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RHEUMATOLOGY

doi:10.1093/rheumatology/keu163
Advance Access publication 24 April 2014

Original article

High expression levels of the B cell chemoattractant CXCL13 in rheumatoid synovium are a marker of severe disease

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Abstract

Objective. The B cell chemoattractant chemokine ligand 13 (CXCL13) is emerging as a new biochemical marker in RA. This study was undertaken to dissect the relationship between CXCL13 expression levels in the synovium and clinico-pathological variables relevant to RA pathogenesis and outcome.

Methods. Synovial tissues from 71 RA patients were evaluated by immunohistochemistry. Thirty paired samples were used for comparative gene expression analysis by quantitative real-time PCR. CXCL13 levels were analysed in relation to cellular, molecular and clinical features of inflammation, lymphocyte activation and joint damage.

Results. In patients with early disease (<12 months duration), CXCL13 expression correlated significantly with synovial markers of local disease activity and systemic inflammation. Such correlation was less evident in established RA. Notably, the association with lymphocyte infiltration and with expression of B/T cell–related activation and proliferation genes, such as activation-induced cytidine deaminase, IFN-γ and IL-2, remained highly significant independent of disease duration and local disease activity. Patients featuring the highest levels of CXCL13 were more frequently ACPA positive and IgG ACPA titres were increased in the high CXCL13 expression group. Furthermore, the frequency of erosive disease on radiographs was significantly higher in the upper tertile of CXCL13 expression ($P = 0.01$ with adjustment for disease duration and ACPA). Accordingly, synovial CXCL13 and the local receptor activator of nuclear factor κB ligand (RANKL)/osteoprotegerin (OPG) ratio significantly co-varied ($\rho = 0.52$, $P < 0.01$), independent of the level of local inflammation.

Conclusion. Synovial CXCL13 appears to be a marker of a more severe pattern of RA disease, characterized by increased lymphocyte activation and bone remodelling beyond the level of conventional markers of inflammation.

Key words: rheumatoid arthritis, synovium, CXCL13, B lymphocytes, biomarkers.

BASIC SCIENCE

Introduction

Management of RA has steadily improved in recent decades, mostly due to enhanced understanding of the pathophysiology of the disease, which has been translated into effective therapies. However, despite the remarkable successes achieved, the clinical complexity and the varied response to therapy leave multiple challenges in RA management. The wide heterogeneity in clinical manifestations, courses and outcomes of the disease continues to be largely unexplained, hampering the possibility of tailored approaches. At present, with the

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Submitted 28 October 2013; revised version accepted 28 February 2014.

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TABLE 1 Main demographic and clinical characteristics of the study population

	Patients ($n = 71$)
Age, mean (S.D.), years	55.4 (13.9)
Female, *n* (%)	49 (69)
Disease duration, median (IQR), months	48 (11–120)
Number of tender joints, median (IQR)	4 (1–13)
Number of swollen joints, median (S.D.)	9 (5–18)
DAS, mean (S.D.)	3.25 (1.14)
ESR, median (IQR), mm/h	27 (17–56.8)
CRP, median (IQR), mg/dl	1.5 (0.4–4.1)
HAQ, mean (S.D.)	1.1 (0.76)
RF positive, *n* (%)	43 (60.6)
ACPA positive, *n* (%)	36 (50.7)
Erosive disease, *n* (%)	44 (62)
Patients on corticosteroids, *n* (%)	44 (62)
Patients on DMARDs, *n* (%)	51 (71.8)[a]
MTX	29 (40.8)
HCQ	19 (26.8)
SSZ	9 (12.7)
Patients on more than one DMARD, *n* (%)	6 (8.5)
Number of previous DMARDs, median (IQR)	0 (0–1)

IQR: interquartile range. [a]Twenty patients (28.2%) with disease duration <12 months were DMARD free.

exception of ACPA [1], most of the parameters used to accommodate the variance of RA are based on non-specific demographic, clinical and laboratory features [2, 3]. Markers embedded in the underlying disease mechanisms represent potentially superior tools for the pathophysiological and clinical stratification of RA. Analysis of the primary involved tissue, such as the synovial membrane, may help in both generating novel biomarkers through non-targeted, data-driven approaches and evaluating the impact of hypothesis-driven parameters [3–5].

Using a computer model representing the biology of the rheumatic joint, the B cell attracting chemokine CXC chemokine ligand 13 (CXCL13) [or B lymphocyte chemoattractant (BLC) or B cell attracting chemokine 1 (BCA-1)] has been recently identified as a new potential serologic marker for severity in RA [6]. Serum levels of CXCL13 have been further confirmed to be associated with synovial inflammation on clinical examination [7, 8] and with synovitis persistence on imaging [7]. Although investigation of the sources of circulating CXCL13 is ongoing [8], the involvement of the chemokine in the pathology of synovitis in RA has long been acknowledged. Synovial ectopic expression of CXCL13 can be induced in a variety of cell types, including follicular dendritic cells, a proportion of $CD14^+/CD68^+$ monocytes/macrophages, and a subset of memory T cells [9–11]. CXCL13 produced locally is associated with the formation of extra-nodal lymphoid aggregates, which represent the primary environment for ectopic B lymphocyte interactions with other cell subsets [12] as well as for local affinity maturation of the immunoglobulin genes [13, 14]. Experimental evidence in physiologic conditions and in different pathological settings indicate that, in addition to mediating recruitment and clustering of B cells through its cognate receptor CXCR5 [15], CXCL13 enhances B cell receptor-mediated B cell activation [16], guides migration of $CXCR5^+CD4^+$ follicular helper T cells [17] and possibly impacts on other CXCR5-bearing cells, including dendritic cells [18] and bone cells [19, 20]. Supporting a role for CXCL13 as an important mediator of autoimmune arthritis, neutralization of CXCL13 or CXCR5 gene deletion significantly reduced disease severity in animal models of arthritis [21–23].

