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Basic science

The course of cytokine and chemokine gene expression in clinically suspect arthralgia patients during progression to inflammatory arthritis

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Abstract

Objectives: Autoantibody responses increase years before the onset of inflammatory arthritis (IA) and are stable during transitioning from clinically suspect arthralgia (CSA) to IA. Cytokine and chemokine levels also increase years before IA onset. However, the course in the at-risk stage of CSA during progression to disease or non-progression is unknown. To increase the understanding of processes mediating disease development, we studied the course of cytokine, chemokine and related receptors gene expression in CSA patients during progression to IA and in CSA patients who ultimately did not develop IA.

Methods: Whole-blood RNA expression of 37 inflammatory cytokines, chemokines and related receptors was determined by dual-colour reverse transcription multiplex ligation-dependent probe amplification in paired samples of CSA patients at CSA onset and either at IA development or after 24 months without IA development. ACPA-positive and ACPA-negative CSA patients developing IA were compared at CSA onset and during progression to IA. Generalised estimating equations tested changes over time. A false discovery rate approach was applied.

Results: None of the cytokine/chemokine genes significantly changed in expression between CSA onset and IA development. In CSA patients without IA development, G-CSF expression decreased ($P = 0.001$), whereas CCR6 and TNIP1 expression increased ($P < 0.001$ and $P = 0.002$, respectively) over a 2 year period. Expression levels in ACPA-positive and ACPA-negative CSA patients who developed IA were similar.

Conclusion: Whole-blood gene expression of assessed cytokines, chemokines and related receptors did not change significantly from CSA to IA development. This suggests that changes in expression of these molecules may not be related to the final process of developing chronicity and may have occurred preceding CSA onset. Changes in gene expression in CSA patients without IA development may provide clues for processes related to resolution.

Keywords: clinically suspect arthralgia, cytokines, chemokines, gene expression, MLPA, inflammatory arthritis

Rheumatology key messages

- Whole blood gene expression of 37 cytokines/chemokines did not change from CSA onset to IA development.
- This suggests that changes in cytokines/chemokines might not relate to the final process of IA development.
- Changes in gene expression in CSA patients not developing disease hint at mechanisms for resolution of inflammation.

Introduction

RA is a chronic autoimmune disease that develops gradually [1]. In the majority of patients, the onset of clinically apparent inflammatory arthritis (IA) is preceded by a phase of arthralgia. A combination of signs and symptoms that is suspect for progression to RA can be recognized by rheumatologists and is called clinical suspect arthralgia (CSA) [2]. Approximately 20% of patients with CSA will progress to IA; the period between symptom onset and IA development is generally 6–12 months [3]. To date, the final hit or final process in the pathophysiology determining progression from CSA to IA is still unclear.

In the pathophysiology of disease development, autoantibody responses occur and increase years before the onset of IA. Their levels are stable during the transition from CSA to IA [4]. In addition, the levels are equally high in autoantibody-positive CSA patients who, over time, do not develop IA [4–6]. Together, this suggests that autoantibody

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maturation occurs before the onset of joint symptoms and does not relate to the final hit of IA development.

In contrast to the autoantibody response, which has been extensively studied in the phases preceding IA, less is known about the course of inflammatory mediators such as cytokines and chemokines in this phase. Some nested case–control studies at the protein level found upregulation of different inflammatory markers in the pre-IA phase compared with healthy controls [7–9]. A nested case–control study included blood samples from 85 patients preceding IA onset and showed upregulation of several cytokines and chemokines compared with control subjects [10]. Sokolove *et al.* [11] reported in a longitudinal study an increase and stabilization of multiple cytokine and chemokine levels years before IA onset. However, the above-mentioned studies did not show whether the changes in cytokine expression occurred already in the asymptomatic pre-IA phase or occurred after symptom development, thus during progression from CSA to clinical arthritis. The relationship with symptom onset is relevant because symptoms can be recognized in a clinical setting where intervention is possible. Ideally, intervention in this stage is targeted at processes that are fundamental for progression to chronic disease. In addition, none of the nested case–control studies included a control group in a similar at-risk stage that ultimately did not develop IA. A control group in a similar at-risk stage is relevant to identify processes that are specific for progression from arthralgia to chronic disease.

In addition to the studies on protein levels of inflammatory markers, there are a few studies focusing on gene expression signatures and pathways in a pre-RA phase. Some of these studies included patients in a symptomatic at-risk stage [12–15]. These studies looked at expression at a single time point during pre-RA in relation to IA development, one of which was published by our group [15]. However, none of these studies had a longitudinal design with serially collected samples and therefore did not study the course of gene expression from symptom onset towards IA development.

