Serum CXCL13, BAFF, IL-21 and IL-22 levels are related to disease activity and lymphocyte profile in primary Sjögren's syndrome

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ABSTRACT

Objective. *To investigate the utility of serum BAFF, IL-17, IL-18, IL-21, IL-22, CXCL13, TNF-R2 and PD-L2 as biomarkers of disease activity in primary Sjögren's syndrome (pSS), their relationship with lymphocyte subpopulations and their accuracy to discriminate pSS from Sicca syndrome.*

Methods. *We conducted an observational study on 66 pSS patients and 48 controls (25 with* Sicca *syndrome and 23 healthy volunteers). Serum levels of BAFF, IL-17 A/F, IL-18, IL-21, IL-22, CXCL13, TNF-R2 and PD-L2 were measured using a multiplex immunoassay. Lymphocyte subpopulations were analysed by flow cytometry. Disease activity of pSS was assessed with ESSDAI at study inclusion.*

Results. *Patients with pSS presented higher serum CXCL13 (364.7 vs. 205.2 pg/mL), IL-21 (43.2 vs. 0 pg/mL) and BAFF (1646 vs. 1369 pg/mL), and lower PD-L2 levels (1950.8 vs. 2792.3 pg/mL) than controls. ESSDAI was associated with BAFF, IL-18 and IL-22. Patients with ESSDAI >0 exhibited higher CXCL13, IL-21, IL-22 and TNF-R2 concentrations. IL-21 levels correlated with lower memory B-cell and higher naïve B-cell percentages and IL-22 levels correlated with increased circulating activated CD4⁺ T-cells. The combination of serum CXCL13, BAFF and PDL2 levels using the formula [ln(CXCL13)+ln(BAFF)]/ln(PD-L2) exhibit an AUC of 0.854 (95% CI: 0.750-0.919) to discriminate between pSS and* Sicca *syndrome (sensitivity 77.2% and specificity 86.4% using a cut-off of 1.7).*

Conclusion. *CXCL13, BAFF, IL-21, and IL-22 are potential biomarkers of pSS activity and IL-21 and IL-22 are associated with disturbances of lymphocyte subpopulations in pSS. The combination of serum CXCL13, BAFF, and PD-L2 levels allows discrimination between pSS and Sicca syndrome.*

Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease that mainly affects middle-aged women, with a frequency in general population ranging from 0.01 to 0.72% (1, 2). The disease is a prototypical autoimmune disorder characterised by lymphocytic infiltration of salivary and lachrymal glands leading to xerostomia and keratoconjuntivitis *sicca* (1). Most patients present with oral and/or ocular dryness, although a wide number of systemic or extraglandular manifestations (EGM) may appear and may be the first clinical manifestation of the disease (3). Potentially, any organ can be involved, with some severe EGM leading to organ impairment and/or early death (3). Additionally, pSS patients are at increased risk of developing B-cell non-Hodgkin's lymphomas (4, 5), and it has been suggested that such lymphomas arise from autoreactive B cells (6). Pathophysiology of pSS is not yet fully understood. The disease typically targets the exocrine glands that are infiltrated by B and T lymphocytes, with periepithelial infiltrates and focal sialadenitis leading to destruction of the epithelial structures, tissue fibrosis and glandular dysfunction (7). Although polyclonal B cells activation, with hypergammaglobulinemia and a wide variety of circulating autoantibodies, is the most distinctive laboratory finding in pSS, T cells also play a role in pSS pathogenesis (8, 9).

Identification of biomarkers that allow the recognition of patients who are at risk of developing severe disease mani-

festations or lymphoma remains an unmet need in pSS (10). These patients could benefit from a closer monitoring and earlier use of intensive therapies. Furthermore, identification of biomarkers could be useful to help elucidate the complex pathogenesis of pSS.

Classically, β-2-microglobulin (β2MG) has been used as a surrogate biomarker of B cell hyperactivity in pSS due to its wide availability in most clinical laboratories (11-13). However, over the last decade, measurement of serum cytokines and chemokines has become more affordable, and several serum cytokines have been investigated in pSS, especially those related to B cells. Indeed, B-cell activating factor (BAFF), a cytokine of the tumour necrosis factor family (TNF) (14), has been identified as a biomarker that correlates with the European League Against Rheumatism (EULAR) Sjögren's Syndrome Disease Activity Index (ESSDAI) score, which is the most commonly used primary outcome measure in pSS clinical trials (15, 16). BAFF has a crucial role in B cell maturation, plasma cell survival, antibody response promotion and immunoglobulin-class switch (17). Serum levels of β2MG and BAFF have both been associated with disease activity and lymphoma development in patients with pSS (15, 18, 19). Likewise, Interleukin (IL) 21 levels have also been found increased in pSS patients, but its utility as a biomarker has not yet been established (20, 21). Other cytokines such as IL-4, IL-6, IL-12, IL-17, IL-18, IL-22, interferon gamma (IFNγ) and TNF alpha (TNF-α) have also been assessed in pSS (22, 23). Recently, Nishikawa *et al*. have identified five proteins (CXCL13, TNF-R2, CD48, BAFF and PD-L2) that correlated with the ESSDAI score (24). Furthermore, a link between CXCL13, disease activity, and lymphoma development, has been proposed by Nocturne *et al*. (25).

The aim of the present study was to investigate the accuracy of serum BAFF, IL-17, IL-18, IL-21, IL-22, CXCL13, TNF-R2, and PD-L2 levels, as potential biomarkers of disease activity in pSS, as well as their utility to discriminate pSS patients from patients with non-Sjögren S*icca* syndrome.

