Predictive factors for outcome of rheumatoid arthritis

Linden, M.P.M. van der

CHAPTER 5

Identification of CXCL13 as marker for outcome of rheumatoid arthritis using an *in silico* model of the rheumatic joint

M.P.M. van der Linden[1#]
C.M.L. Meeuwisse[2#]
J.A.C. Rullmann[2]
C.F. Allaart[1]
R. Nelissen[2]
T.W.J. Huizinga[1]
A. Garritsen[2]
R.E.M. Toes[1]
C. van Schaik[2]
A.H.M. van der Helm-van Mil[1]

[#] *both authors contributed equally to this work.*

[1] *Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands*
[2] *Merck Sharp & Dohme, Oss, the Netherlands*

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ABSTRACT

Objective

Rheumatoid arthritis (RA) is characterized by inflammation and destruction of joints. The amount of damage is highly variable between RA patients. Prediction of the disease severity using known clinical and serological risk factors is inaccurate. Here we aimed to identify new serological markers for RA severity using an *in silico* computer model of the rheumatic joint.

Methods

An *in silico* computer model of a prototypical rheumatic joint predicted candidate markers associating with erosiveness. From a broader set of candidate markers, four were progressed for validation: Trap5b, NTX, Ang-2 and CXCL13. Serum of 74 RA patients was used to study whether radiological joint destruction (total erosion score (ES) and total Sharp/van der Heijde score (SHS)) after 4-years of disease associated with serum levels at the time of diagnosis. Serum marker levels were determined using ELISAs. For confirmation, baseline serum levels were studied for an association with progression of joint damage over seven years of follow-up in a cohort of 155 early RA patients.

Results

Comparison of high and low quartiles of ES and SHS at 4-years showed a difference in baseline CXCL13 serum level ($p=0.011$ and $p=0.018$ respectively). In the confirmation cohort, elevated baseline CXCL13 levels associated with increased rates of joint destruction during 7 years follow-up, without ($p<0.001$) and with ($p<0.005$) adjustment for CRP levels. Analyzing anti-CCP2-positive and anti-CCP2-negative RA separately yielded a significant result only in the anti-CCP2-negative group ($p<0.001$).

Conclusion

CXCL13 is a novel serological marker predictive for RA severity. This marker was identified with the help of an *in silico* model of the RA joint.

INTRODUCTION

The perspectives of patients with rheumatoid arthritis (RA) have improved by treatment strategies with tight disease control and the availability of new potent biological agents. In order to balance the risks of overtreatment, inducing unnecessary costs and toxicity, and undertreatment, leading to joint destruction that could have been prevented, it is of utmost importance to be able to predict the disease outcome for each patient. At present several markers linked to a severe course of RA are extensively reported on, such as multiple swollen joints at disease onset, relatively high baseline C-reactive protein levels, presence of anti-cyclic citrullinated protein (CCP) antibodies, rheumatoid factor (RF) and erosions at baseline.[1-3] Several attempts have been made to derive adequate prediction models or prediction matrices using these risk markers, but in all cases only less than 50% of the patients could actually be classified.[4-6] This indicates that the currently used risk factors do not allow adequate prediction for individual patients. This, together with the fact that the severity of the disease course is highly variable between patients, underlines the importance to identify new markers for disease outcome in RA.

The severity of RA can be measured objectively by levels of joint destruction on radiographs of hands and feet using validated scoring methods. It reflects the cumulative burden of inflammation over time, and strongly correlates with joint functionality and subsequent disability. Several attempts to predict the radiological damage using serological markers have been made in the last few years. Examples are OPG, RANKL and MMP3 that are all primarily markers that reflect joint tissue remodeling. The baseline OPG/RANKL ratio was observed to be an independent predictor for the level of joint destruction later in the disease course.[7] Such an association is reported for baseline MMP3 levels as well, although its association was dependent on a correlation with traditional risk factors such as anti-CCP antibodies.[8] Importantly, a recent study indicated that these factors insufficiently account for radiological joint damage.[9] This underlines the value of new serological markers.