Here we performed the first longitudinal study on cytokine, chemokine and related receptor gene expression in CSA patients. The ultimate aim of this study was to increase the understanding of the processes in the symptomatic at-risk phase of CSA that are crucial for the development of IA. More specifically, we aimed to study the course of cytokine, chemokine and related receptor gene expression from CSA onset towards progression to IA. In addition, we aimed to determine the course of cytokine, chemokine and related receptor gene expression in CSA patients who were thus at risk but who ultimately did not develop IA.

Methods

Study population

Patients were recruited from the Leiden CSA cohort [2]. In the Leiden CSA cohort, all patients who presented at the outpatient clinical with recent onset (<6 months) arthralgia of the small joints, suspicious for progression to RA according to a rheumatologist, were consecutively included. Baseline visits consisted of physical examination, blood sampling (including PAXgene tubes) and imaging of hands and feet. Autoantibody status was not known at the time of inclusion since general practitioners were discouraged to test for them according to Dutch guidelines. Follow-up research visits took place at 4, 12 and 24 months. In case of joint swelling or gain of symptoms, additional visits were scheduled. Patients were followed for 2 years or until IA development, determined by a rheumatologist at physical examination (66 swollen joint count $\geq$1). Treatment with DMARDs, including corticosteroids, was not allowed during follow-up.

In this study, RNA expression of 37 cytokines and chemokines was measured using blood from PAXgene tubes of CSA patients using multiplex ligation-dependent probe amplification (MLPA). A detailed flowchart of the included patient samples is depicted in Fig. 1. Baseline RNA measurements were available for 495 of 639 consecutive CSA patients (Fig. 1). A total of 22 CSA patients had paired measurements at CSA onset and IA development that were assessed in the same MLPA run and were studied for question 1. From all CSA patients who achieved at least 2 years follow-up and did not progress to IA, 88 had paired samples that were studied in the same MLPA run for question 2 (Fig. 1). Reasons for the absence of a second PAXgene tube are described below the flowchart in Fig. 1 and the missingness was assumed to be random. The baseline characteristics of patients with and without a paired sample did not remarkably differ (Supplementary Table S1, available at *Rheumatology* online).

Measurement of RNA expression levels

RNA isolation

From baseline and follow-up, RNA from whole blood in PAXgene tubes was extracted using PAXgene Blood RNA kits (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturers' protocol [16]. RNA yield was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Dual-colour reverse transcription multiplex ligation-dependent probe amplification (dcRT-MLPA) assays

RNA expression was determined by dcRT-MLPA as previously described [17]. RNA expression analysis included 37 cytokines, chemokines and related receptors: CXCR5, CCL11, CCL17, CCL18, CCL2, CCL22, CCL3, CCL4, CCR6, CCR7, CSF2, G-CSF, CXCL10, CXCL3, CXCL4, CXCL7, CXCL9, IFNG, IL-10, IL-12A, IL-12B, IL-13, IL-15, IL-18, IL-1A, IL-1B, IL-1Ra, IL-2, IL-22RA1, IL-23A, IL-6, IL-7R, IL-8, IL-9, TGF-β, TNF and TNIP1. Data were analysed using GeneMapper 5 software (Applied Biosystems, Waltham, MA, USA). Results from target genes were calculated relative to the geometric average signal of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [18]. Signals below the noise cut-off (log2 transformed peak area $\leq$7.64) were assigned the threshold value. Further analyses were performed on log2 transformed data.

RNA measurements on baseline blood samples were performed in two runs. The first run comprised blood samples from patients included between April 2012 and March 2015 (n = 236). The second run comprised blood samples from patients included between March 2015 and January 2019 (n = 259). Follow-up measurements (either at the time of arthritis development or after 2 years) were performed in either MLPA run 1 or 2.

Before running the blood samples of the included CSA patients, we performed a separate MLPA run in which we determined the expression of genes that are reported to be differently expressed in individuals at risk for RA (IFN-γ, IL-13, CXCL9, CCL11 and IL-1Ra) compared with healthy controls

