RHEUMATOLOGY

Original article

Unique Sjögren's syndrome patient subsets defined by molecular features

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Abstract

Objective. To address heterogeneity complicating primary SS (pSS) clinical trials, research and care by characterizing and clustering patients by their molecular phenotypes.

Methods. pSS patients met American–European Consensus Group classification criteria and had at least one systemic manifestation and stimulated salivary flow of $\geqslant 0.1$ ml/min. Correlated transcriptional modules were derived from gene expression microarray data from blood ($n = 47$ with appropriate samples). Patients were clustered based on this molecular information using an unbiased random forest modelling approach. In addition, multiplex, bead-based assays and ELISAs were used to assess 30 serum cytokines, chemokines and soluble receptors. Eleven autoantibodies, including anti-Ro/SSA and anti-La/SSB, were measured by Bio-Rad Bioplex 2200.

Results. Transcriptional modules distinguished three clusters of pSS patients. Cluster 1 showed no significant elevation of IFN or inflammation modules. Cluster 2 showed strong IFN and inflammation modular network signatures, as well as high plasma protein levels of IP-10/CXCL10, MIG/CXCL9, BLyS (BAFF) and LIGHT. Cluster 3 samples exhibited moderately elevated IFN modules, but with suppressed inflammatory modules, increased IP-10/CXCL10 and B cell–attracting chemokine 1/CXCL13 and trends toward increased MIG/CXCL9, IL-1α, and IL-21. Anti-Ro/SSA and anti-La/SSB were present in all three clusters.

Conclusion. Molecular profiles encompassing IFN, inflammation and other signatures can be used to separate patients with pSS into distinct clusters. In the future, such profiles may inform patient selection for clinical trials and guide treatment decisions.

Key words: Sjögren's syndrome, precision medicine, biomarkers, interferon

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CLINICAL SCIENCE

Rheumatology key messages

- Transcriptional modules separate Sjögren's patients into distinct clusters characterized by patterns of immune dysregulation.
- Molecularly distinct clusters of Sjögren's patients share similar demographics and clinical features.
- Molecular profiles spanning multiple immune pathways may support rational clinical trial design and treatment selection.

Introduction

Primary SS (pSS) is an autoimmune disorder characterized by severe, persistent dryness of the mouth, eyes, and other mucous membranes. These symptoms result from exocrine gland dysfunction that is associated with infiltrating mononuclear cells and systemic autoimmunity. In nearly half of pSS patients, systemic autoimmunity produces one or more extraglandular symptoms, such as arthritis, vasculitis, lung disease and lymphoma. Despite disabling manifestations of systemic autoimmunity, pSS is primarily treated symptomatically [1], and options for disease-modifying treatment are limited.

Trials of disease-modifying immunomodulatory treatments in SS have largely failed to meet their primary endpoints, perhaps in part because of the clinical endpoints used and the clinical and immunologic heterogeneity of subjects meeting entry criteria. All of these factors can potentially affect the probability of any given patient responding to a particular intervention. For example, baseline gene expression data in an open-label trial of 15 patients with pSS correlated with response to rituximab [2]. In addition, the response to belimumab in another pSS trial was independently associated with low numbers of NK cells in the saliva and blood [3], and cellular immune phenotypes in the blood identified pSS patient subsets with higher disease activity and glandular immune infiltration [4]. These findings suggest that molecular phenotyping of pSS patients may support the identification of subjects who will respond to therapy and may ultimately lead to success in advancing disease-modifying therapies to the clinic [5]. However, little is known about the different immunologic subsets of patients with this disease.

Autoantibody profiles have been historically considered to identify subsets of pSS patients. In an international cohort of 886 pSS patients meeting 2002 American–European Consensus Group classification criteria, 76% exhibited anti-Ro/SSA antibodies and 49% anti-La/SSB antibodies [6]. Individuals with anti-Ro/SSA and/or anti-La/SSB antibodies demonstrated increased infiltration of the exocrine glands, reduced salivary and lacrimal duct function and an increased likelihood of extraglandular manifestations [7]. Although less common, anti-centromere and anti-mitochondrial autoantibodies in pSS correlate with additional clinical signs such as Raynaud's syndrome and major organ damage [7].