The present study aimed to identify new serological markers that are predictive for the severity of the disease course of RA. A computer model representing the biology of the rheumatic joint was used to select candidate markers for bone erosiveness. These markers were tested in baseline serum of RA patients with 4-years radiological data in order to study for an association *in vivo*. A second cohort of RA patients with long-term follow-up data was used in order to confirm the predictive ability of these markers for the disease outcome of RA, measured by the rate of joint destruction as well as the chance of sustained DMARD-free remission.

PATIENTS AND METHODS

Identification of markers

In silico model

An *in silico* computer model of a prototypical articular joint in a patient with RA was created previously (Rheumatoid Arthritis (RA) PhysioLab® platform, Entelos, Foster City, CA, USA).[10,11] This RA PhysioLab platform represents the biology behind RA on the level of synovial tissue inflammation, cartilage destruction and bone erosion. The model integrates relevant *in vitro* and clinical data into a computer-based platform to reproduce disease characteristics of RA. When run, the model simulates disease or biological response to treatment in a prototypical joint representative of affected joints of RA patients. The platform models the life cycle of inflammatory cells, endothelium, synovial fibroblasts, chondrocytes, and bone cells, as well as their products and interactions. During simulation experiments with the computer model the interplay between these cells and processes result in a reproduction of RA disease characteristics: self-perpetuating inflammation and breakdown of cartilage and bone. These characteristics are represented by numerical read-outs in the model that closely resemble accepted read-outs in the clinic, such as ACR-response, DAS28 and bone erosion progression rate. For example, bone erosion is computed as the net loss of bone volume due to bone synthesis and resorption, which in turn depend on density and activation state of osteoblasts and osteoclasts. Computed treatment responses are in line with clinically observed responses.[11] RA is a multi-factorial disease with a heterogeneous manifestation. To capture this heterogeneity the computer model uses the Virtual Patient concept. Different settings for selected (combinations of) model parameters are used in parallel for the simulations. This results in a range of disease activities and therapy outcomes. Every combination of such pre-defined parameter sets represents a Virtual Patient (VP). Examples of these settings are an increased production rate of TNFα by TNFα producing cells or a decrease of the effect of MTX on MTX-sensitive pathways in the model (see reference for a more detailed description).[12] We used RA PhysioLab version 3.2 and a set of 120 distinct VPs to predict candidate serological markers associating with localized bone loss, representing erosiveness. Therefore erosiveness is the main outcome measure used in this study. Since the erosion score is part of the Sharp/van der Heijde score, the method used to score radiological joint damage, the total SHS is assessed as well.

Patients

Discovery cohort

Patients included in the BeSt cohort for whom baseline serum was available were used to test the association of serological markers with the level of joint destruction at 4 years of followup (total erosion score (ES) and total Sharp/van der Heijde score (SHS)) (n=74). The BeSt-cohort (a

Dutch acronym for "Behandel Strategieën", treatment strategies), included patients with recent-onset RA with a disease duration of 2 years or less that were randomly allocated to one of four treatment groups with different DAS-guided combinations and applications of DMARDs.[13] At the time this study was initiated, the four years of follow-up was the maximal available follow-up duration. Applied treatments consisted of sequential disease-modifying antirheumatic drug monotherapy (group 1), step-up combination therapy (group 2), initial combination therapy with tapered high-dose prednisone (group 3), and initial combination therapy with the tumor necrosis factor antagonist infliximab (group 4) respectively.