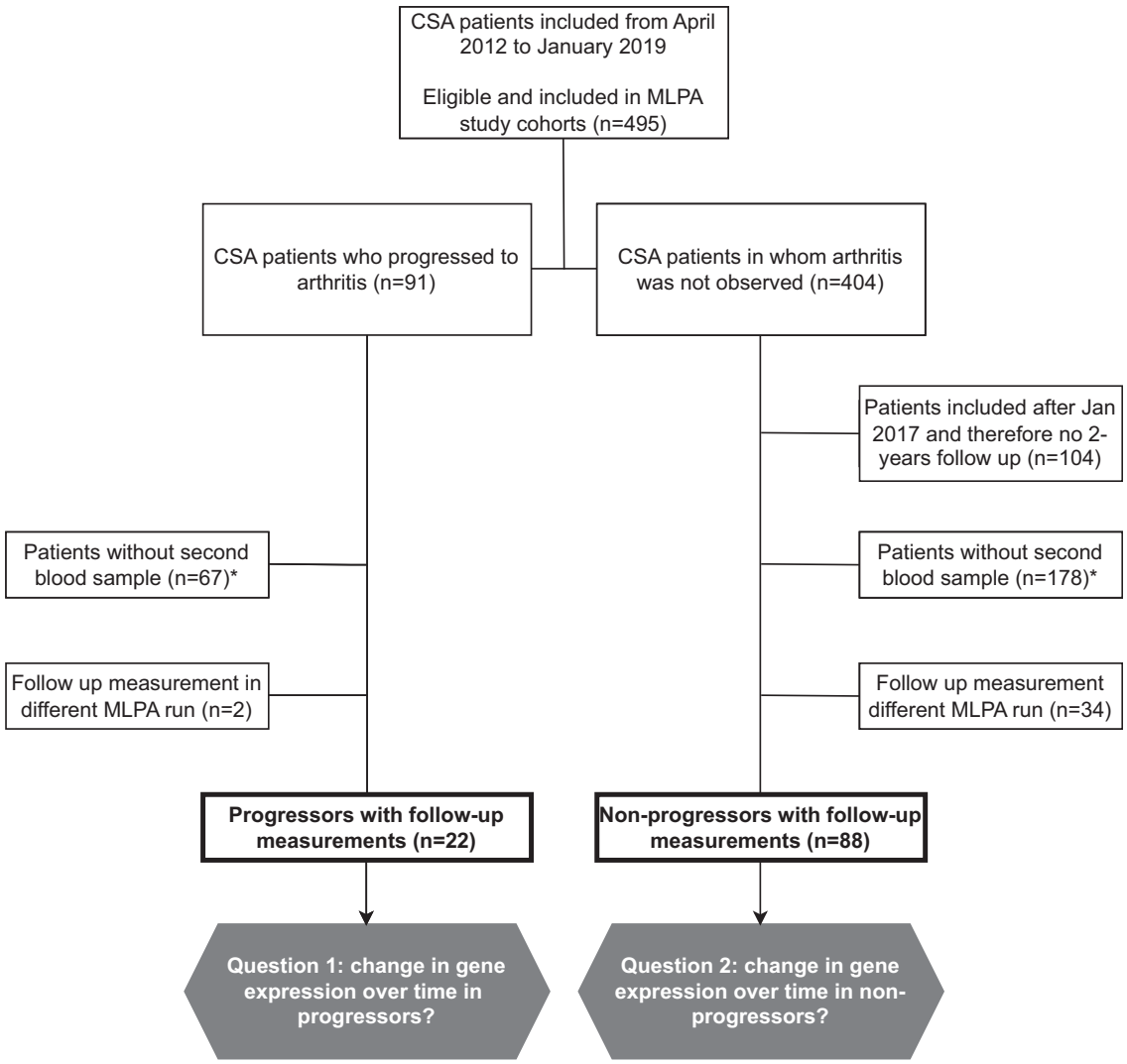

Figure 1. Flowchart of included patients in the two study groups. Between April 2012 and January 2019, 639 patients were included in the Leiden CSA cohort. PAXgene tubes were not available from 44 patients at baseline. A total of 98 patients were included in a randomized placebo-controlled trial and were excluded because of possible DMARD treatment. RNA measurements failed for two patients. In total, baseline RNA measurements were obtained from 495 eligible patients. In this flowchart the flow of paired measurements from CSA patients who progressed to IA (progressors) and from CSA patients who did not progress to IA (non-progressors) are displayed. *Missingness was due to no PAXgene collection in the beginning of the CSA study, no 2 year visit (only in the non-progressors group due to resolution of symptoms or lost to follow up) or no PAXgene collection at the research visit due to logistic reasons or patient's preference

[10, 11]. This cross-sectional comparison was done as preliminary work to evaluate whether our findings were in line with results from the literature. Included was whole blood RNA from four CSA patients who progressed to IA, six CSA patients who did not progress to IA and five healthy controls. The healthy controls were recruited on a voluntary basis. Subjects had no history of autoimmune disease, were >18 years of age and gave written informed consent. This revealed increased expression of IFN-γ, IL-13, CXCL9, CCL11 and IL-1Ra in CSA patients (both CSA patients who progressed and CSA patients who did not progress to IA) compared with healthy controls (Supplementary Fig. S1, available at *Rheumatology* online). This finding is in line with earlier studies and validated our assay [10, 11].

