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CXCL13 as biomarker for histological involvement in Sjögren's Syndrome

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ABSTRACT

Objectives: Sjögren's syndrome (SS) is an autoimmune condition characterised by systemic B cell activation, autoantibody production and ectopic germinal centers (GC) formation within salivary gland (SG). The extent of SG infiltrate has been proposed as biomarker of disease severity. Plasma levels of CXCL13 correlate with GC activity in animal models and disease severity in SS, suggesting its potential use as a surrogate serum marker to monitor local B cell activation. Aim of this study was to evaluate the potential role of CXCL13 as biomarker of SG pathology in two independent SS cohorts.

Methods: 109 patients with SS were recruited at Sapienza University of Rome (Italy) (n=60), or at Queen Elizabeth Hospital in Birmingham and Barts Health NHS Trust in London (n=49). Both sera and matched minor SG biopsy were available. Sicca (n= 57) and healthy subjects (n=19) sera were used as control.

Results: CXCL13 serum level were higher in SS patients compared to controls. Correlations between its serum levels and a series of histomorphological parameters, including size of the aggregates and the presence GCs, were observed.

Conclusion: Our data foster the use of CXCL13 to monitor the extent of local pathology in SS and its validation in longitudinal clinical studies.

Key Words:

Sjögren's Syndrome; Biomarkers; Histopathology; Cytokines and inflammatory mediators; Lymphocytes.

Key messages:

- In SS, serum CXCL13 correlate with the extent of salivary gland inflammation and lymphoid organization.
- CXCL13 correlates with the focus score and with novel quantitative and qualitative histological parameters.
- Longitudinal studies are recommended to confirm CXCL13 as a surrogate biomarker for SS histology.

INTRODUCTION

Sjögren's syndrome (SS) is an autoimmune disease characterised by aberrant B cell activation, dryness and systemic manifestations [1]. The presence of germinal centres (GC) within salivary gland (SG) ectopic lymphoid structures (ELS) has been associated with autoreactive B cell survival, increased systemic disease activity [2], and lymphoma development in some [3,4] but not all studies [2,5].

ELS within SGs are histologically referred to as foci. The number of foci/4mm^2 of glandular tissue is reported as the focus score (FS) [6]. Despite obvious limitations related to potential biases in its calculations and its inability to capture the extent and degree of organization of SG infiltration [7], FS calculation is routinely used in clinical practice and known to correlate with the presence of autoantibodies, ocular and oral dryness [8]. High FS also correlates with presence of GCs and systemic manifestations including lymphoma development [9].

The analysis of SG biopsies has been implemented in clinical trials to monitor response to therapy and local efficacy of biological compounds. Whilst acceptable as part of the diagnostic algorithm, the use of repeated SG biopsies in clinical trials may deter some patients from participation. Until now, there are no clear indications that serum biomarkers can be used to monitor the extent of SG infiltration and whilst there are some indications that a correlation exists between SG histopathological features and autoantibody titres [8], no attempts have been made to identify serum biomarkers that could robustly monitor local disease state in SS.

The CXCL13-CXCR5 (chemokine (C-X-C motif) receptor 5) axis plays a central role in regulating B cell aggregation and organization, including movements within and outside the GC and aggregation of ELS [10]. Local protein and mRNA expression of CXCL13 within the SG has been previously associated with the degree of cellular organization of the ELS in patients with SS and in animal models of the disease [11,12]. More recently, CXCL13 serum levels, measured with commercially available kits, have been used as biomarkers of SS disease activity [13]. However, the correlation between CXCL13 serum levels and the degree and organisation of cellular infiltration in the SG has not previously been studied.