At baseline blood samples were taken for routine diagnostic laboratory screening and serum was stored at -70 °C. There were no significant differences between baseline patient characteristics of patients with and without serum data available, apart from slightly higher numbers of swollen joints in the group with missing data (data not shown). All 4-years radiographs were scored independently by two trained readers blinded to the patient's identity, treatment group and sequence of the films. The mean score of the two readers was used for the analysis. The interobserver correlation coefficient (ICC) was 0.96.[14]

Confirmation cohort

The second set of RA patients was used to confirm findings between serological marker and severity of the disease course. This set comprised 155 early RA patients that were included in the Leiden Early Arthritis Clinic (EAC) cohort between 1993 and 2006 and for whom both baseline serum as well as yearly taken radiographs were available. No significant differences were observed between baseline patient characteristics of patients with and without serum available (data not shown). The Leiden EAC is a large prospective cohort as previously described.[15] Patients were referred by general practitioners when arthritis was suspected and included in the EAC cohort if arthritis was confirmed at physical examination and symptom duration was less than 2 years. At inclusion, patients were inquired about their joint symptoms and subjected to a physical examination. At baseline blood samples were taken for routine diagnostic laboratory screening and serum was stored at -20 °C (start of the cohort) or -70 °C. The EAC patients studied were not included in the BeSt cohort.

Radiographs of hands and feet were taken at baseline and consecutive years and were scored chronologically by an experienced reader (MPMvdL) as previously described.[16] ICCs were 0.91 for all radiographs, 0.84 for baseline radiographs, and 0.97 for the radiographic progression rate. To encompass a reliable sample size, radiographic follow-up data were restricted to a maximum of 7 years. As mentioned, the total erosion score (ES) was the main outcome measure; in addition, the total SHS was assessed. The present study had a power of 96% to detect a difference of 15 SHS points (SD25) at the seven year time point with an alpha of 0.05. Disease remission was assessed as a second outcome measure in order to further substantiate the findings. Remission was defined in its most stringent form as the persistent absence of synovitis for at least one year after cessation of DMARD therapy and the identification of remission by the patient's rheu-

matologist.[17] The remission status could be reliably ascertained in 152 out of 155 RA patients. Most patients who achieved remission had a follow-up monitoring for longer than the minimally required 1 year; the median time of observation after discontinuation of DMARDs was 2.5 years.

Biomarker measurement

Serum measurements for biomarker levels in both independent patient cohorts were performed by ELISA according to the manufacturers' instructions. Measured biomarkers were cross-linked N-teleopeptide of type I collagen (NTX) (Osteomark Ntx, Unipath Limited, Bedford, United Kingdom, 1:5 dilution), Tartrate-resistant acid phosphatase (TRAP) 5b (BoneTRAP® Assay SB-TR201A, Immunodiagnostic Systems (IDS) Ltd., Boldon, United Kingdom), Angiopoietin 2 (1:3 dilution) and Chemokine (C-X-C motif) ligand 13 (CXCL13) (both R&D systems, Minneapolis, MN, USA).

Statistical analysis

RA PhysioLab platform

RA PhysioLab version 3.2 was used for the simulations. The patient cohort consisted of 120 Virtual Patients (VPs) with different underlying pathophysiologies. Data for all VPs were analyzed after simulation of 1 year untreated disease. Erosiveness in each VP was determined by the volume of bone loss during the period of simulation. The bone loss values were categorized in quartiles and the lowest and highest quartile were compared. In total 150 simulation variables, including the concentrations of all mediators, were investigated for association to erosiveness. For the statistical analysis, Student's t, Wilcoxon rank sum and Kolmogorov-Smirnov from R version 2.4.1 were used.

Discovery cohort

To investigate whether the *in silico* model accurately predicted that erosiveness during the disease course was associated with baseline biomarker serum levels, the 4-years ES and SHS scores were categorized into quartiles. The lowest and highest quartiles were compared for differences in baseline serum level using the Mann-Whitney U test. In addition, in all RA patients the correlations between ES and SHS on a continuous scale and the serum levels were assessed using a non-parametric Spearman correlation test and a linear regression analyses on log transformed radiological data with adjustment for treatment strategy (randomization arm).

Confirmation cohort

The association between baseline CXCL13 serum levels and the rate of joint destruction during 7 years of follow-up was assessed using a repeated measurement analysis on log-transformed radiological data, correcting for age, gender and applied treatment strategy as previously described.[16] The repeated measurement analysis is performed using a multivariate normal regression model

that, on longitudinal data, evaluates the progression rates over time and takes into account the correlation between the measurements within one subject. In order to test whether biomarker levels were associated with joint destruction independent of inflammation, adjustments for C-reactive protein (CRP) were made as well. To test for associations between baseline CXCL13 levels and baseline clinical characteristics, analyses were performed using the non-parametric Spearman correlation test.

