACR-EULAR classification for primary Sjögren's syndrome.

	Univariate analysis		Multivariate analysis		
Biomarker	**Nagelkerke R^2**	**P**	**Model 1 P**	**Model 2 P**	**Model 3 P**
FLCκ	0.374	<0.001	ns	0.035	ns
FLCλ	0.254	<0.001	ns	ns	ns
IgG	0.521	<0.001	<0.001	-	0.001
Anti-SSA*	0.605	<0.001	-	<0.001	<0.001

*Anti-SSA status was entered as a binary variable. All other independent variables were entered as continuous variables. Ns: Not significant.

PART II

T CELL-DEPENDENT B CELL HYPERACTIVITY: TARGET FOR TREATMENT?