METHODS

Patients and samples

Unselected consecutive patients with SS, meeting 2002 AECG criteria [14], for which both serum samples and matched minor SG biopsy were available, were recruited in the Sjogren's Clinic at Sapienza University of Rome (Italy) (Italian Cohort, n=60), or in the Sjogren's Clinics based at Queen Elizabeth Hospital Birmingham and Barts Health NHS Trust in London (British cohort, n=49). Patients' demographics, clinical and laboratory features are described in Supplementary Table S1, available at *Rheumatology* online. Sicca patients, as well as age- and sex-matched healthy subjects (HS), from both cohorts were used as controls (n= 57 and n=19, respectively). All materials and data collected in this study derive from routine clinical practices. SG biopsies have been performed for diagnostic purposes, in particular in cases presenting with negative autoantibodies. All subject provided informed consent to participate in this study and approval by the local Ethical Committee was obtained for the 3 participating centres. No significant differences in histological parameters were observed between the two cohorts, with the exception of a tendency toward a higher prevalence of GCs in the British cohort (Supplementary Table S2, available at *Rheumatology* online); a lower prevalence of autoantibodies in the Italian cohort was also observed (Supplementary Table S1, available at *Rheumatology* online).

SG biopsies were performed for diagnostic purposes and peripheral blood samples were collected either at the time of surgery or, for the Birmingham cohort, at the first presentation with the biopsy being collected at a subsequent visit. Paraffin-embedded SGs were processed for Haematoxylin and Eosin (H&E) and immunohistochemistry (IHC) staining. Samples were analysed at a single cutting level according to standard of practice. Immunostaining were performed on sections sequential to those used by the pathologist for the routine histological analysis. GCs were defined as follow: nodular aggregates presenting a clear evidence of a light and dark zone, confirmed by positive IHC staining for CD21 long isoform (CD21L) on sequential section. SG tissue from 25 SS patients (one SG per patient) from the Italian cohort was also stored in OCT freezing medium at -80°C. Seven micron-thick frozen SG sections were sequentially cut, in RNA-free conditions, on Superfrost slides and stained as described [15] (supplementary material, section Methods, available at *Rheumatology* online). Five 30 microns sections, sequential to the first slide, were also cut and collected in RNA free Eppendorf for RNA isolation and qPCR. For ELISA, Immunostaining and qPCR methods, see supplementary material, section Methods, available at *Rheumatology* online.

Image analysis

Stained slides were scanned by Zeiss Axio Scan. The perimeter of the glandular tissue and foci were manually drawn using Zeiss software (Figure 1a). The relative areas were digitally calculated as well as segregated foci (Figure 1c; comparison with mixed infiltrate in Figure 1b) and GCs (Figure 1d)

were detected in order to collect the following parameters: FS, percentage of GCs, mean foci area, percentage of infiltration, percentage of segregation; see supplementary material, section Methods, available at *Rheumatology* online

Statistical analysis

For the statistical analysis, Mann-Whitney U test and Spearman's rank correlation tests were used. Two-tailed p values < 0.05 were considered significant. Statistical analysis was performed using GraphPad software.

RESULTS

In both cohorts, FS values correlated with the percentage of infiltration (Figure 1e and 1f) and more moderately with the percentage of segregated foci, GCs and mean foci area (Figure 1g-l). Percentage of infiltration and mean foci area showed a correlation with the presence of segregated foci and GCs in both cohorts (Supplementary Figure S1 a-h, available at *Rheumatology* online). This finding suggest how the larger are the infiltrates and the higher is the percentage of infiltration, the more organised the infiltrates are in terms of segregation and presence of GCs. CXCL13 serum levels were higher in all SS patients [90.3 (84.2) pg/ml], compared to both sicca patients [61.9 (38.6) pg/ml), p=0.0005] and HS [36.5 (40.18) pg/ml, p<0.0001] (Supplementary Figure S2, available at *Rheumatology* online). No differences in average CXCL13 levels were detected between the two cohorts [83 (95.2) pg/ml and 103 (64.9) pg/ml, respectively] (Supplementary Table S2, available at *Rheumatology* online). In both cohorts, serum levels of CXCL13 were found to correlate with the percentage of SG infiltration, FS and mean foci area (Figure 2a-d). Moreover, higher serum levels of CXCL13 were observed in patients with histologic evidence of segregated foci and GCs (Figure 2g-2j). Higher serum levels of CXCL13 were detected in patients with positivity for anti-Ro/SSA antibodies (p=0.0007, Italian cohort and p=0.0294, British cohort) as well as rheumatoid factor (RF) (p=0.0037, Italian Cohort and p=0.0033, British Cohort). In the Italian cohort, CXCL13 serum levels were also higher in patients with anti-La/SSB antibodies (p=0.0050) (Supplementary Figure S3, available at *Rheumatology* online). Both in the Italian and in the British cohorts, a positive correlation was observed between CXCL13 serum levels and Immunoglobulins (g/l) (p=0.006 r=0.380 and p=0.015 r=0.343, respectively) and RF (U/ml) (p=0.001 r=0.443 and p=0.0002 r=0.514, respectively) (Supplementary Figure S3, available at *Rheumatology* online). All of the above mentioned histological and laboratory correlations were maintained even pooling the two cohorts together. We also divided all the SS patients in two subgroups according to a diagnostic cut off value of