Analysis of sustained DMARD-free remission was performed by comparing Kaplan Meier curves and by Cox regression analysis, correcting for age and gender, taking into account the differences in follow-up times among patients. For patients who achieved remission, the dependent variable was "time-to-event", indicating the time until reaching remission. For non-remission patients the time to last follow-up was used.

SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) was used. P-values <0.05 were considered significant. All reported p-values are two-sided.

RESULTS

RA PhysioLab prediction results

After simulation of one year of untreated disease the virtual patient cohort was categorized on a numerical read-out representing erosiveness. For the identification of candidate biomarkers we focused on model variables related to proteins for reasons of biomarker detection feasibility. Other variables like those related to cell densities were not taken into account for the analyses. We identified a set of proteins of which the concentrations were significantly different between the lowest and highest erosiveness quartiles in each of three statistical tests (Student's t, Wilcoxon rank sum, Kolmogorov-Smirnov; p-value < 0.0001). Four of these mediators were selected for follow-up: Ang-2, NTX, Trap5b and CXCL13. Selection of these 4 mediators was based on pragmatic criteria: assay availability and presence (NTX, Trap5b) or absence (Ang-2, CXCL13) of supportive literature linking the protein to bone erosion. The ability of CXCL13 to differentiate between high and low erosive virtual patients is illustrated in Figure 1. In the RA PhysioLab platform, most mediators are tracked as synovial quantities. For the four mediators selected transport between synovium and serum is modeled only for NTX. For Ang-2, Trap5b and CXCL13 the difference in mediator concentration between the erosiveness quartiles relates to synovial tissue concentrations; for NTX to synovial tissue and serum concentration.

Identification and replication in two independent cohorts

Baseline characteristics

Baseline characteristics of the two sets of RA patients are presented in Table 1.

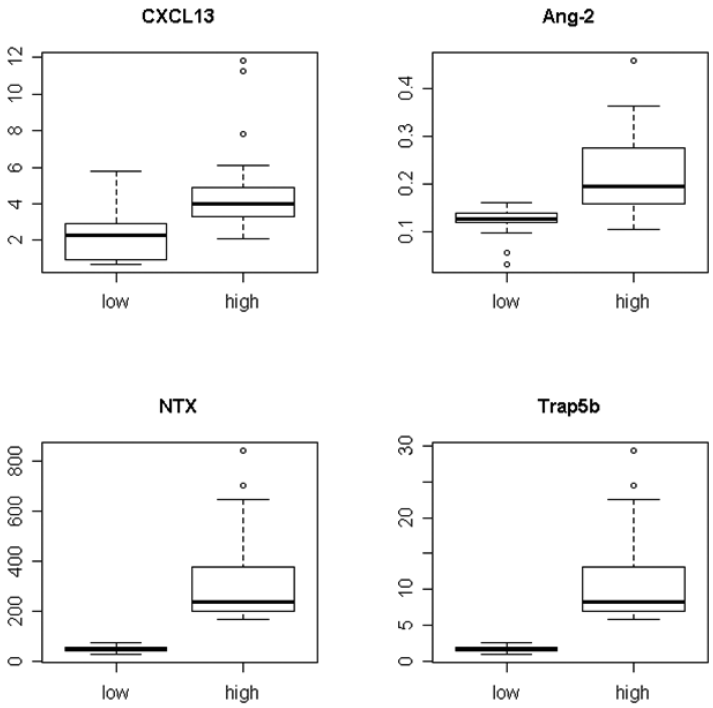

Figure 1. Boxplots of simulated synovial concentrations tested across 120 distinct Virtual Patients. For each mediator the synovial concentrations (ng/ml) are shown in the low and high erosive groups as defined in the text. The box indicates the lower and upper quartiles, the whiskers extend to the most extreme data point which is no more than 1.5 times the interquartile range from the box