Methods

Design and study population

A cross-sectional study was performed between April 2014 and September 2017. Patients with pSS were consecutively recruited from the outpatient's clinic of the Systemic Autoimmune Diseases Unit of Vall d'Hebron University Hospital (Barcelona, Spain). Control group was composed of patients with non-Sjögren *Sicca* syndrome (n=25) from the same clinic, and healthy volunteers (HV) (n=23) recruited from the hospital staff in the same period. All patients with pSS and none of the patients with *Sicca* syndrome met the 2002 American-European Consensus Group criteria for pSS (26). Exclusion criteria included past or active lymphoma and ongoing infection. Clinical Research Ethics Committee of Vall d'Hebron University Hospital approved this study (PR(AG)35/2014) and informed written consent was obtained from all subjects before study inclusion.

Clinical and serological assessment

We collected clinical data including duration of the disease, subjective xerostomia and xeropthalmia, objective xerostomia and xeropthalmia, and presence of EGM such as arthritis, cutaneous vasculitis, peripheral nervous system (PNS) involvement, renal disease, and interstitial lung disease (ILD), according to the ESSDAI definitions (27). Objective xerophthalmia was assessed by ophthalmological evaluation with Schirmer's test, Tear Breakup time and corneal staining (SICCA Ocular Staining Score), at diagnosis and subsequently according to ocular findings. The last available routine evaluation was recorded. Objective xerostomia was evaluated by salivary gland scintigraphy at diagnosis in all cases. Salivary gland scintigraphy was repeated over the next 5 years from diagnosis in those patients with an initial I-II stage. Minor salivary gland (MSG) biopsy was routinely performed at diagnosis in all patients who gave informed consent.

Biological variables including presence of hypergammaglobulinemia, anti-Ro and anti-La antibodies, anti-nuclear antibodies (ANA) or rheumatoid factor (RF), erythrocyte sedimentation rate (ESR), immunoglobulin G (IgG) levels, C3 and C4 levels, cryoglobulins, and serum β2MG were assessed at study entry. Treatment with hydroxychloroquine, steroids, immunosuppressive drugs, or intravenous immunoglobulins (IVIG) over the last 6 months, and/or rituximab over the last 12 months was also recorded. Disease activity in pSS patients was routinely assessed using the ESSDAI score, and both baseline ESSDAI (at diagnosis) and at study inclusion were collected (27). ESSPRI was also collected in patients with pSS(28).

Serum cytokines

Serum samples were obtained from all subjects and stored at -80°C until analysis.

IL-17 A/F, IL-18, IL-21, IL-22, CXCL13 (C-X-C motif chemokine 13), TNF-R2 (TNF receptor 2) and PD-L2 (Programmed Death Receptor 1 Ligand 2) were determined using a personalized ProcartaPlex Multiplex Immunoassay (ThermoFisher Scientific, Waltham, MA, USA) following the manufacturer's instructions Briefly, after thawing and spinning serum samples, 25 µl of serum was incubated with shaking and at room temperature with the following materials and times: antibody beads for 2 hours, detection antibody for 30 minutes and Streptavidin–Phycoerythrin for 30 minutes. Data were acquired on a Luminex™ MAGPIX™ Instrument System (DiaSorin, Saluggia, Italy) and analysed with ProcartaPlex Analyst 1.0 software (Affymetrix, Thermofisher, ThermoFisher Scientific, Waltham, MA, USA).

BAFF concentrations were measured by ELISA using the commercially available Quantikine kit (R&D Systems, Minneapolis, MN, USA), following manufacturer's instructions.

All cytokines are expressed in pg/mL. Lower limits of quantitation for each cytokine are shown in Supplementary Table S1.

Lymphocyte subpopulations

Peripheral blood samples were collected with K3-EDTA anticoagulant at the time of study inclusion in all patients. Immu-

Table I. Characteristics of patients and controls.

	Sjögren's syndrome n=66	Sicca syndrome n=25	Healthy volunteers n=23	*p*-value
Age (years)	64.3 [48.1-70.7]	67.4 [63.6-72.0]	46.1 [41.1-53.5]	0.0001
Female sex	63 (95.5%)	25 (100%)	22 (95.7%)	0.6437
Duration of symptoms (years)	12.0 [8.1-16.4]	10.3 [8.4-15.4]		0.9362
Subjective xerostomia	62 (93.9%)	24 (96.0%)		1.000
Subjective xerophthalmia	65 (98.5%)	22 (88.0%)		0.0615
Pathologic salivary scintigraphy	66 (100.0%)	22 (88.0%)		0.0189
Objective xerophthalmia	65 (98.5%)	19 (76%)		0.0015
Positive MSG biopsy	37/41 (90.2%)	0/19		
Anti-Ro60	46 (69.7%)	0		
Anti-La	28 (44.4%)	0		
Anti-nuclear antibodies	66 (100%)	12 (48%)		<0.0001
Rheumatoid factor	42 (63.6%)	2 (8%)		<0.0001
Immunoglobulin G (mg/dL)	1317.5 [1046-1827]	974 [928-1129]		0.0004
ESR (mm/h)	32 [17-46]	21 [10-33]		0.1322
ESSDAI at baseline	10 [8-28]			
ESSDAI at inclusion	2 [0-4]			
Moderate or high score (≥5)	9 (13.6%)			
ESSDAI biological domain	1 [0-1]			
ESSPRI at inclusion	6 [4.00-7.7]			
Extraglandular manifestations¶	29 (43.9%)	1 (4%)		<0.0001
Arthritis	11 (16.7%)	1 (4%)		
Cutaneous vasculitis	7 (10.6%)	1 (4%)		
PNS involvement	7 (10.6%)	0		
Interstitial lung disease	6 (9.1%)	0		
Renal involvement	4 (6.1%)	0		
Active treatment	19 (28.8%)	1 (4%)		0.0103
Hydroxychloroquine	11 (16.7%)	0		
Corticosteroids	12 (18.8%)	1 (4%)		
Immunosuppressive drugs	4 (6.1%)*	0		
Rituximab	1 (1.5%)	0		
IVIG	1 (1.5%)	0		