Similar to other systemic autoimmune rheumatic disorders, pSS also has been associated with a type I IFN signature in many patients [8–11]. Further, patients with pSS can be stratified by the relative extent of type I and type II IFN activity [12, 13], and an elevated IFN signature has been associated with increased rates and higher titres of anti-Ro/SSA and La/SSB autoantibodies [14]. IFN gene expression in the minor salivary glands has been associated with extraglandular features such as arthritis, arthralgia and RP [13]. However, IFN activation does not consistently correlate with the severity of sicca features of pSS [12], and many autoantibody-positive, IFN-positive pSS patients have no major extraglandular involvement.

A better understanding of molecular heterogeneity in pSS is necessary for more effective clinical trial design and individualized therapeutic selection. To address this need, we assessed a broad panel of whole blood gene expression signatures, circulating immune and inflammatory mediators and autoantibody profiles in patients with pSS to identify different immunologic phenotypes that may be useful for future research, clinical trials and, potentially, clinical care.

Methods

Study design

This study evaluated patient samples from the baseline visit in a study of baminercept for treatment of SS (ClinicalTrials.gov identifier NCT01552681) [15]. Individuals with pSS ($n = 47$) were enrolled at nine sites through the National Institute of Allergy and Infectious Diseases–funded Autoimmunity Centers of Excellence programme and appropriate samples for the present analyses were available for 47 of these subjects. The study complies with the Helsinki Declaration and was approved by each site's institutional review board (Cedars-Sinai, Stanford, St. Francis Medical Center/ University of Connecticut, University of Chicago, Johns Hopkins, University of Rochester Medical Center, Duke University Medical Center, Oklahoma Medical Research Foundation, University of Pittsburgh). Patients provided written informed consent prior to participation. Inclusion criteria included age between 18 and 75 years, fulfilment of at least three of the four revised European criteria proposed by the American–European Consensus Group for pSS, stimulated salivary flow $\geqslant 0.1$ ml/min and the presence of one or more systemic non-life-threatening SS manifestations. Disease activity was measured by the EULAR SS Disease Activity Index (ESSDAI) [16] and visual analogue scales (VASs; range 0–100) for physician and patient global assessments for dryness, fatigue and joint pain [15]. Healthy controls for gene expression profiling were derived from the Oklahoma Immune Cohort [17]. Study data for patients are available through ImmPort under study SDY823.

Gene expression profiling

Blood was collected into PAXgene blood RNA tubes (PreAnalytiX, Hombrechtikon, Switzerland) and total cellular RNA was isolated and purified (PAXgene Blood RNA kit, Qiagen, Valencia, CA, USA). RNA quality and quantity were determined using the Agilent 2100 Bioanalyzer and Agilent RNA 6000 Nano system (Agilent Technologies, Santa Clara, CA, USA). After depletion of globin mRNA (GLOBINclear-Human Kit, Thermo Fisher Scientific, Waltham, MA, USA), RNA was amplified and transcribed *in vitro* using the TargetAmp-Nano Labelling Kit for Illumina Expression BeadChip (Epicenter Technologies, Madison, WI, USA) and cDNA was hybridized to the 12-sample HumanHT-12 v4.0 Expression BeadChip (Illumina, San Diego, CA, USA). Chips were scanned using the Illumina iScan system. Quality control of gene expression data was performed with GenomeStudio version 2011.1 (Illumina) according to manufacturer's protocol.

Autoantibody detection

The presence and concentration of serum autoantibodies against Ro/SSA composite (52 kDa Ro and/or 60 kDa Ro), La/SSB, centromere B, chromatin, Scl-70, dsDNA, ribosomal P, Sm, SmRNP, nRNP composite (nRNP A and/or nRNP 68) and Jo-1 were assayed using bead-based multiplex assays on a BioPlex 2200 platform (Bio-Rad Technologies, Hercules, CA, USA) as previously described [18]. Autoantibodies were quantified using an antibody index value based on the fluorescence intensity of each of the autoantibody specificities, with a manufacturer-recommended positive cut-off of 1 on the antibody index (range 0–>8). Anti-dsDNA was quantified with a manufacturer-recommended positive cut-off of 10 IU/mL.

Soluble mediator detection

Serum levels of B lymphocyte stimulator (BLyS) and LIGHT were assessed by ELISAs per the manufacturers' protocol (Human BAFF/BLyS/TNFSF13B Quantikine ELISA, R&D Systems, Minneapolis, MN, USA; Human CD258/LIGHT Ready-Set-Go, Thermo Fisher Scientific/Invitrogen, Waltham, MA, USA). B cell–attracting chemokine 1 (BCA-1)/CXCL13 was quantified by singleplex xMAP assay per the manufacturer's protocol (ProcartaPlex Human BLC/CXCL13 Simplex assay, Thermo Fisher Scientific/Invitrogen).