CXCL13 (101.3 pg/ml) calculated by the receiver operating characteristic curve [sensitivity 44.95%, specificity 82.4% (likelihood 2.562)]. An association with autoantibody production (anti-SSA, anti-SSB, RF, hypergammaglobulinemia) as well as histological features (GCs and segregated foci) was identified in patients with CXCL13 serum levels above 101.3 pg/ml (Supplementary Figure S4, available at *Rheumatology* online). In this subgroup of patients, no association was identified with specific clinical features. CXCL13 gene expression was also assessed in 25 frozen SGs. Clinical, serological and histological data regarding the 25 SGs used for qPCR are reported in the online Supplementary Table S3, available at *Rheumatology* online. Median SG area was 5 (1) mm^2 [9/25 (36%) had a total area lower than 4 mm^2]. In these patients, tissue expression of CXCL13 was found to correlate moderately with the mean foci area, and percentage of infiltration (Supplementary Figure S5a and S5b, available at Rheumatology online). In addition, higher expression of CXCL13 transcripts was observed in SGs with segregated foci and GCs (Supplementary Figure S5e and S5f, available at *Rheumatology* online). Higher local expression of CXCL13 was also associated with the presence of anti-Ro/SSA antibodies (Supplementary Figure S5g, available at *Rheumatology* online). Interestingly, serum levels of CXCL13 did not correlate with local mRNA levels of the same cytokine (Supplementary Figure S5d, available at *Rheumatology* online).

DISCUSSION

In this study, we demonstrate, for the first time, that CXCL13 serum levels correlate with the extent of SG inflammation and lymphoid organization in patients with SS. In particular, the association with the presence of ectopic GCs, and with subsequent lymphoma development as reported by others [13], makes CXCL13 an important candidate biomarker of higher disease activity and more severe pathology evolution. Although the association of CXCL13 with lymphoma in SS requires replication in longitudinal cohorts, data from non-human primate models show how blood CXCL13 correlates strongly with GC activity [16] and high baseline levels of CXCL13 strongly associate with non-Hodgkin lymphoma development in a large population-based study [17].

We and others have advocated SG biopsy as an objective outcome measure in SS clinical trials [7]. However, the invasive nature of the investigation may deter some participants, making blood-based correlations attractive. Moreover, considerable variability in infiltration between individual minor SGs from the same patient raises concerns about reliability of histology analyses, especially when limited tissue is obtained. It is therefore conceivable that a blood-based biomarker may more comprehensively assess the extent of SG infiltration, as well as reflect involvement of secondary lymphoid tissue. The moderate correlations we observed between CXCL13 and the various measures