ESSDAI: EULAR Sjögren's syndrome disease activity index; ESSPRI: EULAR Sjögren's Syndrome Patient Reported Index; ESR: erythrocyte sedimentation rate; IGIV: intravenous immunoglobulin; MSG: minor salivary gland; PNS: peripheral nervous system.
Values are expressed as n (%) or as the median and interquartile range [IQR].
¶Several patients had more than one extraglandular manifestation at inclusion.
*Immunosuppressive drugs were methotrexate in 3 patients and mycophenolate in another patient.

nophenotypic analysis was performed using a standard stain/lysis procedure. Briefly, 100 µL of whole blood sample was stained for 15 minutes at room temperature in the dark with saturating amounts of monoclonal antibodies in a 4–8-colour panel (Suppl. Table S2). The antibody clones, fluorochromes and their source are specified in Supplementary Table S3. Afterwards, erythrocyte lysis was performed with 1 mL of Versa-Lyse Lising Solution (Beckman Coulter, Brea, CA, USA) for 10 minutes. After lysis step, cells were washed in phosphate buffered saline. Acquisition of flow data was performed immediately in a Navios flow-cytometer (Beckman-Coulter, Brea, CA, USA) and analysis software was Kaluza (Beckman-Coulter, Brea, CA, USA). Total leukocytes and lymphocytes were gated using a $CD45^+$/SSC scattergram. Main lymphocyte populations were defined accordingly with the Human Immune Phenotyping Consortium (29). Gating strategies are illustrated in Supplementary Figure S1. Absolute counts of lymphocyte subsets were calculated using the percentages obtained by flow cytometry and the leucocyte count obtained from a haemocytometer the same day (number of cells/µL).

Statistical analysis

Categorical variables are expressed as number and percentage, whereas continuous variables are expressed as the median and interquartile range [IQR], unless otherwise specified. Fisher's exact test, Mann-Whitney U-test and Kruskall-Wallis test were used as appropriate to compare groups. All analyses were two-tailed. Pearson correlation coefficient was used to assess correlations. The relationship between ESSDAI and serum cytokines was evaluated using Poisson regression and Pearson correlation.

The diagnostic accuracy of serum cytokines that were significantly different in pSS, as well as their ratios was evaluated using the area under the curve (AUC) of receiver operating characteristic (ROC) curves. The 95% confidence intervals (CI) of AUC were calculated with binomial exact method. Sensitivity and specificity 95% CI were calculated with Wilson method. When using combinations of cytokines, we applied logarithmic transformations to allow the sum of cytokines with different order of magnitude.

In all analyses, *p*-values <0.05 were considered statistically significant. Graphics and statistical analyses were performed using GraphPad Prism 8.2.1