Table 1. Patient characteristics at baseline

	Discovery cohort (n=74)	Replication cohort (n=155)	p-value
Age at inclusion (yrs), mean (SD)	53.5 (14.4)	56.4 (13.6)	0.232
Female, N (%)	45 (60.8)	108 (69.7)	0.184
SJC, mean (SD)	9.27 (3.89)	8.95 (6.78)	0.107
CRP (mg/l), mean (SD)	32.0 (40.0)	31.0 (36.5)	0.774
Anti-CCP2-positive, N (%)	42 (56.8)	89 (58.2)	0.840

CRP: C-reactive protein; SJC: 66-swollen joint count. The p-value reflects if both cohorts are significantly different for the indicated variable

Discovery cohort

To study the results on markers and erosiveness predicted by the *in silico* model for an association *in vivo*, baseline serum levels for four biomarkers predicted *in silico* were determined. The mean (SD) levels were 4658.4 (1596.0) pg/ml, 51.9 (24.5) nM BCE/l, 2.19 (1.14) U/l and 166.7 (86.0) pg/ml respectively for Ang-2, NTX, Trap5b and CXCL13.

To analyze whether these biomarkers accurately predicted that erosion scores and the total level of joint destruction during the disease course were associated with baseline serum levels, the lowest and highest quartiles of 4-years erosiveness were studied in relation to the markers' serum levels (Table 2). This revealed a significant difference for CXCL13 (p=0.011 for the total erosion score (ES) and p=0.018 for the total Sharp/van der Heijde score (SHS). Similarly, significant correlations between the CXCL13 levels and the ES (p=0.022, ρ=0.267) and the SHS (p=0.014, ρ=0.286) were observed when performing the analysis on continuous data of all RA patients. In addition, analyzing joint destruction data in all RA patients using linear regression analysis revealed that baseline CXCL13 levels remained significantly associated with the 4-year ES and SHS (β=1.002 (95%CI 1.000-1.005), p=0.049 and β=1.002 (95%CI 1.000-1.005), p=0.033 respectively) after adjustment for treatment strategy. For Ang-2, NTX and Trap5b, no significant associations were observed (Table 2). Taken together these data indicate that out of 4 serum markers predicted by the *in silico* model one marker was actually observed to associate with joint destruction in patients using the analysis workflow described.

Table 2. Baseline marker serum levels compared for low and high ES and SHS at 4 years of disease duration

		Discovery cohort (n=74)			
		Ang-2 (pg/ml), mean (SD)	NTX (BCE/l), mean (SD)	Trap5b (U/l), mean (SD)	CXCL13 (pg/ml), mean (SD)
4 year ES	1st quartile	4361.2 (1401.9)	48.47 (16.41)	2.45 (0.94)	137.7 (80.4)
	4th quartile	5286.8 (1611.8)	62.89 (40.66)	2.61 (1.41)	189.8 (66.1)
	p-value	0.121	0.548	0.717	0.011
4 year SHS	1st quartile	4444.0 (1606.7)	51.61 (15.64)	2.41 (0.99)	139.6 (80.6)
	4th quartile	5279.2 (1634.5)	55.83 (23.23)	2.08 (1.32)	186.9 (64.9)
	p-value	0.190	0.800	0.373	0.018

ES: Erosion score; SHS: Total Sharp-van der Heijde score. Note that the SHS is composed of erosion and joint space narrowing scores. Differences in levels between quartiles 1 (low) and 4 (high) were compared using the non-parametric Mann-Whitney U test

Confirmation cohort

For confirmation, baseline serum CXCL13 levels were measured in a second set of patients, yielding a mean concentration of 155.5 (98.9) pg/ml. Baseline serum levels were categorized as low or high based on quartile distribution and were studied in association with the rate of progression in joint destruction over 7 years. Using repeated measurement analysis, higher CXCL13 levels associated with significantly higher progression rates in ES (p<0.001) as well as in SHS (p<0.001; analyses performed on log-transformed data) (Figure 2). For the erosion score, the increase per year in the original scale was 1.12, 1.08 and 1.18 times greater for CXCL13 quartiles 2, 3 and 4, respectively, than for quartile 1. For the total SHS, a 1.11, 1.09 and 1.18 times greater increase per year was observed compared to the lowest quartile. Over a period of 7 years this resulted in