Statistical analyses

Generalized estimated equations were used to test average changes in cytokine and chemokine expression levels over time within patients who progressed to IA and separately in patients who did not progress to IA. In patients who progressed to IA, a subanalysis of gene expression over time was conducted in patients who were diagnosed with RA (i.e. with a clinical diagnosis and who fulfilled the 1987 and/or 2010 criteria for RA and/or started a DMARD at IA development; $n = 18$). In an additional subanalysis, patients who progressed to IA were stratified for ACPA status. This was done because studies on risk factors suggest that autoantibody-positive and autoantibody-negative RA are subgroups with differences in the underlying pathophysiology [19]. Logistic regression models were used to test baseline differences in gene expression levels between ACPA-positive ($n = 40$) and ACPA-negative ($n = 51$) patients who progressed to IA. All analyses were corrected for the two different MLPA runs, as patients from different MLPA runs were included in the same analyses. No other corrections were made. The false discovery rate (FDR) was used to correct for multiple testing. *P*-values

≤0.05 were considered statistically significant. Stata version 17.0 (StataCorp, College Station, TX, USA) was used for the analyses.

Ethics

The study was approved by the local medical ethical committee Leiden-Den Haag-Delft (P11.210). All patients gave written informed consent.

Results

Study population

Baseline characteristics of the patients who were studied for the two research questions are presented in Table 1.

Cytokine, chemokine and receptor gene expression over time from CSA onset to IA development (question 1, Fig. 2)

The median time between presentation with CSA and IA-development was 4.1 months (IQR 3.4–8.3). After correction for multiple testing and MLPA run, none of the 37 cytokine and chemokine gene expression levels significantly changed over time towards IA development. The course over time is depicted in Fig. 2. *P*-values are presented in Supplementary Table S2, available at *Rheumatology* online.

Cytokine, chemokine and receptor gene expression over time in CSA patients who did not progress to IA (question 2, Fig. 3)

Patients who presented with CSA and over time did not progress to IA were studied with paired samples with a 2 year interval. The expression level of G-CSF had decreased after 2 years of follow-up ($P < 0.001$). The expression levels of CCR6 and TNIP1 had both increased after 2 years of follow-up ($P < 0.001$ and $P = 0.002$, respectively; Fig. 3 and Supplementary Table S2, available at *Rheumatology* online). The other 34 genes did not change significantly. To explore whether changes in CCR6, G-CSF and TNIP1 were correlated over time, we made pairwise comparisons for the three genes (Supplementary Fig. S2, available at *Rheumatology* online). Changes in CCR6 and G-CSF were statistically significantly correlated but the correlation coefficient was weak ($R^2 = 0.42$, $P < 0.001$); changes between the other genes were not correlated.

Subanalyses

Subanalysis in CSA patients who progressed to RA

Sensitivity analysis in CSA patients who progressed to RA showed similar results as observed for CSA patients progressing to IA (Supplementary Fig. S3, available at *Rheumatology* online).

Subanalysis in ACPA-positive vs ACPA-negative CSA patients who developed IA

We also compared differences at CSA onset and over time between CSA patients who developed ACPA-positive and ACPA-negative IA. The baseline characteristics of these patients are presented in Supplementary Table S3, available at *Rheumatology* online. At CSA onset, expression of the 37 candidate genes did not differ between ACPA-positive and ACPA-negative patients. Although IL-13 and CCR7 gene expression levels were lower in ACPA-positive compared with ACPA-negative patients, results were not significant after correction for multiple testing (Supplementary Table S4, available at *Rheumatology* online). The course of gene expression of cytokines and chemokines over time during progression to ACPA-positive and ACPA-negative IA is shown in Supplementary Fig. S4, available at *Rheumatology* online. Visual inspection revealed no obvious differences. Because of the low number of patients in the ACPA-positive group, no statistical tests were performed.

Discussion

In this longitudinal study we aimed to increase the understanding of the processes involved in the progression from the CSA at-risk stage to clinically apparent IA by studying the course of expression of cytokines, chemokines and related receptor genes and relate this to the course of gene expression in CSA patients who did not develop IA. We observed that gene expression levels of cytokines, chemokines and related receptors did not change over time from CSA to IA development. In addition, in CSA patients not developing IA, G-CSF, CCR6 and TNIP1 changed over the course of 2 years.