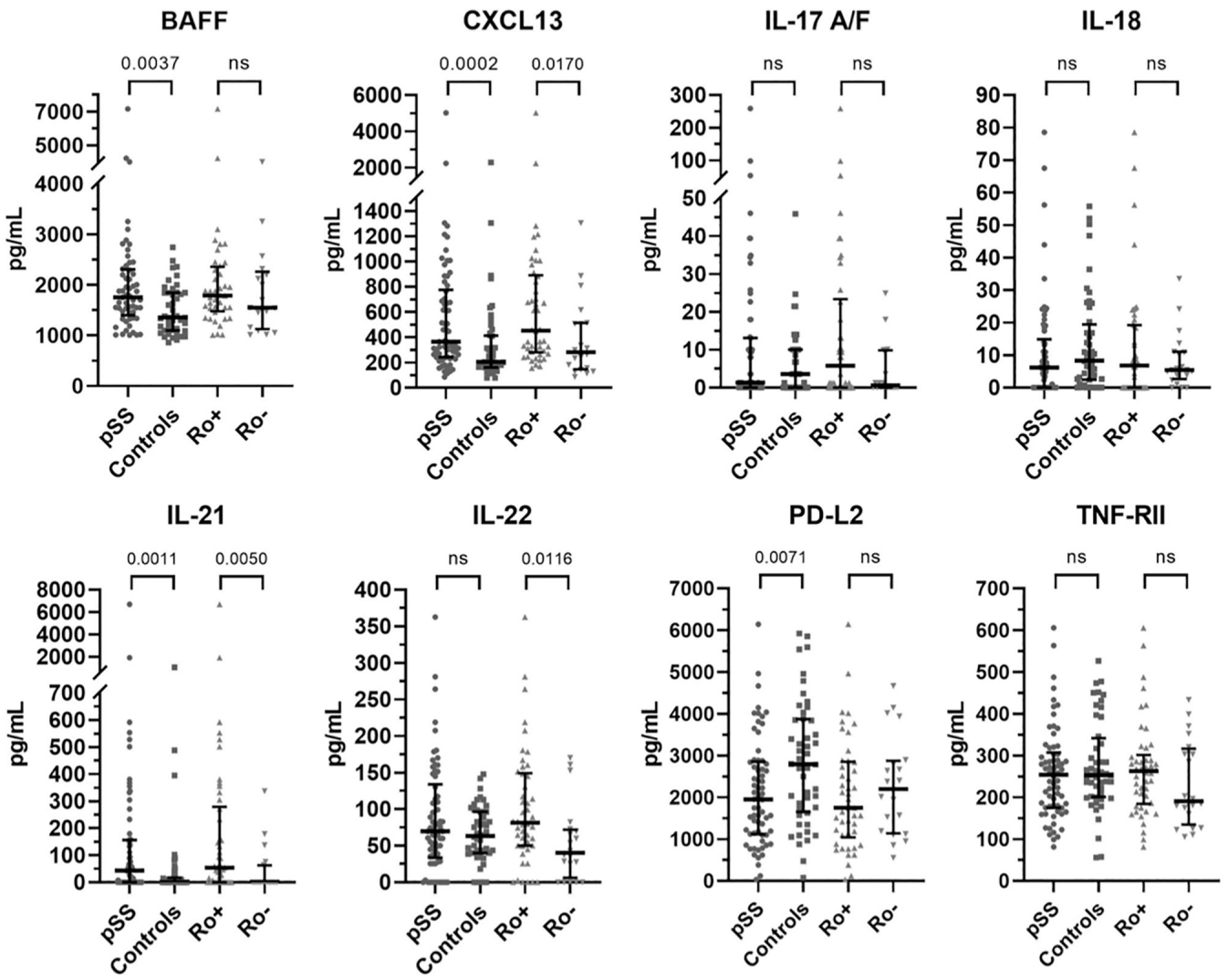

Fig. 1. Concentrations of serum cytokines in patients with primary Sjögren's syndrome and controls.
Concentrations of serum cytokines in patients with primary Sjögren's syndrome (pSS) (n=66), controls (25 *Sicca* syndrome and 23 healthy controls), seropositive pSS patients (Ro+) (n=46) and seronegative pSS patients (Ro-) (n=20) are shown in each scatter plot, with median and interquartile range.
All concentrations are shown in pg/mL.
BAFF: B-cell activating factor; CXCL13: C-X-C motif chemokine 13; IL-17 A/F: Interleukin 17 A/F heterodimer; IL-18: Interleukin 18; IL-21: Interleukin 21; IL-22: Interleukin 22; PD-L2: programmed death 1-ligand 2; TNF-RII: tumour necrosis factor receptor 2.

(GraphPad Software, San Diego, CA, USA) and Stata 15.1 (Stata Corp., College Station, TX, USA).

Results

Patients

Sixty-six patients with pSS and 48 controls (25 patients with *Sicca* syndrome and 23 HV) were enrolled. Characteristics of patients and controls are detailed in Table I. Median age was similar in patients with pSS and *Sicca* syndrome (64.3 *vs*. 67.4 years respectively, p=0.2671), but HV were younger (median 48.7 years, p=0.0001). Almost all subjects were women. Forty-six (69.7%) patients with pSS had positive anti-Ro60 antibodies while 28 (44.4%) had also positive anti-La antibodies. Nineteen (28.8%) pSS patients were receiving immunomodulant or immunosuppressive treatment at study inclusion. One patient with *Sicca* syndrome presented an isolated flare of arthritis and cutaneous vasculitis at disease onset that was treated with corticosteroids. This patient had positive ANA and RF at low titres only at baseline, with negative anti-citrullinated protein antibodies and negative Extractable Nuclear Antigen Antibodies Panel at baseline and in repeated tests and did not develop any other EGM over 10 years of follow-up.

Differences in serum cytokines between pSS patients and controls

Patients with *Sicca* syndrome and HV did not exhibit significant differences in serum cytokines concentrations (Suppl. Table S4), so a unique control group was considered for comparisons. Compared to controls, patients with pSS presented significantly increased CXCL13, IL-21 and BAFF concentrations, whereas PD-L2 was significantly decreased (Fig. 1).