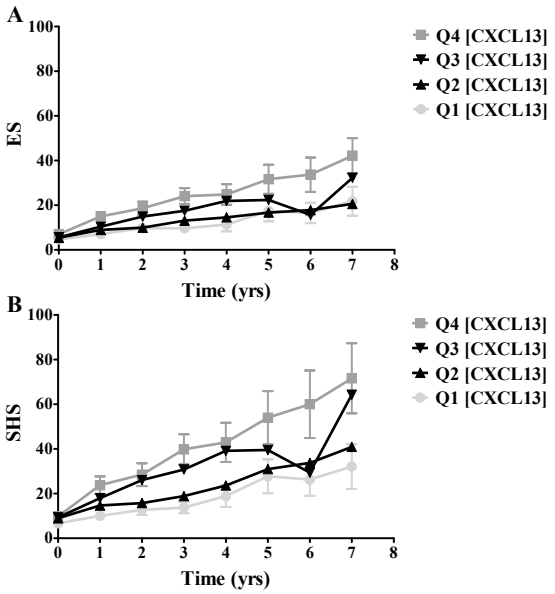

Figure 2. CXCL13 level and progression in radiographic joint damage in RA patients in the confirmation cohort. Erosion (panel A: ES) and Sharp/van der Heijde (panel B: SHS) scores (n=155). The number of patients and levels (mean (SD)) for CXCL13 were n=37, 59.2 (15.9) pg/ml, n=40, 107.6 (13.8) pg/ml, n=39, 158.9 (17.4) pg/ml and n=39, 292.3 (90.9) pg/ml for quartiles 1, 2, 3 and 4 respectively

2.2 (95%CI 1.5-3.3), 1.8 (95%CI 1.2-2.6) and 3.1 (95%CI 2.1-4.7) times larger progression rates for the ES and 2.1 (95%CI 1.4-3.3), 1.9 (95%CI 1.2-2.8) and 3.2 (95%CI 2.1-4.9) times larger progression rates for the SHS. Since categorical analysis generally results in less discriminative ability, the CXCL13 level was also included in the repeated measurement analysis as a continuous variable. Also here, higher CXCL13 levels associated significantly with higher progression rates of ES and SHS (both $p<0.001$). This analysis was also used to determine the variance explained by CXCL13. This showed that 7% of the total variance in progression in ES was explained by CXCL13.

Clinical associations of CXCL13

To study clinical factors that possibly influenced the observed association of CXCL13 with the rate of joint destruction in the confirmation cohort, baseline patient characteristics were analyzed in relation to baseline CXCL13 serum levels. Significant correlations were found for CXCL13 level and CRP level ($p<0.001$, $\rho=0.429$), ESR level ($p=<0.001$, $\rho=0.300$) and the number of swollen joints ($p=0.023$, $\rho=0.255$). In addition, mean CXCL13 levels were significantly higher in serum samples from anti-CCP2 positive patients than anti-CCP2 negative patients (172.0 (104.7) pg/ml vs.134.8 (87.3) pg/ml, $p=0.008$). Similar results were observed for IgM-RF, yielding levels of 172.9 (104.8) pg/ml vs. 120.2 (77.7) ($p<0.001$) for IgM-RF positive compared to IgM-RF negative patients respectively.