Serum levels of other inflammatory mediators were assessed using a custom multiplex panel (ProcartaPlex, Thermo Fisher Scientific/Invitrogen) on the Bioplex 200 Luminex xMAP plate reader (Bio-Rad Technologies) as previously described [19]. The analytes that passed quality control included IL-1α, IL-7, IL-21, IL-2RA, IFN-γ-inducible protein 10 (IP-10)/CXCL10, regulated upon activation normal T cells expressed and secreted/CCL5, macrophage inflammatory protein 1α/CCL3, macrophage inflammatory protein 1β (MIP-1β)/CCL4, monocyte chemoattractant protein-1/CCL2, monocyte chemoattractant protein-3/CCL7, monokine induced by IFN-γ (MIG)/CXCL9, stromal derived factor 1α, eotaxin-1/CCL11, sE-selectin, ICAM-1, VCAM-1, VEGF-A, sCD40L, TNFRI, TNFRII, TNF-related apoptosis-inducing ligand, leukaemia inhibitory factor, plasminogen activator inhibitor 1, PDGF-BB, leptin, resistin and stem cell factor. A bridge control serum sample was included on each plate (Cellect human AB serum, catalogue no. 2931949, lot no. Q8823, MP Biomedicals, Solon, OH, USA) to control for plate-to-plate variation of soluble mediator assays. The mean interassay coefficient of variance of these assays (10.5%) was within that previously shown for bead-based assays [20].

The limit of blank, limit of detection and limit of quantification were determined and used for quality control as previously described [21]. Samples and standards below the limit of detection were considered undetectable. Analytes with >60% undetectable rate were excluded from subsequent analyses. For analytes passing quality control, concentrations were interpolated from five-parameter logistic non-linear regression standard curves or assigned a value of 0 if a sample was below the limit of detection.

Statistical analysis

Analyses were performed with R version 3.3.3 (R Foundation for Statistical Computing, Vienna, Austria). Proportions were compared by χ^2 or by Fisher's exact test when fewer than five observations were expected in any category. Continuous variables were compared between two groups by t test if normally distributed or by Mann–Whitney if not normally distributed by the Shapiro–Wilk test. *P*-values were adjusted for multiple comparisons by the false discovery rate method. Variables with no more than five unique values (VCAM-1) were analysed as categorical variables. Heat maps and radar plots present modified Z-scores.

For gene expression, background-subtracted expression data were log2 transformed and normalized with the rank-invariant method using the lumiR package [22]. System-based modular analysis was performed at the group level and individual-level module scores (M1–M6) were calculated using second-generation modular frameworks as previously described [23–25]. Briefly, transcription levels of predefined sets of co-regulated module genes for each patient were compared with the average transcription levels of those genes in healthy controls to determine the activity level of each module. These co-expression module scores were used for subsequent random forest clustering models.

To identify molecularly similar patient clusters, random forest was used to classify patients based on their modular expression scores (M1–M6) by simultaneously assessing an ensemble of independent decision trees, as previously described [21]. Random forest was performed in R (version 4.6-12; https://cran.r-project.org/), with mtry = 3, mtree = 4000 and the dissimilarity matrix defined as $\sqrt{1 - similarity}$, where the similarity matrix was the average similarities by repeating random forest clustering 100 times [26]. Batch effects were removed using the empirical Bayes methods when gene expression data sets were combined [27].

Results

Molecular signatures distinguishing pSS patient clusters

Analysis of previously defined transcriptional modules [23–25] revealed molecular heterogeneity among pSS patients (Fig. 1). Based on these modules, random forest identified three distinct clusters of pSS patients (Fig. 1A).