Differences in serum cytokines betweensubgroups of patients with pSS

We compared serum concentrations of cytokines between pSS subgroups

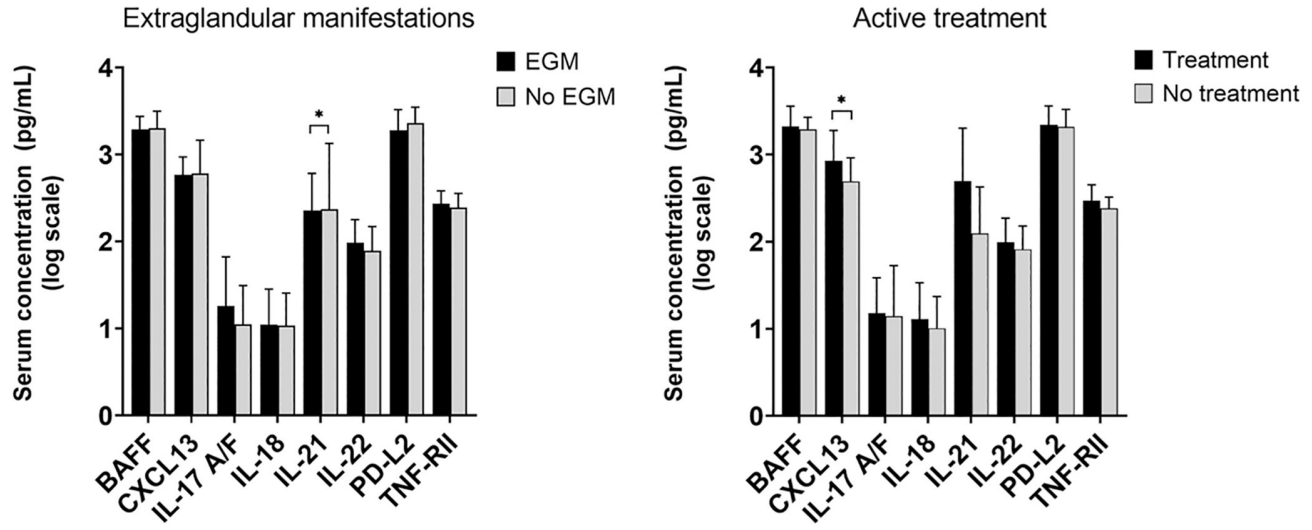

Fig. 2. Serum concentrations of cytokines in primary Sjögren's syndrome according to the presence of extraglandular manifestations and treatment status. Concentrations of serum cytokines in patients with primary Sjögren's syndrome are shown in logarithmic scale according to presence of extraglandular manifestations (left graph) and to treatment status at study entry (right graph). Bars show the mean and standard error of the mean after logarithmic transformation.
BAFF: B-cell activating factor; CXCL13: C-X-C motif chemokine 13; IL-17 A/F: Interleukin 17 A/F heterodimer; IL-18: Interleukin 18; IL-21: Interleukin 21; IL-22: Interleukin 22; PD-L2: programmed death 1-ligand 2; TNF-RII: tumour necrosis factor receptor 2; EGM: extraglandular manifestations.

according to the presence of antibodies against Ro60, EGM, and treatment status (Fig. 2). Seropositive pSS patients exhibited significantly increased CXCL13, IL-21 and IL-22 (p=0.0170, p=0.0050 and p=0.0116, respectively) while BAFF, IL-17 A/F, IL-18, PD-L2 and TNF-R2 were similar in both groups (Fig. 1).

Serum cytokines did not show relevant differences between patients with or without extraglandular involvement (Fig. 2). When we analysed if there was any relationship between serum cytokines and specific EGM, we found that CXCL13 was increased in patients with ILD (1143.3 *vs*. 330.7 pg/mL, p=0.0043), with a positive correlation between CXCL13 levels and the presence of ILD (r=0.354, p=0.0035).

Patients on active treatment showed similar serum levels of cytokines than those not receiving treatment, except for CXCL13 levels, that were higher in treated patients (848.8 *vs*. 492.1 pg/mL, p=0.0154) and IL-21 levels that were also higher in patients receiving treatment (76 *vs*. 14.9 pg/mL, p=0.0677).

Association between serum cytokines and markers of disease activity in pSS

We assessed correlation of serum cytokines with the ESSDAI score at study inclusion, the biological domain of ESSDAI, ESSPRI, ESR, IgG, RF, β2MG and the duration of disease (Fig. 3A). CXCL13 levels were significantly correlated with β2MG levels (r=0.374, p=0.0020) and with biological domain of ESSDAI (r=0.351, p=0.0039), and exhibited a weak correlation with ESSDAI at study inclusion (0.329, p=0.0071). IL-21 levels correlated with ESR (r=0.390, p=0.0016), IgG level (r=0.553, p<0.0001) and RF positivity (r=0.684, p<0.0001). The cytokine that best correlated with other biomarkers of disease activity was IL-22, which was correlated with ESR (r=0.474, p=0.0001), IgG levels (r=0.511, p<0.0001), RF (r=0.385, p=0.0018), β2MG (r=0.398, p=0.0009) and the biological domain of ESSDAI (r=0.370, p=0.0022). TNF-R2 showed correlation only with β2MG (r=0.425, p=0.0004). No correlations were found between serum cytokines and C3 or C4 levels or presence of cryoglobulins.

In the Poisson regression model (Suppl. Table S5), ESSDAI was associated with serum levels of BAFF (OR 1.00020, p=0.0017), IL-18 (OR 1.00971, p=0.0167) and IL-22 (OR 1.00191, p=0.0397), but not those of CXCL13 (OR 1.00016, p=0.0641). Of note, patients with ESSDAI >0 exhibited higher values of CXCL13 (696.9 *vs*. 359.8 pg/mL, p=0.0091), IL-21 (311.1 *vs*. 49.8 pg/mL, p=0.0461), IL-22 (98.4 *vs*. 59.1 pg/mL, p=0.0464) and TNF-R2 (278.8 *vs*. 207.4 pg/mL, p=0.0164).

Serum cytokine levels interrelationship in pSS

We analysed the relationship between the different cytokines evaluated in this study (Fig. 4). CXCL13 correlated with IL-21 (r=0.425, p=0.0004), IL-22 (r=0.515, p<0.0001) and TNF-R2 (r=0.471, p<0.0001), but not with BAFF (p=0.3262). IL-22 also correlated with IL-17 A/F (r=0.743, p<0.0001), IL-18 (0.577, p<0.0001), IL-21 (r=0.519, p<0.0001) and TNF-R2 (r=0.656, p<0.0001). IL-17 A/F and IL-18 also showed correlation (r=0.529, p<0.0001).