Expression levels of CXCL13 in the synovium vary widely among patients with RA [9–11], but the regulatory determinants and the effects of this variation on synovial pathobiology and clinical features of RA are currently unclear. In the clinical setting for CXCL13 to function as a candidate biomarker, it is critical to have a better understanding of whether tissue gradients of CXCL13 could reflect differences in the underlying pathology and eventually impact the clinical features of the disease or simply represent another non-specific marker of inflammation. Here we investigated this hypothesis, analysing the biomolecular and clinical characteristics of inflammation, immune cell activation and bone damage in RA in relation to the expression levels of CXCL13 in the joint.

Patients and methods

Patients

Synovial tissue was obtained from 71 consecutive RA patients fulfilling the 1987 ACR criteria. All patients gave informed consent for synovial sampling and the study was approved by the Ethical Committee of the IRCCS Policlinico San Matteo Foundation, Pavia, Italy (20070001302). Demographic and clinical data at the time of tissue collection are reported in Table 1. None of the patients were being treated or had been previously treated with biologic agents. The presence of IgM RF and IgG ACPA was determined by nephelometry using the N Latex RF system (Dade Behring, Marburg, Germany) and a second-generation ELISA (Axis-Shield, Dundee, UK) respectively, according to the manufacturers' recommendations. Erosive disease in the overall cohort was defined when at least one typical bone erosion [24] was seen on the hands and feet on plain radiographs evaluated by two independent experienced rheumatologists (C.M. and R.C.) blinded to histological and molecular findings. In the subgroup of patients with a disease duration <12 months who were DMARD free ($n = 20$), bone erosions were additionally evaluated according to the Sharp–van der Heijde score (SHS) [25].

Synovial tissue collection

Synovial tissues were obtained from synovectomy, arthroscopic or US-guided biopsy from an actively inflamed knee ($n = 58$), wrist or MCP joint ($n = 13$). According to current recommendations, at least six samples were taken

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from different sites during arthroscopic biopsies and eight during US-guided biopsies [26, 27]. Specimens were either immersed in a 5:1 (v:v) mixture of RNAlater for RNA extraction (see below) or fixed in 10% formalin for 24 h, embedded in paraffin, sectioned at 4 μm and processed for histology and immunohistochemistry.

Immunohistochemistry

The expression of CXCL13 and cellular markers was analysed by immunohistochemistry on consecutive sections as previously described [28]. Briefly, formalin-fixed, paraffin-embedded tissue sections were deparaffinized and rehydrated through graded ethanols. After heat retrieval for 35 min at 96°C in Target Retrieval Solution (S1700; DAKO, Glostrup, Denmark), sections were blocked with Protein Block Serum Free (X0909; DAKO) for 10 min. The following primary antibodies were used: anti-CXCL13 (polyclonal; R&D Systems, Minneapolis, MN, USA), anti-CD68 (PG-M1; DAKO) for macrophages, anti-CD20 (L26; DAKO) for B cells, anti-CD3 (polyclonal; DAKO) for T cells and anti-CD138 (MI15; DAKO) for plasma cells. Sections were then incubated with the appropriate biotinylated secondary antibody for 30 min followed by streptavidin biotin complex–alkaline phosphatase (AK-5000; Vector Laboratories, Burlingame, CA, USA) or, for CD68 macrophages, by streptavidin biotin complex–horseradish peroxidase (PK-6100; Vector Laboratories) for an additional 30 min. Stainings were developed using the New Fuchsin Substrate Kit (K0625; DAKO) or, for CD68 macrophages, liquid 3,3′-diaminobenzidine in chromogen solution (K3467; DAKO). Concentration- and isotype-matched control antibodies were included as a negative control and tonsil sections were included as a positive control.

Histological and immunohistochemical evaluation

Samples were blindly analysed by two independent observers (S.B. and B.V.) in a randomized order on two different occasions. All the evaluations were standardized by analysis of a separate set of slides prior to analysis of the study samples. The analysis included all areas of each biopsy section. Samples without intact lining were ignored.

Because of the high heterogeneity of RA synovium and the low frequency of $CXCL13^+$ cells, quantification of positive cells was performed on hot spots, defined as areas containing the highest density of positive cells [29]. Two hot spots per synovium were selected. In each spot, positive cells were counted in 10 consecutive fields at 400× magnification. One field corresponded to 0.55 mm^2, and thus one spot corresponded to 11 mm^2. The number of positive cells per two hot spots was averaged and the results were expressed as the number of positive cells per square millimetre. $CXCL13^+$ cell density was then categorized as low or high based on tertile distribution.