These clusters had significantly different IFN modular network signatures. Looking at the IFN modules (M1.2, M3.4, M5.12), Cluster 1 had the weakest IFN modular network signature and Cluster 2 had the strongest IFN modular network signature (Fig. 1B, C, and Supplementary Fig. 1, available at *Rheumatology* online). Other key modular network signatures were strongest in Cluster 2 and weakest in Cluster 3, including inflammation (M3.2, M4.2, M4.13,

Fig. 1 Clustering pSS patients with molecular signatures
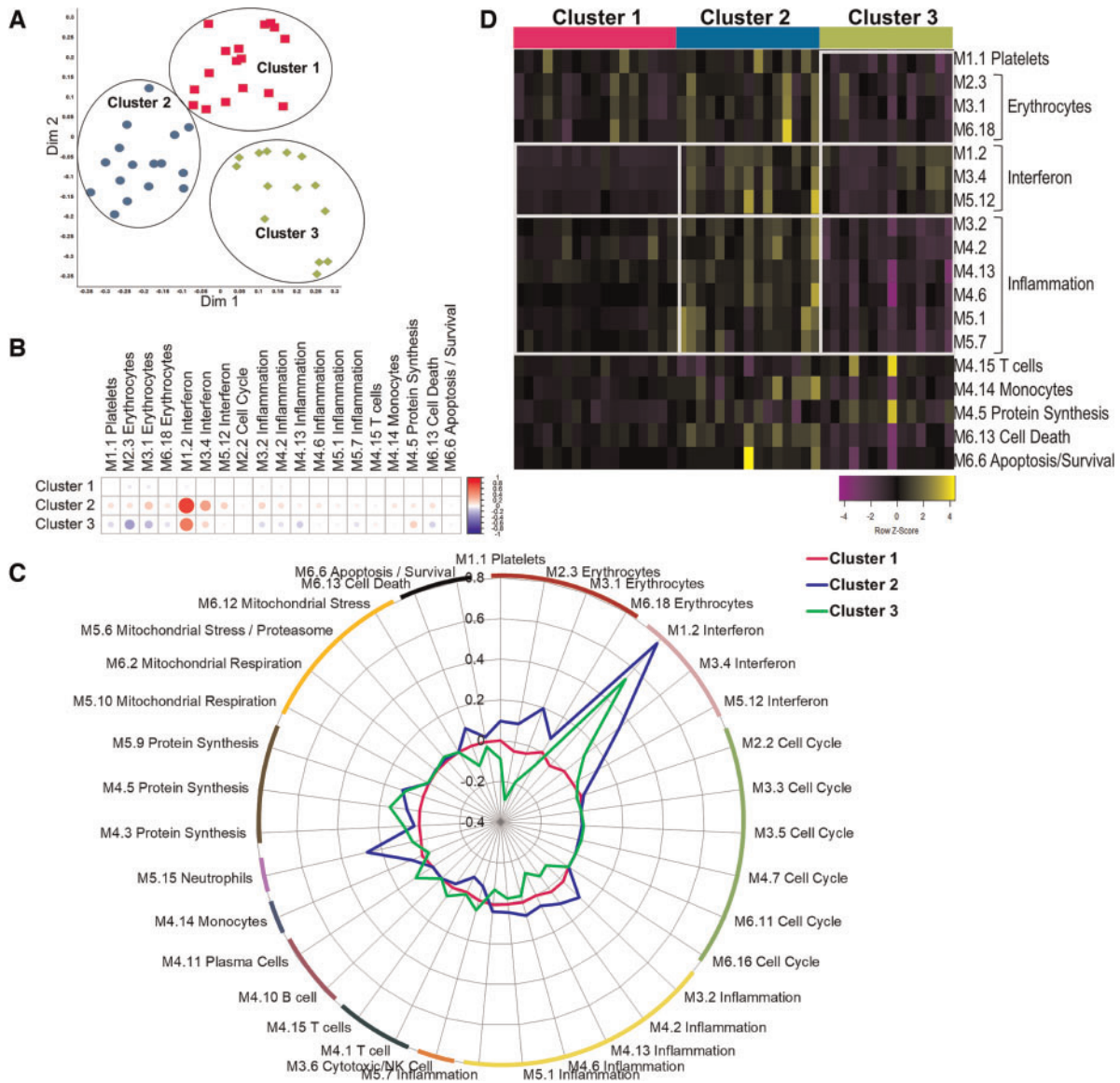
(A) Random forest using expression module scores identified molecularly similar pSS patient subsets. Each point in the multidimensional scaling plot represents a patient. The distance between points represents dissimilarity between subjects. (B) The size of each circle represents the absolute value of the median module score. Red indicates an increase (positive scores); blue indicates a decrease (negative scores). (C) Median modified Z-scores are shown for each module. (D) Columns represent individual patients, grouped by cluster; rows show relative activation (modified Z-scores) of select modules. Purple indicates less activation and yellow more activation.