of SG inflammation are encouraging towards the use of serum CXCL13 as a surrogate biomarker of immunological activity in the SG and a possible predictor of disease evolution, aside from its value on monitoring disease activity. Nonetheless, it is interesting that there is no clear correlation between serum and local levels of CXCL13. This finding suggests that serum CXCL13 levels might not simply reflect a local production but also a systemic production. A limitation of this latter experiment has to be however considered. mRNA analysis was performed on a limited amount of frozen glandular tissue [only one sg per patient with a median sg area of 5 (1) mm^2]. Given the heterogeneous distribution of the SG infiltrate within the different glands, this small sampling might introduce a significant bias in the analysis.
In the context of a clinical trial, we have also recommended collecting measures of SG infiltration additional to the FS, given that disease improvement might be also reflected by a reduction in size and organization of the foci rather than number [7].

The additional histological measures that we propose in this study appear attractive not only to overcome problems related to a limited amount of tissue, which might impair FS calculation, but also to provide qualitative information regarding the infiltrates. Here we have demonstrated that both the FS and the new histological parameters are correlated with CXCL13 serum levels. However, longitudinal studies would be required to test these additional histological parameters. The ability of CXCL13 to function as a surrogate biomarker for histological or clinical endpoints in SS requires further testing too. Furthermore, evaluation of CXCL13 saliva levels in relation to SG histological features, would also be of interest in future studies .

In a small open label study, abatacept treatment of SS patients induced reduction in CXCL13 levels and clinical systemic disease activity [17] and a trend towards fewer GCs but with no FS changes [18]. CXCL13 levels and clinical outcomes were both markedly improved following treatment with the anti-CD40 agent Iscalimab (CFZ533) in a double blind randomised trials, but no data are available on histological changes [20]. Conversely, a decrease in CXCL13 did not appear to correlate with clinical response in a randomised clinical trial using Pi3Kdelta inhibitor, leaving space for further investigation.
Despite these controversies on its use as biomarker of disease activity, our study, clearly demonstrate the ability of CXCL13 to reflect the degree of local disease activity in SS. Finding were confirmed in two separate cohorts, analysed independently by two individuals. Our data do not demonstrate that serum CXCL13 can replace biopsy as a diagnostic tool for SS, nor we are

advocating such use; however, they support the use of this biomarker in SS, favouring its measurement in longitudinal clinical trials and cohorts.

FIGURES

Figure 1. Histological analysis and correlation between focus score and novel parameters. Minor SG (H&E staining) analysed with Zeiss program: red line to circumscribe and calculate the salivary gland total area (mm2), green line to circumscribe and calculate the foci area (mm2) (a). Minor SG IHC staining for T (CD3+; brown) and B (CD20+; pink) showing non segregated (b) and segregated focus (c). Minor SG germinal centre stained with H&E and IHC for T (CD3+; brown) and B (CD20+; pink) and FDC (CD21; brown) (d). Correlation and linear regression analysis between focus score (FS) and different histological parameters; % infiltration (e and f) , % of segregated foci (g and h), % of germinal centre (i and j) and mean foci area (k and l) in the Italian (e, g, i and k) and British cohort (f, h, j and l) of SS patients. Each symbol (x) represents one patient. Dashed line represents the linear regression. Spearman r correlation coefficient and p-value (two-tailed) are presented on each graph.

Figure 2. Correlation and association between CXCL13 serum levels and histological parameters. Correlation and linear regression analysis between CXCL13 serum levels (pg/mL) and % infiltration (a and b), focus score (FS) (c and d) and mean foci area (mm2) (e and f) in the Italian (a, c and e) and British cohort (b, d and f) of SS patients. Each symbol (x) represents one patient. Dashed line represents the linear regression. The Spearman r correlation coefficient and the p-value (two tailed) are presented on each graph. CXCL13 serum levels (pg/mL) in SS patients with segregated and non-segregated foci (g and h) and in those with germinal centres (GC+) and without germinal centres (GC-) (i and j) in minor SG in the Italian (g and i) and British cohort (h and j). Data are presented as box and whiskers plots; individual data points are shown. P-values are displayed in each graph; two-tailed unpaired Mann-Whitney U test.

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