Each tissue was assigned a score for hyperplasia of the synovial lining layer (0: 1–2; 1: 3–4; 2: 5–6; 3: >7 cell layers) [30]. Infiltration with $CD68^+$ sublining macrophages, $CD3^+$ T lymphocytes and $CD138^+$ plasma cells was evaluated semi-quantitatively (0–3) according to validated methods [31]. $CD20^+$ B cell infiltration was assessed as an aggregational gradient. B cell aggregates were first graded according to their radial cell count, adapting published methods [32]. The B cell aggregational score was then defined semi-quantitatively, combining the size and sublining density distribution of $CD20^+$ B cell aggregates: 0 = no B cell aggregates/low-density (<3 aggregates in the whole biopsy area) grade (G)1 B cell aggregates; 1 = high-density G1/low-density G2; 2 = high-density G2/low-density G3; 3 = high-density G3 B cell aggregates. Validation studies performed in our laboratories confirmed a highly significant correlation between this scoring system and CD19 mRNA expression assessed in the same samples by real-time PCR [$\rho = 0.71$, $P = 0.0004$ by Spearman's rank correlation (not shown)]. All semi-quantitative scores showed excellent intra- and interobserver agreement ($\geqslant 0.85$) and never differed by >1 point. When discordant scores were obtained, the mean of the two scores was used.

Quantitative real-time PCR

From 30 of the 71 patients, paired paraffin and RNA samples were available and used for comparative immunohistochemistry analysis and real-time quantitative PCR (qPCR). The main demographic and clinical features of the subgroup of patients used for qPCR analyses were comparable to the overall population. The median disease duration was 48 months [interquartile range (IQR) 10–108], 46.7% of the patients were ACPA positive and 63.3% had erosive disease on radiographs.

Each specimen was divided into two parts: one was formalin fixed and paraffin embedded and the second was stored in RNAlater (Ambion, Austin, TX, USA) at −80°C for RNA extraction and qPCR analysis, as previously described [13]. Briefly, total RNA was extracted using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands), with on-column DNase I digestion to avoid genomic DNA contamination. cDNA was generated from 1 μg of RNA using the Thermoscript RT-PCR System for First-Strand cDNA Synthesis (Invitrogen, Carlsbad, CA, USA). A detailed list of the genes investigated and the specific primers and probes is available in supplementary Table S1, available at *Rheumatology* Online. The reverse transcription (RT)-PCR was run in triplicate with an equal loading of 20 ng of cDNA per well. The results were analysed after 40 cycles of amplification using an Realplex PCR machine (Eppendorf, Hauppauge, NY, USA). Relative quantification was measured using the comparative CT (threshold cycle) method after normalization for β-actin expression levels. cDNA from tonsil was used as a positive control.

Statistical analysis

Demographic and clinical features were described as mean (S.D.), median (IQR) or relative frequency, as appropriate. One-way analysis of variance (ANOVA) tests, Kruskal–Wallis tests and chi-squared statistics were used to compare histopathological and

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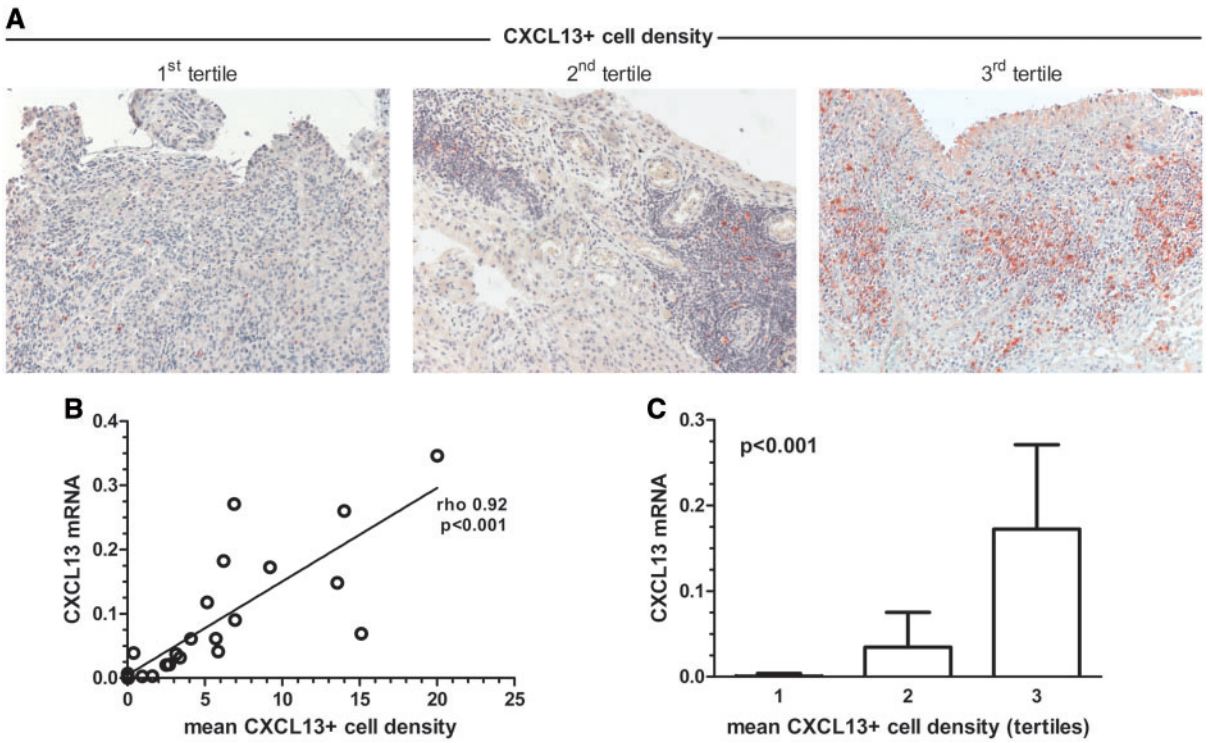
FIG. 1 Synovial CXCL13 expression in patients with RA