M4.6, M5.1, M5.7), platelet/erythrocyte (M1.1, M3.1, M6.18) and cell death/apoptosis/survival (M6.13, M6.6) signatures.

Although these patterns were also observed at the individual level, the profiles varied between individuals within the same cluster (Fig. 1D). For example, many patients in Cluster 2 had both a strong IFN modular network signature and a strong inflammation modular network signature, but a few had only one of these signatures. Further, two subsets of patients were apparent in Cluster 3, one with an intermediate IFN modular network signature and another with a low IFN modular network signature. However, both subsets within Cluster 3 exhibited low activity of the inflammation modules. Together, these results highlight the molecular heterogeneity of pSS patients, particularly in IFN and inflammation modules.

Demographics were similar between clusters (Table 1). ESSDAI scores were fairly homogeneous across all patients, with ESSDAI scores >6 in six patients (12.8%) and ESSDAI scores >10 in four patients (8.5%). ESSDAI scores trended slightly higher in Cluster 2 compared with the other clusters (overall $P = 0.19$, $P = 0.24$ vs Cluster 1, $P = 0.073$ vs Cluster 3; Table 1). Clusters showed no significant differences at baseline in patient-reported dryness, fatigue, joint pain or global disease activity; physician-assessed global disease activity or IgG levels (Table 1). The slightly higher joint pain in Cluster 1 was not significant (Table 1). Clusters showed no significant differences in the use of HCQ (overall $P = 0.546$) or

corticosteroids (overall $P = 0.766$) (Supplementary Table 1, available at Rheumatology online).

Autoantibodies in clusters of pSS patients defined by molecular signatures

To compare autoantibodies among clusters, we used a multiplex assay that simultaneously detects 11 ANA specificities, including anti-Ro/SSA and anti-La/SSB. Across all clusters, Ro/SSA and La/SSB were the predominant ANA specificities, and anti-Ro/SSA and anti-La/SSB were present in all clusters (Table 1, Supplementary Fig. 2, available at Rheumatology online). Rates of La/SSB positivity trended higher in Cluster 2 than in Cluster 1 (overall $P = 0.085$, Cluster 2 vs 1 $P = 0.056$) and Cluster 2 had significantly higher levels of La/SSB compared with Cluster 1 {median antibody index in Cluster 1: 0.2 [interquartile range (IQR) 0.2–0.5], Cluster 2: 1.5 [IQR 0.375–8.0], Cluster 3: 0.2 [IQR 0.2– 5.88]; overall $P = 0.027$; Cluster 2 vs Cluster 1 $P = 0.008$; Supplementary Fig. 2A, C, available at Rheumatology online}. However, extremely high levels of Ro/SSA and La/SSB occurred in all clusters (Supplementary Fig. 2B, C, available at Rheumatology online).

Differences in serum levels of soluble mediators between pSS patient clusters

Next we tested whether pSS patient clusters defined by expression modules also showed differences in systemic measures of inflammation, such as cytokines, chemokines and other soluble mediators of inflammation. Levels of

TABLE 1 Demographics and baseline clinical characteristics of the study population