Relationship between serum cytokines and lymphocyte subpopulations in pSS

We evaluated the relationship between serum cytokines levels and the absolute and relative lymphocyte counts, as well as between the relative count of $CD4^+$ T-cells, activated $CD4^+$ T-cells, B-cells, naïve B-cells, and memory B-cells (Fig. 3B). CXCL13, IL-17 A/F and IL-22 levels were negatively correlated with the percentage of lymphocytes (r=-0.424, p=0.0004; r=-0.396, p=0.0011; and r=-0.336, p=0.0063, respectively). IL-17 A/F and IL-22

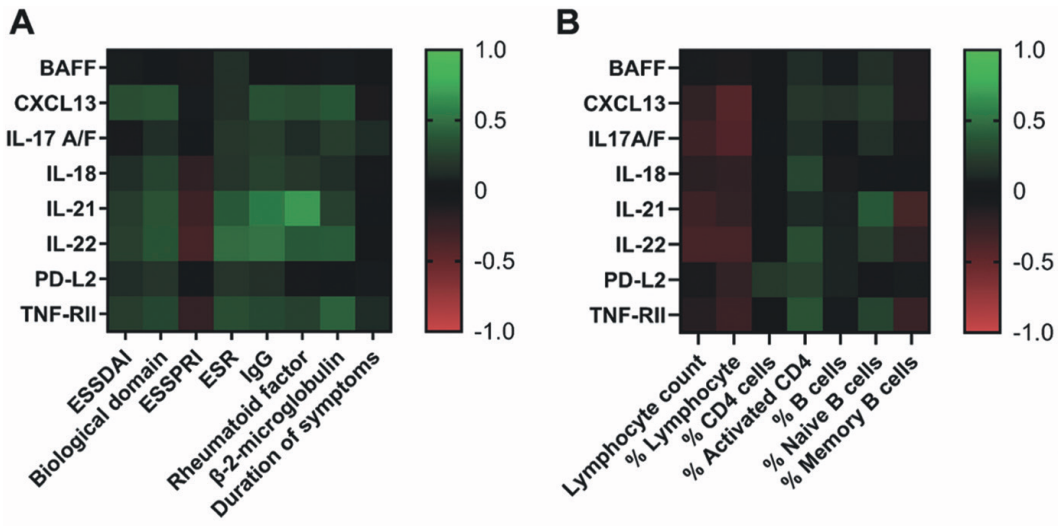

Fig. 3. Correlation of serum cytokines to disease activity and circulating lymphocyte subpopulations in primary Sjögren's syndrome.
A: Heat map of Spearman correlations between serum cytokines and disease activity measures and surrogates.
B: Heat map of Spearman correlations between serum cytokines and circulating lymphocyte count and lymphocyte subpopulations.
BAFF: B-cell activating factor; CXCL13: C-X-C motif chemokine 13; IL-17 A/F: Interleukin 17 A/F heterodimer; IL-18: Interleukin 18; IL-21: Interleukin 21; IL-22: Interleukin 22; PD-L2: programmed death 1-ligand 2; TNF-RII: tumour necrosis factor receptor 2; ESSDAI: EULAR Sjögren's syndrome disease activity index; ESSPRI: EULAR Sjögren's Syndrome Patient Reported Index; ESR: erythrocyte sedimentation rate; IgG: immunoglobulin G.

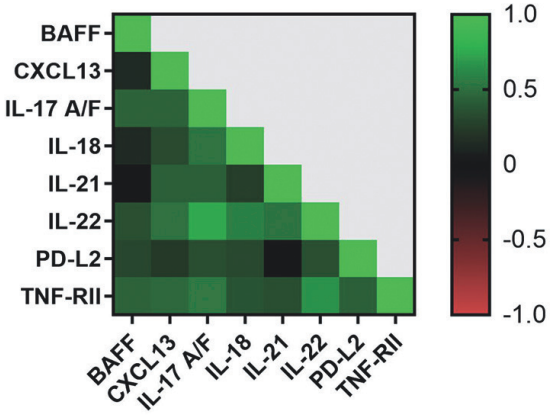

Fig. 4. Relationship between serum cytokines in primary Sjögren's syndrome.
Heat map of Spearman correlations between serum cytokines in primary Sjögren's syndrome.
BAFF: B-cell activating factor; CXCL13: C-X-C motif chemokine 13; IL-17 A/F: interleukin 17 A/F heterodimer; IL-18: interleukin 18; IL-21: interleukin 21; IL-22: interleukin 22; PD-L2: programmed death 1-ligand 2; TNF-RII: tumour necrosis factor receptor 2.

levels were also negatively correlated with the absolute count of lymphocytes (r=-0.306, p=0.0133, and r=-0.334, p=0.0067 respectively). IL-21 showed the best correlation with B-cell subpopulations percentages, exhibiting a negative correlation with memory B-cells (r=-0.344, p=0.0062) and exhausted B-cells (r=-0.422, p=0.0008) and a positive correlation with naïve B cells (r=0.379, p=0.0024). The percentage of activated $CD4^+$ T cells also correlated with IL-22 (r=0.331, p= 0.0110) and TNF-R2 (r= 0.349, p=0.0072).

Diagnostic accuracy of serum cytokines to discriminate between pSS and Sicca *syndrome*

Serum cytokines that were significantly different between pSS group and control group, as well as their combinations, were assessed as diagnostic biomarkers of pSS. As summarised in Table II, BAFF, CXCL13 and PD-L2 showed the best accuracy to identify patients with pSS. CXCL13 exhibited an AUC of 0.779 (95% CI: 0.681-0.860) to discriminate between pSS and *Sicca* syndrome; using a cut-off of 240 pg/mL sensitivity was 77.3% (95% CI 65.8–85.7) and specificity 68% (95% CI: 48.4–82.8). The ratio

Table II. Diagnostic accuracy of BAFF, CXCL13, IL-21 and PD-L2 to discriminate between *Sicca* syndrome and Sjögren's syndrome.