Quantification of synovial CXCL13$^+$ cells in the overall cohort ($n = 71$) was performed according to the hot spot method of analysis. Manual cell counting on paraffin sections was validated against CXCL13 mRNA quantitative expression on paired samples ($n = 30$). CXCL13$^+$ cell density was further categorised based on the tertile distribution and reassessed for CXCL13 mRNA. (**A**) Representative examples of different degrees of synovial CXCL13$^+$ cell density (tertiles 1–3) in specimens from three independent patients with RA are shown. Original magnification 100×. (**B**) Scatter diagram showing the CXCL13$^+$ cell count plotted against CXCL13 mRNA expression in single samples (empty circles) and the corresponding regression line. (**C**) Bar graph showing the mean (S.D.) CXCL13 mRNA expression levels stratified according to tertiles of synovial CXCL13$^+$ cell density.

immunohistochemistry scores, mRNA levels and clinical, laboratory and radiographic data among different CXCL13 tertiles, when appropriate. Correlations among mRNA levels were computed by Spearman's rho correlation coefficients. The association of high CXCL13 with erosive disease on radiographs was evaluated by means of univariable and multivariable logistic models fitted to account for potential confounders (disease duration, ACPA positivity). MedCalc for Windows, version 9.4.2.0 (MedCalc Software, Mariakerke, Belgium), was used for analysis. *P*-values <0.05 were considered significant.

Results

Heterogeneity of synovial CXCL13 expression and validation of CXCL13$^+$ cell quantification

The distribution of CXCL13$^+$ cells in the synovium was evaluated in all 71 patients included in the study. The mean CXCL13$^+$ cell density was extremely variable across different samples, ranging from 0 to 44.6 cells/mm^2 [median 7.2 cells/mm^2 (IQR 1.8–12.6)] (Fig. 1A). Confirming the reliability of our manual counting, cell density computed by the hot spot analysis was strongly correlated with CXCL13 mRNA ($\rho = 0.92$, $P < 0.001$) (Fig. 1B). Also, CXCL13 mRNA levels progressively increased when cell density was categorized as low or high based on the tertile distribution (Fig. 1C). Although CXCL13$^+$ cells were slightly increased in samples obtained from the small joints of the hands and wrists compared with the knees (12.1 *vs* 8.7 cells/mm^2), the difference was not statistically significant. Similarly, no differences in CXCL13 expression were observed in relation to disease duration.

CXCL13 expression and synovial tissue biomarkers of disease activity

In the overall cohort, increasing CXCL13$^+$ cell density scores (categorized based on the tertile distribution) showed a progressive increase in the degree of CD68$^+$ sublining macrophage infiltration (Fig. 2A) and the lining layer thickness (Fig. 2B), two validated histological markers of local disease activity and systemic inflammation

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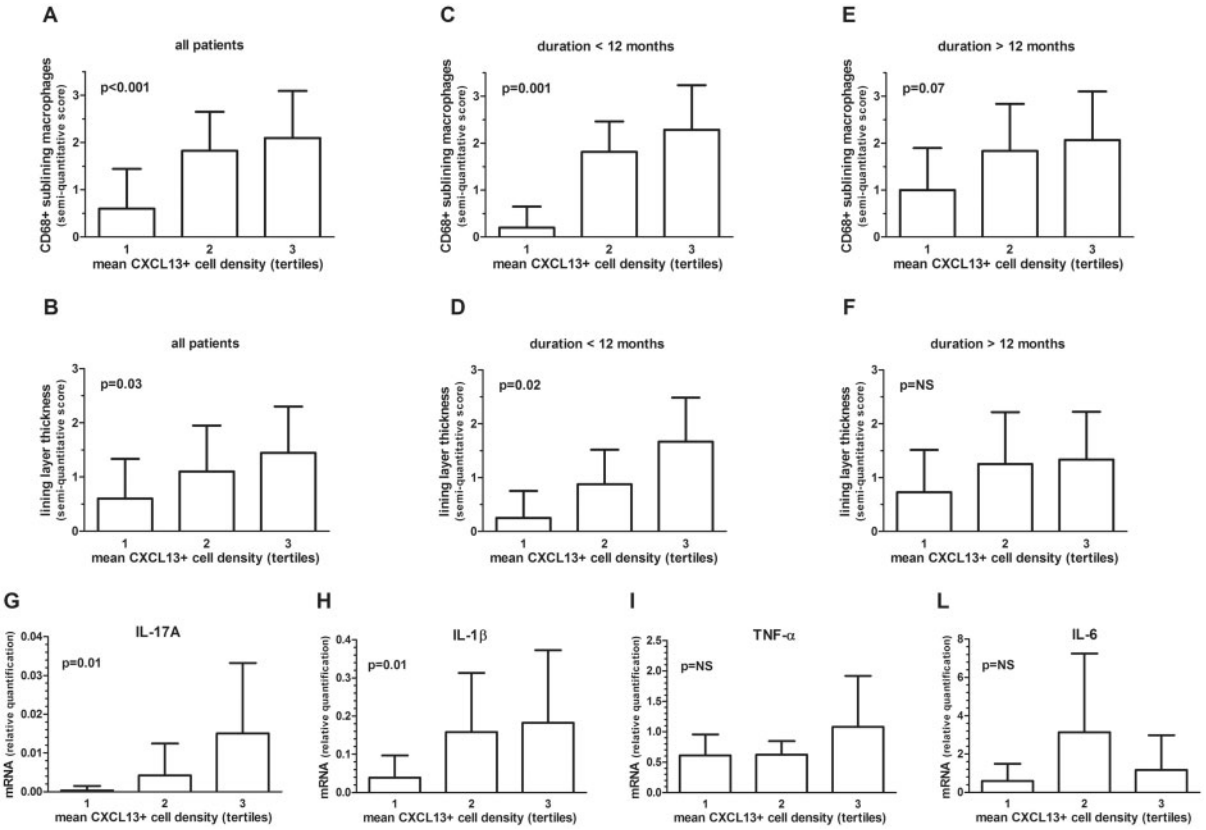
FIG. 2 Association of CXCL13 expression levels with synovial inflammatory markers