Characteristics	All pSS patients (n = 47)	Cluster 1 (n = 17)	Cluster 2 (n = 16)	Cluster 3 (n = 14)	P-value[a]
Age, years, median (IQR)	53 (47–59)	52.8 (42.4–63.1)	51.9 (41.1–62.6)	51.2 (39.5–62.9)	0.92
Female, n (%)	40 (85.1)	15 (88.2)	11 (91.7)	14 (100)	0.77
Race, n (%)					0.70
European American	34 (72.3)	11 (64.7)	12 (75.0)	11 (78.6)	
African American	4 (8.5)	2 (11.8)	1 (6.2)	1 (7.1)	
Hispanic	5 (10.6)	3 (17.6)	1 (6.2)	1 (7.1)	
American Indian	2 (4.2)	1 (5.9)	1 (6.2)	0 (0.0)	
Asian	2 (4.2)	0 (0.0)	1 (6.2)	1 (7.1)	
Disease Activity Scores, median (IQR)[b]					
ESSDAI	2 (1–4.5)	2 (0–4)	3.5 (2–5.25)	2 (0.25–2.75)	0.19
Physician Global Assessment	51 (37.5–51)	52.0 (42.0–65.0)	50.0 (40.8–56.0)	52.0 (18.8–73.8)	0.73
Dryness VAS	72 (63–80.4)	70.9 (53.9–87.9)	74.3 (54.6–88.0)	73.8 (58.2–89.4)	0.88
Fatigue VAS	70.0 (59.5–80.9)	76.0 (59.0–86.0)	72.4 (63.2–79.2)	64.5 (49.2–78.8)	0.47
Joint pain VAS	55 (33–67.3)	56.2 (35.8–76.7)	48.7 (19.9–77.4)	48.1 (26.7–69.6)	0.56
Patient Global Assessment	71 (62–79)	71.7 (56.5–83.9)	71.7 (55.3–88.1)	69.0 (47.8–90.2)	0.88
IgG, mg/dL, median (IQR)	1200 (988–1720)	1140 (884–1445)	1280 (1150–1908)	1265 (834–1823)	0.51
Anti-Ro/SSA, n (%) positive	28 (59.8)	8 (47.1)	11 (68.8)	9 (64.3)	0.45
Anti-La/SSB, n (%) positive	20 (42.6)	4 (23.5)	10 (62.5)	6 (42.8)	0.09

P-values compare the three clusters. [a]Normally distributed variables (age, dryness VAS, Patient Global Assessment) were compared by analysis of variance. Variables that were not normally distributed (ESSDAI, Physician Global Assessment, Fatigue VAS) were compared by Kruskal–Wallis test. Proportions were compared by χ^2 (anti-La/SSB) or by Fisher's exact test if they did not meet the assumptions of χ^2 (gender, race, anti-Ro/SSA). [b]Higher scores indicate more disease activity. The VAS and Global Assessment scales have a minimum of 0 and maximum of 100.

several soluble mediators varied between clusters (Fig. 2, Supplementary Table 2, Supplementary Fig. 3, available at *Rheumatology* online). Cluster 2 showed the highest levels of the TNF superfamily members LIGHT and BLyS. Levels of the IFN-induced mediators IP-10/CXCL10 and MIG/CXCL9 appeared highest in Cluster 2, which had a strong IFN gene expression signature, and intermediate in Cluster 3, which had a moderate IFN signature. Clusters 2 and 3 also had high levels of BCA-1/CXCL13. In Cluster 3, levels of IL-1α, IL-21, IL-2RA, MIP-1β/CCL4 and sE-selectin trended higher than in the other clusters (Fig. 2, Supplementary Table 2, available at *Rheumatology* online). In addition, several mediators differed between pSS patients and age, race and gender cohort-matched healthy controls (Supplementary Table 3, Supplementary Fig. 4, available at *Rheumatology* online). These observations reinforce both the immunologic heterogeneity among pSS patients and the concept that pSS can involve systemic dysregulation of multiple immune pathways.

Discussion

Immunologic heterogeneity in pSS poses a challenge when assembling patient cohorts for research or clinical trials. This study identified molecular profiles that differ among pSS patients. These profiles can be used to separate pSS patients into distinct subsets characterized by patterns of immune dysregulation.

Differences in inflammation, IFN and leucocyte modules identified three distinct clusters of pSS patients, despite relative homogeneity in ESSDAI scores across this cohort. It is unclear how these or other modular network signatures might vary among patients with higher ESSDAI scores, particularly given the inconsistent relationship between clinical manifestations and IFN signatures [8, 12, 13]. These results reinforce that clinical disease activity does not perfectly correlate with underlying immune dysfunction, perhaps due in part to limitations of current measures of clinical disease activity. Larger studies and broader discussions within the field may enable the development of more nuanced clinical assessments that align more closely with the underlying mechanisms of disease. In addition, multimodal molecular-based tests are needed to directly survey immune pathways that may influence disease progression and response to treatment.

Two different patterns of immune activation emerged in this study of pSS patients. In Cluster 2, the combination of a strong IFN modular network signature and elevation of IFN-related soluble mediators (IP-10/CXCL10, MIG/CXCL9) demonstrated IFN activation in these patients. However, the specific IFNs involved cannot be determined from the IFN modular network signature. Furthermore, Cluster 2 exhibited increased levels of the TNF superfamily members sCD40L/CD154, LIGHT and BLyS, as well as slight elevations in autoantibodies. LIGHT and CD154 synergize to increase IFN-γ production by T cells [28], and both type I [29] and type II [30] IFN are capable of upregulating BLyS. In activated immune cells, CD154 [31], LIGHT [32] and BLyS [33] are proteolytically cleaved to soluble forms that promote inflammation [34–37] and antibody production [36–38], which in turn may contribute to pSS pathogenesis.