Our finding that gene expression levels of 37 cytokines, chemokines and related receptors did not change during the transition from arthralgia to IA suggests that changes in the genes/molecules studied do not relate to the final hit or final process of developing IA. Previous nested case–control studies revealed that increases in cytokine and chemokine levels occurred several years before the diagnosis of RA [7–10, 20]; information on symptom onset was not included here.

Table 1. Baseline characteristics of CSA patients included in the main analyses

Characteristics	Progressors[a] with paired measurements ($n = 22$)	Non-progressors[b] with paired measurements ($n = 88$)
Age, years, mean (S.D.)	49 (12)	44 (12)
Gender, female, *n* (%)	17 (77)	71 (81)
Symptom duration, weeks, median (IQR)	19 (7–53)	23 (14–46)
Tender joint count, median (IQR)	5 (3–7)	5 (2–10)
ACPA positivity, *n* (%)	6 (27)	9 (10)
RF positivity, *n* (%)	7 (32)	14 (16)
Increased CRP, *n* (%)	7 (32)	10 (11)
MRI-detected subclinical inflammation, *n* (%)	14 (64)	31 (36)

[a] CSA patients who progressed to IA with paired measurements.
[b] CSA patients who did not progress to IA with paired measurements over 2 years.

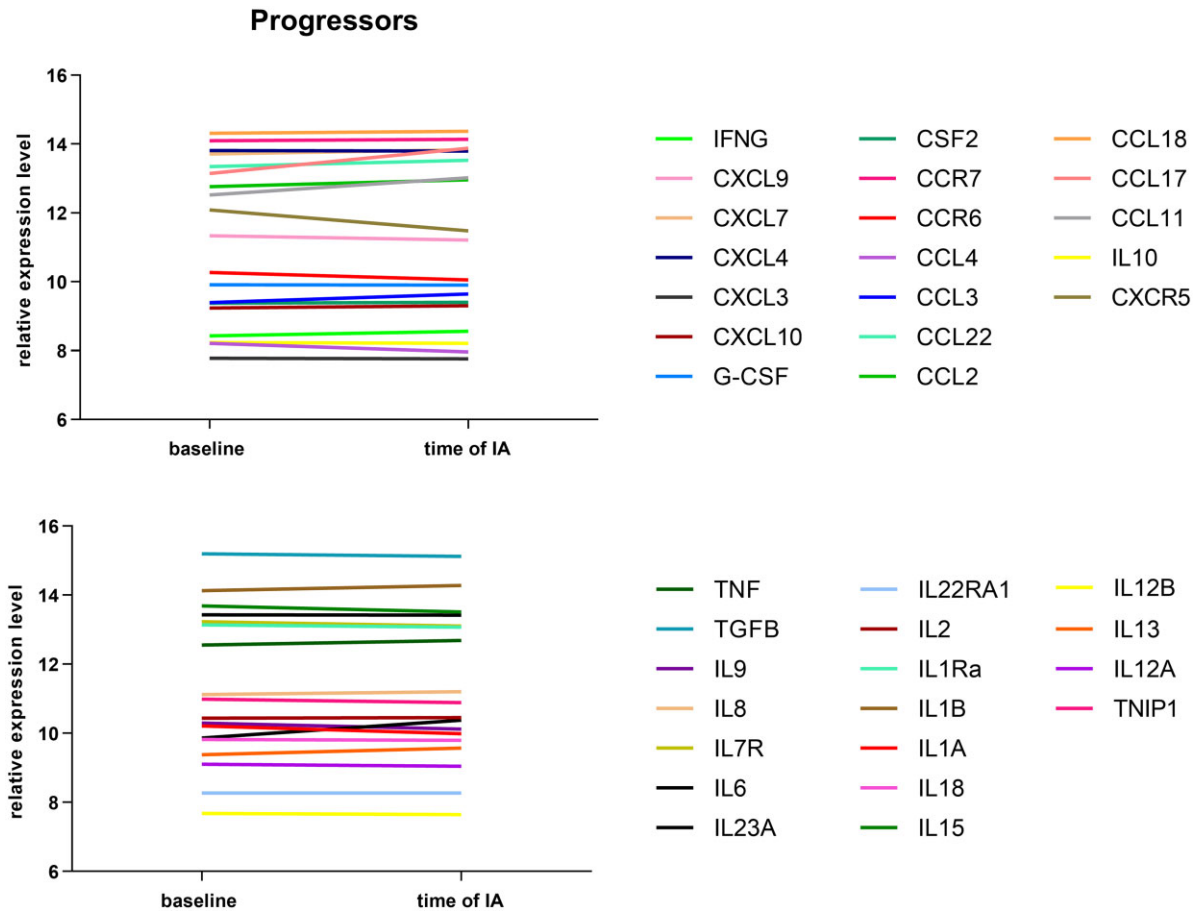

Figure 2. Modelled course of gene expression of 37 cytokines, chemokines and related receptors in CSA patients who progressed to IA. Cytokines, chemokines and related receptors were measured at baseline and at the time of IA development and for reasons of clarity are presented in two plots. No statistically significant changes were observed during follow-up

However, CSA usually develops ≈6–12 months before the onset of clinical arthritis [3]. Therefore the combination of our and previous findings makes it presumable that increases in (measured) cytokines/chemokines most likely take place before symptom development and, based on our data, levels do not change afterwards (during progression to IA).

Interestingly, the current data on cytokines/chemokines resemble the data on autoantibody maturation as measured in a longitudinal study in CSA showing that measured autoantibody levels and the number of isotypes and fine specificities were already increased at CSA onset and did not mature any further during progression to IA [4]. In the absence of observed evident changes in markers from the systemic circulation during progression from CSA to IA, it can be hypothesized that the