CXCL13 in relation to other serological markers

It was studied whether CXCL13 serum levels associated with ES and SHS independently of the known serological markers CRP and anti-CCP2. First CRP was entered as adjustment variable in the repeated measurement analysis. Also here CXCL13 was significantly associated with the ES (p=0.001) and the SHS (p=0.004). In addition, since anti-CCP2 positive and anti-CCP2 negative RA are considered to be separate subsets of the disease with differences in underlying pathogen mechanisms, the mentioned analyses were repeated in the anti-CCP2+ and anti-CCP2− subsets. In anti-CCP2 negative patients, high CXCL13 levels were also significantly associated with larger ES and SHS progression rates (p<0.001 and p=0.001 respectively). After adjustment for CRP level, the association remained significant for the ES (p=0.002), but significance was lost for the SHS (p=0.10). In anti-CCP2 positive patients no associations were observed for either the ES or SHS (data not shown).

Sustained DMARD-free remission

To further substantiate the CXCL13 findings we investigated a different outcome measure for RA severity and studied the effect of CXCL13 level on the achievement of DMARD-free remission. In addition to an observed association of CXCL13 levels and joint damage, comparison with the achievement of remission showed that higher CXCL13 levels were associated with significantly lower chances of achieving remission. Compared to the first quartile CXCL13, hazard ratios of 3.3 (95%CI 1.0-11.1) (p=0.049), 4.1 (95%CI 1.1-14.8) (p=0.034) and 6.3 (95%CI 1.4-29.2) (p=0.019) for not achieving remission were observed for the 2nd, 3rd and 4th quartile of CXCL13 respectively (Figure 3). With an additional adjustment for CRP, the hazard ratios were respectively 4.1 (95%CI 0.8-21.2), 2.6 (95%CI 0.7-10.2) and 2.6 (95%CI 0.7-9.0). Because only 3 patients achieved remission in the anti-CCP2 positive RA group, no stratified analysis was performed here.

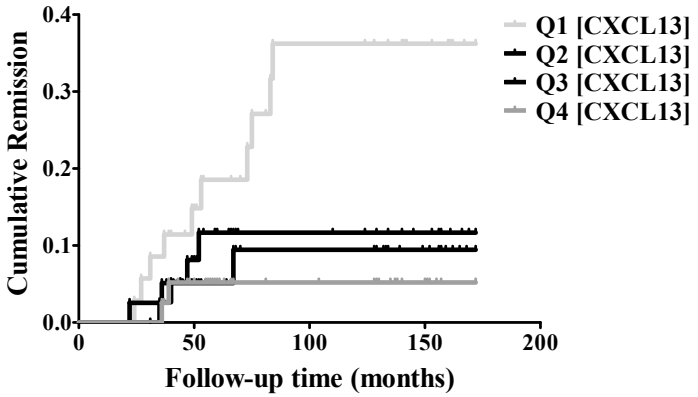

Figure 3. CXCL13 level and cumulative DMARD-free remission in RA patients in the replication cohort. The number of patients and levels (mean (SD)) for CXCL13 were n=35, 60.0 (15.9) pg/ml, n=39, 107.2 (13.7) pg/ml, n=39, 158.9 (17.4) pg/ml and n=39, 292.3 (90.9) pg/ml for quartiles 1, 2, 3 and 4 respectively. In total 19 patients achieved DMARD-free remission

DISCUSSION

The heterogeneous nature of RA severity is presently incompletely understood and hampers accurate disease prognoses on the individual patient level. This underlines the need for new disease markers with improved prognostic potential of marker sets in order to guide adequate treatment regimes. Also, exploration of new markers will enhance our understanding of involved pathophysiological processes. Exploration of suitable new markers was initiated by candidate marker prediction using the RA PhysioLab computer model of the rheumatic joint. The value of PhysioLab simulation approaches has been shown previously in RA[11] as well as in other diseases.[18-20] The PhysioLab simulation platform allows to perform question based simulation experiments from which the results contribute to hypotheses driven, focused follow-up experimental research. For this approach it is assumed that the scope of the computer model resembles real disease and patient population behavior as well as possible. In this study we identified a new biomarker, CXCL13, which showed an association between baseline serum level and the level of joint destruction at 4 years of disease, thereby validating the association predicted by the *in silico* model. Moreover, in an independent cohort relatively high serum CXCL13 levels were also associated with an enhanced progression of the rate of joint destruction over 7 years and a decreased chance of achieving DMARD-free remission, thereby confirming the association between CXCL13 and disease severity.