Cluster 3 did not show an inflammation modular network signature but exhibited moderate elevations in the IFN modules and levels of IP-10/CXCL10 and MIG/CXCL9. In addition, slight elevations in the T cell and cytotoxic/NK cell expression modules, along with a trend toward elevated levels of IL-21 [39], suggest that dysregulated T cell pathways may contribute to disease in this cluster. Further, IL-21 induces the differentiation of naïve and memory B cells into plasma cells [40], consistent with the active plasma cell module. Together, this suggests IL-21 may help drive the autoantibodies in this subset of pSS patients.

The final subset of pSS patients in this study (Cluster 1) satisfied the inclusion criteria for the parent clinical trial yet showed no increases in the IFN modules and minimal activity of inflammation-related gene modules. Although they present clinical features such as arthritis and/or elevated ESSDAI scores, such patients may not be ideal candidates for clinical trials of IFN, inflammation or immune-modifying therapies. Pathology in these patients may arise from other pathways, at least at the studied time point.

Indeed, it is not clear whether these subsets represent truly distinct disease subsets or different stages of disease that vary over time. Answering this question would require longitudinal assessments in a prospectively collected cohort. In addition, our sample size was limited, and larger studies are needed to confirm these whole blood findings, refine the identification of pSS patient subsets and fully delineate the pathogenic pathways in various pSS patient subsets. For example, this study analysed modular network signatures that were previously defined in patients with lupus. In particular, the relevance of the inflammation signature in Cluster 2 is not clear. Cluster 2 did have the highest levels of TNF superfamily members (LIGHT and BLyS), consistent with inflammation, but it is possible that inflammation modules may not reflect all aspects of inflammatory cytokines/chemokines. Additional studies are needed to confirm these signatures at the cellular and molecular level in pSS patient subsets. Further, this study was not designed to dissect the roles of type I and type II IFNs in the different patient subsets. Moreover, pSS may involve pathogenic mechanisms evident in salivary glands but not peripheral blood, such as epithelial dysfunction, altered muscarinic receptors and fibrosis or other damage. However, this study was not designed to address these possibilities. Future studies may require analyses of cellular immune composition and responses within specific cell subsets, genetic differences, epigenetic signatures and functional assays of IFN activity.

These results demonstrate the feasibility of defining pSS patient subsets using a broad panel of molecular markers. Patients in different subsets may have different responses to disease-modifying treatments, and profiles that correlate with treatment effects may be useful for

Fig. 2 Cytokines, chemokines and other soluble mediators of inflammation in molecularly defined clusters of patients with pSS
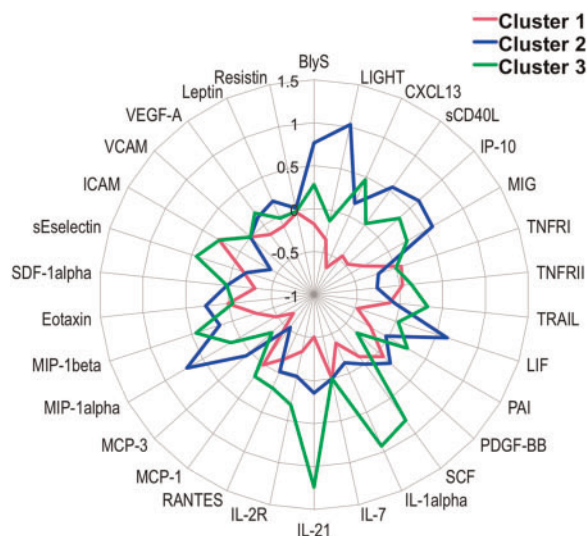
Cytokines, chemokines and other soluble mediators of inflammation were measured by a multiplex bead-based assay. Modified Z-scores are shown as a radar plot.

clinical trial design or treatment selection. In addition, molecular profiles that vary longitudinally may be useful for monitoring disease activity or progression [24]. Further studies are needed to validate these findings and delineate the pathogenic mechanisms in molecularly defined subsets of pSS patients.

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Supplementary data

Supplementary data are available at *Rheumatology* online.

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