final decisive hit in the process of IA development occurs locally, e.g. in the joints, in the phase of CSA. However, this is a subject for future studies. In parallel with arthritis development, leprosy is another chronic disease in which the development occurs in several phases. In a longitudinal study in leprosy, the expression of genes encoding several inflammatory proteins increased preceding the onset of symptoms [21]. The order of a systemic immune response before abnormalities in the target organ occur may therefore be a shared trajectory between diseases.

To our knowledge, we are the first to study systemic expression of cytokines and chemokines in a symptomatic at-risk population that ultimately does not develop IA. In CSA patients not developing IA, gene expression of G-CSF had decreased and expression of CCR6 and TNIP1 had increased after 2 years of non-progression. The other studied genes did not change over the course of 2 years. Although healthy controls were not included in the MPLA runs, it is likely that some genes were increased in expression at CSA presentation, since the CSA patients not progressing to IA in our cross-sectional evaluation, which was done before studying serial samples, had higher levels of gene expression compared with healthy controls (Supplementary Fig. S1, available at *Rheumatology* online).

The genes that changed in expression in patients who did not progress from CSA to IA (G-CSF, CCR6 and TNIP1) may hint at processes mediating spontaneous resolution of CSA and related subclinical inflammation. G-CSF expression levels decreased in patients who did not progress to IA. G-CSF is a pro-inflammatory cytokine involved in haematopoiesis that plays a role in inflammatory diseases, including RA [22]. Elevated levels of G-CSF have been detected in synovial fluid and serum of RA patients and are related to disease severity [23]. In a mouse model of RA, G-CSF levels increased over the course of the disease and blocking G-CSF ameliorated disease in mice. G-CSF deficiency protected mice from acute and chronic arthritis [24, 25]. This evidently pro-inflammatory role of G-CSF in established RA is in line with our finding that G-CSF decreased towards non-progression in CSA. Kokonnen *et al.* [10] reported in a nested case–control study an elevated level of G-CSF in pre-RA patients compared with control subjects that did not increase further after diagnosis.