	Sjögren's syndrome n=66	Sicca syndrome n=25	Cut-off	AUC	Sensitivity (%)	Specificity (%)
BAFF (*pg/mL*)	1754 [1452-2290]	1156 [1020-1630]	>1452.0	0.743 (0.636-0.838)	75.4 (62.9-84.8)	68.2 (47.3-83.6)
CXCL13 (*pg/mL*)	364.7 [243.4-764.1]	181.0 [131.8-297.5]	>239.7	0.779 (0.681-0.860)	77.3 (65.8-85.7)	68.0 (48.4-82.8)
IL-21 (*pg/mL*)	43.2 [0-155.2]	0 [0-12.0]	>14.92	0.661 (0.552-0.756)	57.6 (45.6-68-8)	80.0 (60.9-91.1)
PD-L2 (*pg/mL*)	1950.8 [1123.4-2843.9]	2199.0 [1576.1-3850.5]	<1986.7	0.600 (0.496-0.705)	68.0 (48.4-82.8)	51.5 (39.7-63.2)
CXCL13/PD-L2	0.2517 [0.1263-0.3507]	0.0777 [0.0494-0.1419]	> 0.1	0.788 (0.693-0.869)	72.7 (61.0-82.0)	84.0 (65.3-93.6)
(CXCL13 + BAFF) / PDL2	1.084 [0.841-1.967]	0.544 [0.465-0.840]	>0.84	0.809 (0.706-0.890)	75.4 (62.9-84.8)	77.3 (56.6-89.9)
ln ***CXCL13*** **+** ln **BAFF**	1.8157 [1.6951-1.9137]	1.5711 [1.5147-1.6743]	> 1.7	0.854 (0.750-0.919)	77.2 (64.8-86.2)	86.4 (66.7-95.3)
ln ***PDL2***						

Serum cytokines concentrations are expressed as median and 25th and 75th percentiles.

CXCL13 to PD-L2 exhibited better specificity to discriminate between pSS and *Sicca* syndrome with an AUC of 0.788 (95% CI: 0.693–0.869); the cut-off 0.1 yielded a sensitivity of 72.7% (95% CI: 61–82) and a specificity of 84% (95% CI: 65.3–93.6). However, the most accurate score was the combination of CXCL13, BAFF and PDL2 levels using the formula [ln(CXCL13)+ln(BAFF)]/ln(PD-L2), which exhibit an AUC of 0.854 (95% CI: 0.750–0.919) and yielded a sensitivity of 77.2% (95% CI: 64.8–86.2) and a specificity of 86.4% (95% CI: 66.7–95.3) using a cut-off of 1.7.

Discussion

There is an unmet need for reliable serum biomarkers of disease activity in pSS that may guide monitoring and treatment (10). BAFF, CXCL13, TNF-R2 and PD-L2, have been previously related to the ESSDAI score (15, 24). In 2016 Nishikawa *et al*., using a high-throughput proteomic analysis to identify differentially expressed proteins between pSS patients and healthy controls, identified five proteins (CD48, CXCL13, TNF-R2, BAFF and PD-L2) that correlated with ESSDAI scores (24). In our study, we could confirm that BAFF, CXCL13 and PD-L2 levels were different between pSS patients and controls, but not those of TNF-R2. In contrast to previous studies, in our cohort CXCL13 and BAFF were weakly correlated to the ESSDAI score at the study entry. We believe that the main reason for this discrepancy is that our patients had a long-standing disease and ESSDAI may not reflect the disease activity so accurately in its advanced stages, when most clinical manifestations are long lasting (stable for at least 12 months), since stable manifestations score zero on ESSDAI and this may underestimate the overall disease activity. Indeed, in the Nishikawa *et al*. cohort the mean disease duration was 4.7 (SD 6.6) years in the derivation cohort and 3.5 (SD 5.8) years in the validation cohort, compared to 13.2 (SD 7.2) years in our patients.

B-cell activating factor (BAFF), also known as B Lymphocyte Stimulator (BLyS) was one of the first cytokines related to pSS activity (15, 30). In 2013 Quartuccio *et al*. reported a strong correlation between ESSDAI and BAFF(15). Compared to our cohort, their cohort comprised younger patients, with higher disease activity at study entry (median ESSDAI score at inclusion 10 *vs*. 2 in our cohort). More recently, James *et al*. also described a correlation between ESSDAI and BAFF in the largest cohort dealing on this topic (13). However, their cohort also included patients with shorter disease duration (mean 7 (SD 6.1) years *vs*. 13.2 (SD 7.2) years in our cohort) and higher ESSDAI scores at study entry (median 4 [1-7] *vs*. 2 [0-2] in ours). Altogether, our findings suggest that BAFF may be a more accurate biomarker to identify patients with high activity at early stages of the disease.

C-X-C motif chemokine 13 (CXCL13), also known as B lymphocyte chemoattractant (BLC) or B cell-attracting chemokine 1 (BCA-1), is a homeostatic chemokine that regulates B-cell trafficking, and plays a key role in the organization of lymphoid structures as well as in the formation of ectopic germinal centres in pSS (31, 32). Nocturne *et al*. were the first to report the association between CXCL13 and disease activity in pSS (25). Despite our cohort included pSS patients with more long-standing disease than the Nocturne cohort (12 *vs*. 5 years of disease duration), and a smaller proportion of patients with ESSDAI ≥5 (13.6% *vs*. 39.3%), we also found a significant correlation between ESSDAI and CXCL13, confirming the findings of these authors (24, 25). Furthermore, in our study CXCL13 was also associated with surrogate markers of B cell activity such as anti-Ro60 positivity, β2MG levels and lymphopenia. Besides, CXCL13 levels were associated with ILD development, validating the findings suggested by Nishikawa *et al*. (24). This finding could be of interest in patients with ILD of unknown aetiology when pSS is suspected. In addition, serum CXCL13 levels have recently been associated with lymphoma risk development (33), but patients with prior lymphoma were excluded from our study and none of the included patients developed a lymphoma during the subsequent follow-up until March 2021. However, three patients not included in the study due to prior lymphoma development, presented high serum CXCL13 levels (606 pg/mL in one patient with Hodgkin lymphoma, 1082 pg/mL in one patient with parotid MALT lymphoma, and 317.5 pg/mL in one patient with diffuse large B-cell lymphoma) despite being in complete remission, in line with results reported by Traiano *et al*. (33). Finally, despite CXCL13 has been related to the recruitment of circulatory memory B cells into the salivary glands (8), we did not found an association between circulating B cell subpopulations and CXCL13 levels.