Mean (S.D.) expression levels of different cellular and molecular markers of synovial inflammation stratified according to tertiles of CXCL13$^+$ cell density. (**A**–**F**) CD68$^+$ sublining macrophage infiltration (**A**, **C** and **E**) and lining layer thickness (**B**, **D** and **F**) both show progressively incremental levels in the overall cohort (**A** and **B**) due to significant increases in patients with disease duration <12 months ($n = 20$) (**C** and **D**). The association is non-significant in patients with longer duration (>12 months) (**E** and **F**). (**G**–**L**) Overexpression of proinflammatory cytokines in relation to increasing CXCL13 scores is significant for (**G**) IL-17A and (**H**) IL-1β mRNA, but not for (**I**) TNF-α and (**L**) IL-6.

[31, 33, 34]. When patients were partitioned based on disease duration, the associations were highly significant in early disease (<12 months from onset of symptoms) (Fig. 2C and D), while statistical significance was lost for longer duration (Fig. 2E and F). Accordingly, the correlation of CXCL13 mRNA levels with a preselected panel of proinflammatory cytokines shown to be up-regulated in the RA joint was variable, being significant for IL-17A ($\rho = 0.5$, $P = 0.01$) and IL-1β (ρ 0.55, $P = 0.003$) but not for TNF-α and IL-6, as shown in Fig. 2G–L, where the variations of proinflammatory genes in relation to synovial CXCL13$^+$ cell density scores are illustrated. No association was found with IL-10 and TGF-β expression, arguing against a specific relationship between synovial CXCL13 induction and activation of a local anti-inflammatory response. At the clinical level, synovial CXCL13 expression was unrelated to composite indexes of disease activity, such as the DAS, the 28-joint DAS (DAS28) or the SDAI, in either early or established RA.

CXCL13 expression and markers of lymphocyte infiltration and activation

In contrast with the variable association with synovial inflammatory biomarkers, a tight relationship was observed between CXCL13 expression and markers related to lymphocyte effector functions. Increasing CXCL13$^+$ cell scores were characterized by a progressive increase in the degree of B cell and CD138$^+$ plasma cell infiltration (Fig. 3A and B). mRNA levels of activation-induced cytidine deaminase, the enzyme required for initiating the processes of somatic hypermutation and class switch recombination in activated B cells [13], also showed incremental expression closely matching the CXCL13$^+$ cell density score (Fig. 3C) as well as CXCL13 mRNA ($\rho = 0.87$, $P < 0.001$). Increasing CXCL13 expression was also significantly associated with T cell enrichment (Fig. 3D), as well as with tissue levels of the T cell–specific activation and proliferation cytokine IL-2 and the T cell effector factor IFN-γ (Fig. 3E and F). The correlation between