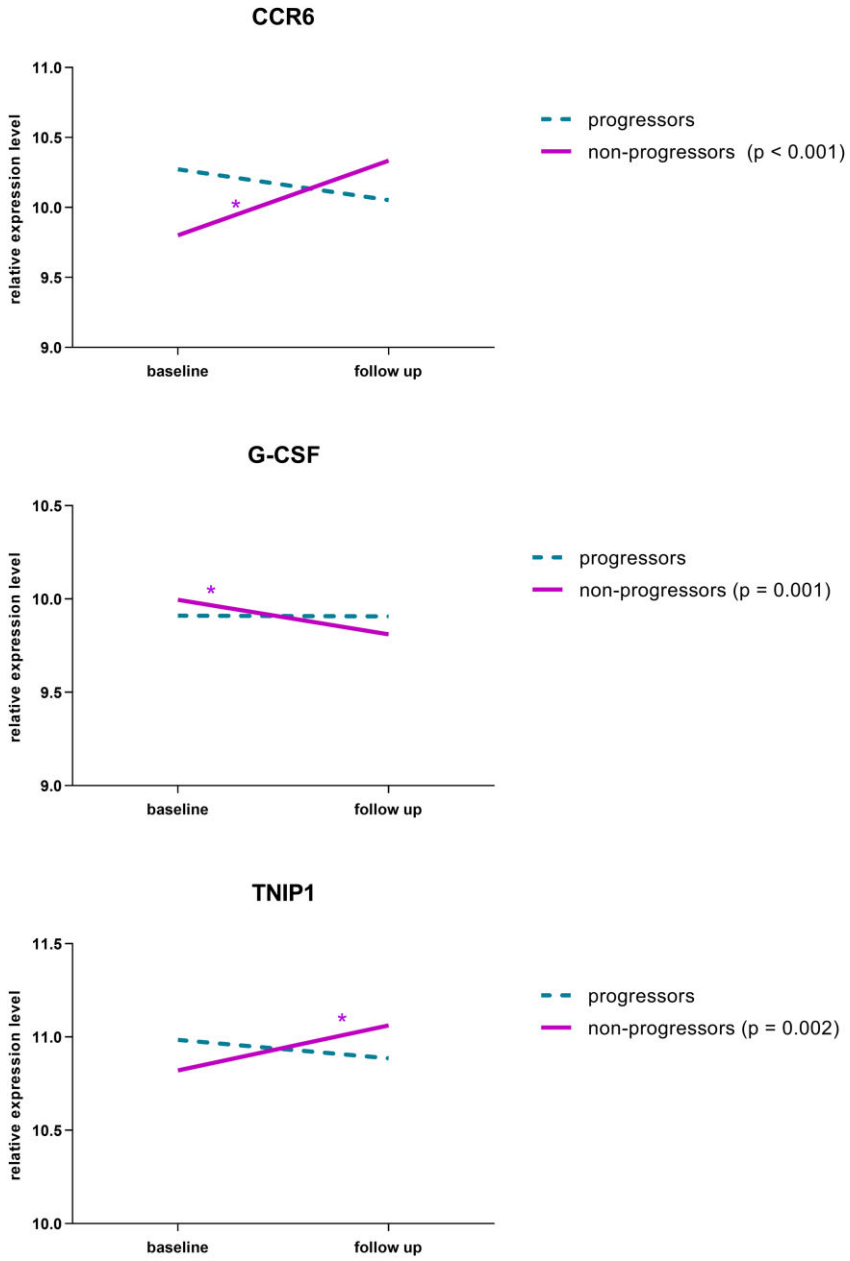

Figure 3. Modelled course of gene expression of CCR6, G-CSF and TNIP1 in CSA patients who did not progress to IA. Cytokines, chemokines and related receptors were measured in paired samples from each patient with 2 year intervals. For comparison, the course of patients who progressed to IA was included, here the second samples were collected at IA development

They did not include symptomatic at-risk patients who did not progress to arthritis, but this is in line with our finding that G-CSF did not change over time in CSA patients who did progress to arthritis.

Gene expression levels of CCR6 increased in CSA patients who did not progress to IA. CCR6 is expressed on different cell types, including T cells (such as Th17, Treg, TCR$\gamma\delta$+ T Cells and ILC3), B cells and DCs [26]. The ligand for CCR6 is CCL20, which is highly expressed at sites of inflammation [27]. Upon binding of CCL20, CCR6$^+$ Th17 cells become activated and migrate along the CCL20 gradient towards the RA joint synovium [27]. Genome-wide association studies revealed that polymorphism in *CCR6* at 6q27 was associated with RA susceptibility [28, 29]. In addition, CCR6 is a marker for Th17 cells and an increased proportion of CCR6$^+$ Th17 cells was found in peripheral blood of treatment-naïve patients with RA as well as in synovial fluid and inflamed synovial tissue of RA patients [30, 31]. Interestingly, CCR6$^+$ Th cells are very plastic and can convert to Treg cells [31, 32]. In addition, not every CCR6$^+$ Th17 cells is pathogenic and exposure to IL-23 is needed for the 'license to kill' [33]. Therefore, higher expression of CCR6 in the blood of CSA patient that do not progress in IA may be due to no migration of CCR6$^+$ cells to the site of inflammation because of a lack of CCL20 or due to the presence of non-pathogenic CCR6$^+$ Th17 cells or even plasticity of these cells into CCR6$^+$ Treg cells. Further research is needed to elucidate the cell type expression of CCR6 in CSA patients who do not progress to arthritis.