IL-21 is a pleiotropic cytokine that belongs to the common cytokine receptor γ-chain-dependent cytokine family (34), and is mainly produced by T follicular helper cells (T_{FH}), operating as a driver of B cell activation and differentiation towards plasma cells (35, 36). In pSS, IL-21 has been related to proliferation of naïve and memory $CD8^+$ T cells, to activation, proliferation and survival of $CD4^+$ T cells, and to the presence of hypergammaglobulinemia (20). In the present study we could confirm that serum IL-21 levels were associated with the abnormal naïve/memory B-cell ratio characteristic of pSS (9) and with surrogate markers of B cell activity such as IgG or the biological domain of ESSDAI. Our findings support the key role of IL-21 produced in the germinal centres of inflamed glands of pSS patients in the recruitment of peripheral memory B cells.

IL-22, a cytokine that belongs to the IL-10 family, is known to regulate mucosal homeostasis and promote epithelial repair following tissue damage (37), and is overexpressed in the salivary glands of patients with pSS. IL-22 is produced by innate lymphoid cells, T cells, and ductal epithelial cells, and has a key role in B-cell recruitment and ectopic germinal centre formation (38, 39). IL-22 has been demonstrated to be involved in the production of lymphoid chemokines like CXCL13 in germinal centres, which in turn orchestrate B-cell clustering, lymphoid aggregation,

and autoantibody production (39). Of note, serum IL-22 has been related with anti-Ro and anti-La antibodies, hypergammaglobulinemia and RF in patients with pSS (40). In our cohort, additionally to previously described relationships, IL-22 was also related to the presence of lymphopenia, increased circulating activated $CD4^+$ T cells, and higher ESSDAI scores, as well as with CXCL13 levels.

Programmed death-1 (PD-1) is a receptor that belongs to the CD28/CTLA-4 family and together with its two ligands (PD-L1 and PD-L2) are key factors in inhibitory T cell signalling, mediating the mechanisms of tolerance and autoimmunity (41). PD-L1 has been extensively investigated over the last years, but PD-L2 function remains unclear. It has been proposed that PD-L2 has a dominant role in mucosal responses to environmental antigens by promoting tolerance (42). Tong et *al*. recently found that patients with systemic erythematosus lupus presented lower levels of soluble PD-L2 than healthy controls (43), and Nishikawa *et al*. proposed the potential utility of PD-L2 as a biomarker of disease activity in pSS (24). In our study we confirmed that patients with pSS had lower serum levels of soluble PD-L2 than controls, but we could not find an association with disease activity. Since oral tolerance is completely abolished in mice deficient in PD-L2 (44), it is possible that insufficient levels of soluble PD-L2 are somehow related to the development or sustenance of autoimmune response in salivary glands of patients with pSS.

Finally, we found that the ratio between CXCL13 plus BAFF to PD-L2 allows the identification of patients with pSS with high specificity. This finding could be useful for seronegative patients in whom pSS diagnosis could be doubtful. This serum biomarker could be useful in patients with *sicca* symptoms and negative antibodies against Ro-60/SSA and La/SSB but who present extraglandular manifestations suggestive of pSS involvement, such as non-specific interstitial lung disease (NSIP), peripheral polyneuropathy, or sensory ganglionopathy, and in whom a salivary gland biopsy is non-diagnostic, not feasible, contraindicated or refused by the patient (45).

Our study presents several limitations. First, the number of patients and controls in our cohort is small, despite larger than in other previous reports (24). Second, patients included in our study had long-lasting pSS, and it is possible that the behaviour of the disease activity biomarkers may be different in patients with initial or earlier stages of the disease. Likewise, it is possible that different biomarkers may be useful for different domains of disease activity (13). Lastly, the ESSDAI score may be less accurate to assess disease activity in patients with long-standing disease because persistent but stable clinical manifestations (>12 months) are scored as zero, which may underestimate disease activity in some patients.

In summary, CXCL13, BAFF, IL-21, and especially IL-22 are useful biomarkers of pSS activity, as all of them correlate with surrogate markers of B cell activity and/or ESSDAI. Furthermore, they may be more accurate indicators of disease activity than BAFF in patients with long-lasting pSS. Additionally, increased levels of IL-21 are related to the abnormal naïve/memory B-cell ratio, characteristic of pSS, and IL-22 levels are associated with increased circulating activated $CD4^+$ T cells. Finally, serum PD-L2 levels are decreased in patients with pSS and the combination of serum CXCL13, BAFF, and PD-L2 levels allows discrimination between pSS patients and *Sicca* syndrome patients. Further studies are needed to confirm our findings and to clarify the role of PD-L2 in pSS and the potential utility of therapies targeting CXCL13 and PD-1 pathways.

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