The cytokine CXCL13 is also known as B lymphocyte chemoattractant (BLC) or B cell-attracting chemokine 1 (BCA-1) and is part of the CXC chemokine family. One of the main effects of CXCL13 implemented in the RA PhysioLab platform is on B cell recruitment, thus supporting a mechanism directly dependent on B cells. CXCL13 serum levels were found to be significantly higher in the serum of RA patients as compared to healthy controls.[21,22] CXCL13 serum levels were also reported to respond to therapeutic intervention with anti-TNFα therapy.[12] Evidence for joint localization of CXCL13 was found, both by the detection of mRNA in inflamed synovial tissue[23] as well as the presence of ectopic lymphoid follicles expressing CXCL13 in the synovium of chronic RA patients.[24] CXCL13 has been reported to attract B lymphocytes and to interact with the receptor CXCR5, which is expressed by B cells as well as follicular B helper T cells.[25-28] High levels of CXCR5 were also found on human osteoblasts and activation by its ligand CXCL13 induced the release of extracellular matrix degrading enzymes. As such, CXCL13 may play an important role in the process of bone remodeling.[29] These data suggest that CXCL13 may have an effect on joint damage in RA that is both dependent and independent of promoting effects on B cells.

Since CXCL13 attracts B cells and CXCL13 levels are reported to be higher in autoantibody positive RA, which was also observed in the present study, and since anti-CCP2 positive and negative RA are subsets of RA with possible differences in the pathogenesis, the effect of CXCL13 was evaluated for anti-CCP2 positive and negative RA separately. In the anti-CCP2 negative group CXCL13 associated significantly with the progression of joint destruction. Adjustment

for baseline CRP levels did not alter this association for the ES, supportive of a CXCL13 effect on joint destruction that is independent of the level of inflammation as expressed by the level of CRP. In anti-CCP2 positive RA, CXCL13 was not independently associated with progression in joint damage. Thus, from a clinical perspective, information on baseline CXCL13 levels seems most valuable in the anti-CCP2-negative sub-population of RA patients.

Analysis of the lowest and highest quartiles of erosiveness in the discovery cohort did not reveal a significant difference for Ang-2. However, when comparing the upper two quartiles with the lower two quartiles, a significant difference was found for Ang-2 (data not shown). This indicates that a second candidate marker from the four markers tested might be of interest for further exploration. In the RA-PhysioLab platform Ang-2 and CXCL13 both have an effect on endothelial lifecycle, which affects all recruitment processes.

To the best of our knowledge this is the first time that a new serum marker for RA was confirmed that was initially predicted by a computer model simulation of the rheumatic joint. This finding illustrates that the present computer model has predictive potential and may be applied to other disease outcomes.

Although the results presented in this manuscript provide a solid foundation for our conclusions, this study also has limitations. Both the discovery and confirmation cohorts consisted of a limited number of patients, which may result in limited power to accurately reach proper conclusions. As a result only relatively large differences in effect sizes may be detected and smaller effects could be missed. A strong association between CXCL13 levels and radiologic progression was observed in two separate cohorts. For Ang-2 the evidence is less conclusive than for CXCL13. Failure to detect an *in vivo* association for the other candidate biomarkers that were tested (NTX, Trap5b) despite their known association to bone biology could indicate that their behavior in a heterogeneous clinical population is not predictive.

Recently, draft validation criteria for a soluble biomarker to be regarded as a valid biomarker reflecting structural damage in RA have been established.[9,30,31] These criteria provide guidance to the types of studies needed to demonstrate the value of CXCL13 as a marker in clinical practice. Our present data reveal that it is a serological marker with a potent CRP-independent predictive value for long-term outcome in RA, thereby providing a rationale for further exploration.

In conclusion, the RA PhysioLab simulation platform has helped in the identification of CXCL13 as a new serological marker for severity of RA as measured by the long-term joint destruction and the achievement of DMARD-free remission in two independent cohorts of RA patients.

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