The third gene for which increased expression was detected after 2 years in CSA patients who did not progress to IA was

TNIP1. Polymorphisms of TNIP1 have been associated with RA, as well as other polymorphisms of genes involved in nuclear factor κB (NF-κB) signalling [34]. TNIP1 encodes TNF-α-induced protein 3 (TNFAIP3)-interacting protein 1, also called A20-interacting protein 1 or ABIN1 (A20-binding inhibitor of NF-κB1). This protein binds the ubiquitin-editing NF-κB inhibitor A20 [35–37]. It can be expressed in every cell. Overexpression of ABIN1 inhibits NF-κB activation by TNF and several other stimuli. Similar to A20, ABIN1 expression is NF-κB dependent, implicating a potential role for the A20/ABIN1 complex in the negative feedback regulation of NF-κB activation [35, 38, 39]. The increased expression of TNIP1 over time in our CSA patients who did not progress to IA is in line with its anti-inflammatory role in the prevention of autoimmune diseases [37].

In our explorative comparison of ACPA-positive and ACPA-negative CSA patients transitioning to IA, cytokine, chemokine and related receptor expression levels did not differ at the time of CSA onset. In addition, the course over time towards IA, evaluated visually, appeared to reveal no differences. Statistical inferences were not made because of the small number of patients in the groups. Most previous studies in pre-RA patients on cytokines included mostly autoantibody-positive patients and did not make direct comparisons to developing autoantibody-negative disease for individual cytokines [8, 10]. If validated in future studies, the similarity in cytokine and chemokine expression levels in ACPA-positive and ACPA-negative patients is interesting since ACPA-positive and ACPA-negative RA have differences in environmental and genetic risk factors but a largely similar clinical presentation at CSA onset and at RA diagnosis [19, 40, 41]. This could be suggestive of a common pathway in clinical arthritis development, despite differences in risk factors and in the severity of the disease after RA diagnosis.

Limitations of our study are the limited number of included patients in the longitudinal analysis of CSA patients who progressed to IA and the absence of healthy control subjects in our main analysis. The limited number of patients may have led to false-negative results, because of a lack of power, regarding the detection of cytokines and chemokines that changed over time. In addition, we studied a relatively small number of inflammatory genes and might therefore have missed other inflammatory genes that perhaps do change during progression from CSA to IA or RA. Another limitation is the fact that without healthy control subjects in our main analysis, we were unable to test for differences between CSA patients and healthy subjects. However, in a small preliminary MLPA run, we were able to compare progressing and non-progressing CSA patients with healthy controls. We demonstrated that observed changes in expression from the literature were also present in the current data, which shows the validity of the findings. Moreover, our control group consisted of CSA patients with similar symptoms but who did not pass the final step in transitioning to IA. In our view this group is valuable to understand the processes related to the final hit of IA development in already symptomatic patients. Finally, a limitation of our study is that the expression levels were measured at the RNA level. Expression at the RNA level does not necessarily translate to protein expression, which is more directly related to the actual activity. However, previous literature reporting increased protein levels of G-CSF in RA patients does not contradict our finding that CSA patients who do not develop IA show a decrease in G-CSF RNA expression. Further research at the protein level of G-CSF, CCR6 and TNIP1, as well as functional assays, can validate our results and explore mechanisms related to the resolution of CSA. In addition, future studies in CSA patients, and more specifically in the joints of CSA patients, are necessary to explore final processes related to the development of arthritis in the symptomatic at-risk population.

Conclusions

In the transition from CSA to IA or RA, no changes in cytokine, chemokine or related receptor gene expression were observed in our study. Hence the results indicate that changes in systemic expression of these cytokines and chemokines are not related to the final hit or process of developing chronic RA. In addition, although not measured here, the data may suggest that changes in expression already occurred preceding the onset of symptoms. Changes in gene expression over time in at-risk patients not developing IA may provide clues to processes related to the resolution of CSA and inflammation, which should be validated and expanded in future studies.

Supplementary material

Supplementary material is available at *Rheumatology* online.

Data availability

The data used in this study are available from the corresponding author upon reasonable request.

Authors' contributions

J.W.H., C.R., E.N., A.G. and A.H.M.v.d.H.-v.M. contributed to the conception and study design. J.W.H. and C.R. analysed and interpreted the data. S.J.F.v.d.E. performed RNA isolation and dcRT-MLPA. J.W.H. wrote the first version of the manuscript and C.R., E.N., S.J.F.v.d.E., P.H.P.d.J., E.L., A.G. and A.H.M.v.d.H.-v.M. revised it critically. A.G. and A.H.M.v.d.H.-v.M. supervised the study. All authors read and approved the final manuscript.

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