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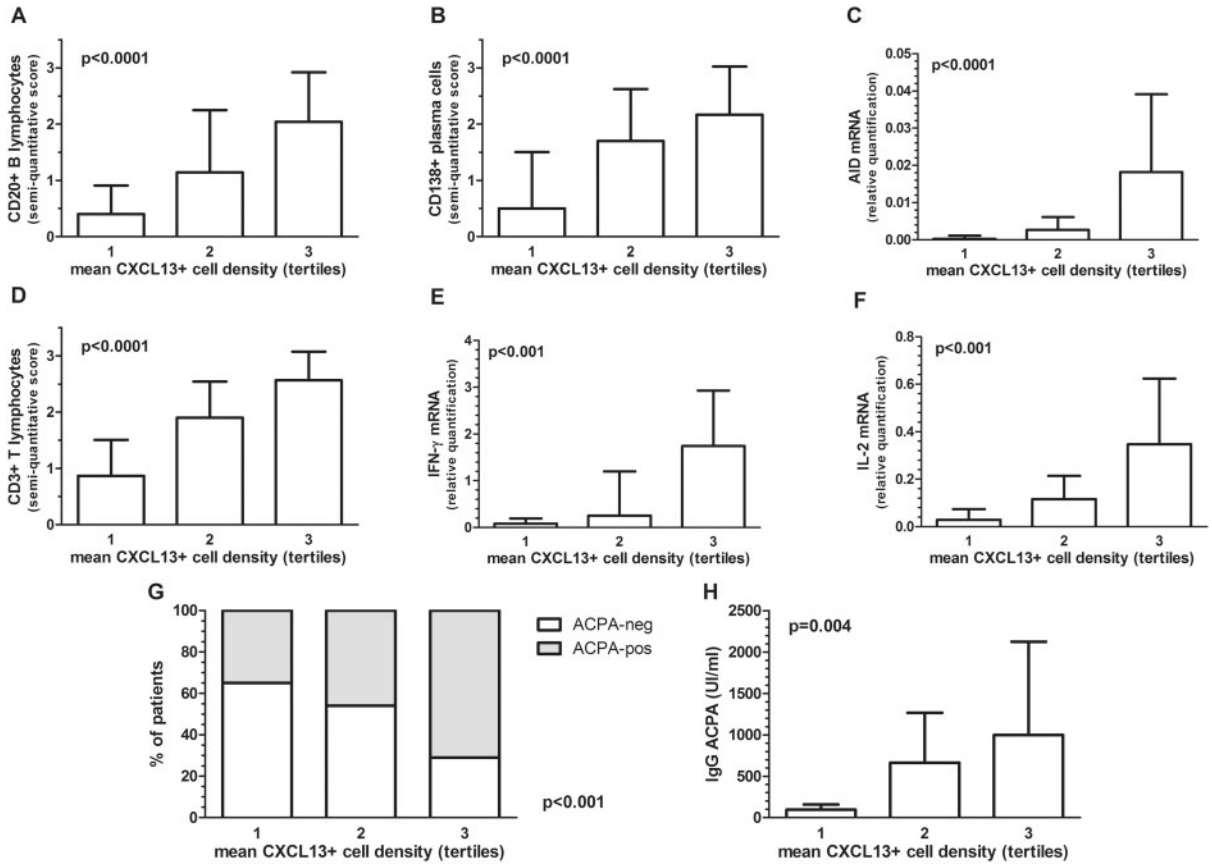
FIG. 3 Association of CXCL13 expression levels with immune cell infiltration and autoantibodies



(**A**–**F**) Mean (S.D.) levels of synovial (**A**) CD20$^+$ B lymphocytes, (**B**) CD138$^+$ plasma cells, (**C**) activation-induced cytidine deaminase (AID) mRNA, (**D**) CD3$^+$ T lymphocytes, (**E**) IFN-γ mRNA and (**F**) IL-2 mRNA stratified according to tertiles of CXCL13$^+$ cell density. All markers show highly significant incremental levels along with increasing CXCL13 expression ($P < 0.001$ for all). (**G**) Histogram showing the frequency of ACPA-positive and -negative patients in each tertile of synovial CXCL13 expression. (**H**) Bar chart showing mean (S.D.) ACPA titres in each tertile of synovial CXCL13 expression in ACPA-positive patients.

lymphocyte infiltration or markers of lymphocyte activation and CXCL13 expression was independent of disease duration and the degree of synovial inflammation as expressed by the CD68$^+$ macrophage score.

To evaluate the possible relationship with clinical parameters of systemic immune deregulation, synovial CXCL13 was analysed in relation to the autoantibody status. As shown in Fig. 3G, ACPA positivity could occur in patients with low or undetectable synovial CXCL13 expression, and high CXCL13 (third tertile) was equally found in a proportion of ACPA-negative patients. However, patients in the third tertile of synovial CXCL13 were more frequently ACPA positive (71% *vs* 46% *vs* 35%, chi-squared trend $P < 0.001$) (Fig. 3G). In ACPA-positive patients, high CXCL13 was also significantly associated with higher autoantibody titres (Fig. 3H).

CXCL13 expression and bone damage

Increasing CXCL13 expression was strikingly associated with a progressive increase in the synovial receptor activator of nuclear factor κB ligand (RANKL)/osteoprotegerin (OPG) ratio. This appeared for both categorical analysis of CXCL13 based on the tertile distribution (Fig. 4A) and for mRNA levels analysed as a continuous variable ($\rho = 0.52$, $P < 0.01$). Notably, this association was partly independent of the degree of synovial tissue inflammation. When only tissue specimens exhibiting the highest levels of local inflammation (CD68$^+$ sublining macrophage score $\geqslant 2$) were analysed, the highest tertile of CXCL13 was still associated with a 3.3-fold increase in the RANKL/OPG ratio compared with the lower tertiles (Fig 4B). Congruently, comparison of the highest and lowest tertiles of CXCL13 in samples with low levels of inflammation (CD68$^+$ score $\leqslant 1$) confirmed a significantly higher RANKL/OPG ratio in the former (Fig. 4C).

In order to determine whether the observed pathological findings translated into clinical features, synovial CXCL13 expression was analysed in relation to radiographic erosions. Although erosive patients were distributed across all the CXCL13 tertiles, the prevalence of

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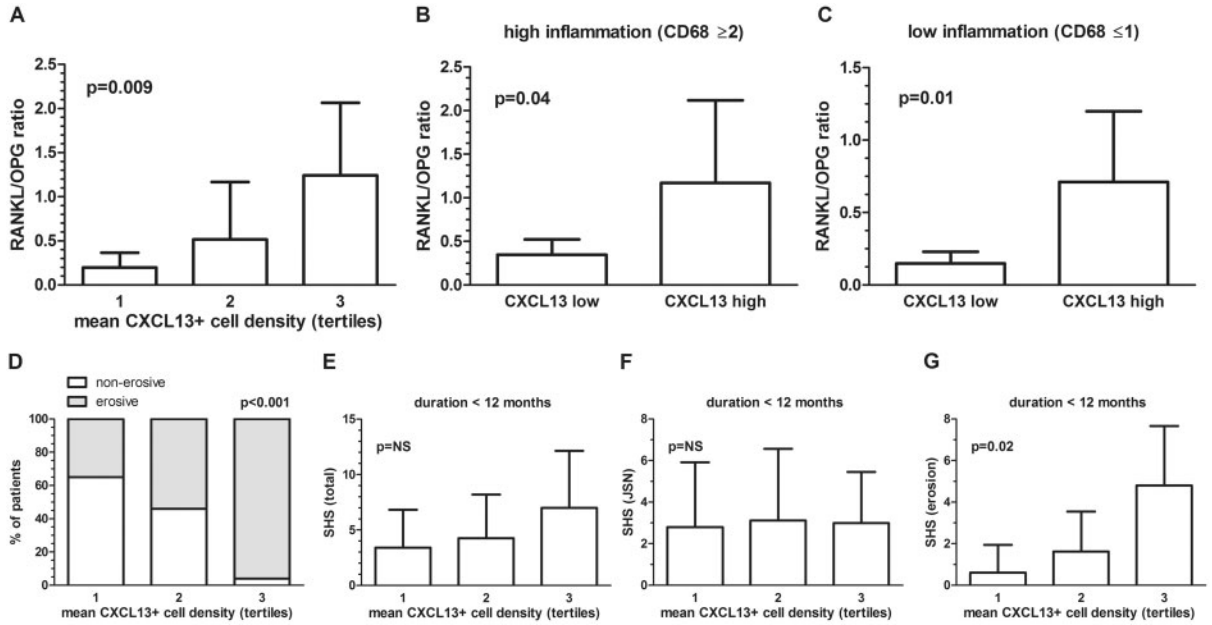
FIG. 4 Association of CXCL13 expression levels with bone damage



(**A**) Bar graph showing the progressive increase in the synovial receptor activator of nuclear factor κB ligand (RANKL)/osteoprotegerin (OPG) ratio along with increasing CXCL13$^+$ cell density tertiles. Bars represent mean (S.D.) values. (**B** and **C**) High CXCL13 expression (third tertile) still displays an increase in the RANKL/OPG ratio compared with lower tertiles (first and second) both in samples with high inflammatory features (CD68$^+$ sublining macrophage score $\geqslant 2$) (**B**) and in samples with low levels of inflammation (CD68 $\leqslant 1$) (**C**). (**D**) Histogram showing the frequency of patients with radiographic erosions in each tertile of synovial CXCL13 expression. (**E**) Mean (S.D.) radiographic total scores, (**F**) joint space narrowing (JSN) and (**G**) erosion subscores according to the Sharp–van der Heijde score (SHS) in different tertiles of CXCL13 expression in patients with a disease duration <12 months. Significant differences are found in the erosion subscore only.

erosive disease progressively increased along with increasing CXCL13 scores (Fig. 4D). Such an increase was highly significant in samples in the third tertile (96%) compared with samples in the second (54%, $P = 0.003$) and first tertiles (35%, $P < 0.0001$), while no differences were found between the second and the first tertiles ($P = 0.3$). The independent association of CXCL13 in the third tertile was maintained when other predictors of erosive disease, such as ACPA positivity and disease duration, were included in a multiple logistic regression model (OR 14.3, 95% CI 1.5, 132.4, $P = 0.01$). In the subgroup of patients with a disease duration of <12 months, although no relationship was observed with the total SHS and the joint narrowing score (Fig. 4E and F), the highest levels of CXCL13 expression appeared to be associated with increased erosion subscores (Fig. 4G).

Discussion

In this study we investigated the clinico-pathological variability of RA in relation to the expression levels of CXCL13 in the joint. The analysis of a large and well-characterized cohort of RA patients heterogeneous for disease duration and activity allowed us to establish that gradients of CXCL13 expression may not reflect *per se* the level of synovial inflammation, as we commonly define it by means of conventional histological/molecular markers. Rather, CXCL13 enrichment appeared linked with an immunologically active lymphoid cell infiltrate and an unbalanced RANKL/OPG ratio that could be maintained beyond the level of synovial inflammation and disease stages. In keeping with such a cellular and molecular milieu, the highest levels of CXCL13 expression were associated with a more severe and erosive disease at the clinical level.

Proinflammatory cytokines play a role upstream of CXCL13 expression and lymphoid tissue organization, as demonstrated in animal models of lymphoid tissue ontogenesis and neogenesis [35–37]. Accordingly, we found a significant association between CXCL13 expression and conventional histological parameters reflecting synovial inflammation in untreated RA patients with early disease. Histopathological features associated with the local expression of CXCL13, such as the presence of ectopic lymphoid structures, were also shown to be related to synovial tissue macrophage infiltration in a previous cohort of early arthritis [38]. However, patient stratification for disease duration revealed that the association with

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most of the inflammatory markers was not maintained regularly in established RA treated with synthetic DMARDs. Similar to our results, the correlation of either CXCL13 or synovial lymphocyte aggregates with cellular and molecular markers of inflammation was found inconsistent in previous cohorts heterogeneous for disease duration and treatment [8, 32, 39, 40]. Thus, although CXCL13 induction, B cell recruitment and follicle formation at peripheral sites are certainly triggered by inflammatory stimuli, their maintenance and resolution dynamics may only partly overlap with acute macrophage-driven inflammatory responses. High degrees of synovial CXCL13 expression may therefore reflect a specific and distinct component of the overall inflammatory process, characterized by more stable and structured biological events [13, 22, 37].

CXCL13 produced within secondary lymphoid tissues plays a critical role in organizing $CXCR5^+$ lymphocytes into follicles and coordinating adaptive immune responses [15–17]. Accordingly, increasing CXCL13 levels in our cohort clearly reflected a gradient of local B cell infiltration associated with a molecular signature indicative of both B and T cell ongoing activation. There is growing evidence indicating that synovial B cells present a strong bias towards citrullinated autoantigen recognition [41], supporting their potential role both as (auto)antigen-presenting cells for local T lymphocytes [12, 41] as well as direct effectors of humoral (auto)responses [13, 14]. In our study population, ACPA positivity occurred in the absence of significant CXCL13 expression and lymphoid cell infiltration; in turn, high CXCL13 was equally found in a proportion of ACPA-negative patients. Nevertheless, samples characterized by the highest levels of CXCL13 were more frequently ACPA positive. Furthermore, by restricting the analysis to ACPA-positive patients and evaluating the autoimmune response in quantitative terms, CXCL13 synovial expression levels were clearly associated with increased ACPA titres. Collectively these data are in line with the concept that while breach of tolerance to citrullinated proteins can occur outside the joint and the autoimmune responses can be maintained in different sites [10, 42–44], synovial B cells can contribute to local ACPA production [13] that may concentrate in the joint and spill over in the circulation with detectably increased titres.

These observations also have relevance from a clinical perspective. Since CXCL13 is not synonymous to ACPA, the two markers may bear different/complementary clinical and prognostic meanings in patients with RA [6, 7]. In early RA, elevated serum levels of CXCL13 at baseline have recently been shown to be associated with increased rates of joint destruction at follow-up [6]. The association of high CXCL13 expression in the synovium and the erosiveness found here, independent of disease duration and the autoantibody status, reinforces the concept that CXCL13 is a marker of disease severity in RA. Supporting our clinical findings, high CXCL13 expression was coupled with an increase in the local RANKL/OPG ratio, whose balance critically regulates bone remodelling in physiologic conditions as well in inflammatory bone loss [45]. Similarly, previous microarray screenings reported a decrease in the expression of genes involved in tissue repair in RA samples enriched in lymphocyte-related transcripts [46]. It is important to emphasize that correlation does not constitute evidence of causation. It is therefore entirely possible that CXCL13 expression in the joints of RA patients might merely represent a marker of a chronic and/or more severe phase of the disease in which mechanisms of joint remodelling are also independently activated. Nonetheless, there are also clues encouraging further studies on the potential causative role of CXCL13 (and downstream lymphoid cell infiltration/organization) in joint remodelling. B cells were recently identified as major producers of RANKL in the synovial fluid of RA patients [47], and rituximab treatment strongly affects the RANKL/OPG system as well as the genes involved in healing processes in the synovium [48, 49]. Furthermore, and perhaps more relevant, autoantibodies produced locally may directly influence bone loss by promoting osteoclastogenesis [50].

In conclusion, local expression of CXCL13 emerges as a marker of severe RA beyond the levels of synovial inflammation and ACPA status. Mechanistic studies are warranted to establish the cause–effect relationship linking CXCL13 expression, local immunity and tissue remodelling. From a clinical perspective, important issues that need to be clarified for CXCL13 to be established as an additional biomarker include the demonstration of whether circulating levels of the chemokine reliably reflect different synovial pathotypes and how well they outperform currently available tests in the clinical assessment and prognostic stratification of RA.

Rheumatology key messages

- In RA patients, variability in synovial chemokine ligand 13 (CXCL13) expression spans beyond conventional features of local inflammation.
- The highest levels of synovial CXCL13 in RA associate with markers reflecting immune cell activity and bone remodelling potential.
- Synovial CXCL13 appears as a biomarker of more severe, erosive RA.

Acknowledgements

C.P. is a recipient of an Arthritis Research UK Experimental Arthritis Treatment Centre Infrastructure Grant (reference 20022).

Funding: This study was supported in part by funding from the Italian Ministry of Health (grant GR-2009-1608032 to A.M.), the Fondazione Italiana per la Ricerca sull'Artrite FIRA Onlus and the IRCCS Policlinico San Matteo Foundation, Pavia, Italy.

Disclosure statement: The authors have declared no conflicts of interest.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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