EXTENDED REPORT

Rheumatoid factor and antibodies to cyclic citrullinated peptides are associated with severe extra-articular manifestations in rheumatoid arthritis

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Objective: To study antibodies to cyclic citrullinated peptides (anti-CCP) and rheumatoid factor in patients with active, severe extra-articular rheumatoid arthritis (ExRA) compared with controls without ExRA.

Methods: 35 consecutive patients with severe ExRA manifestations according to predefined criteria were studied. 70 patients with rheumatoid arthritis, but no ExRA manifestations, individually matched for age, sex and disease duration, served as controls. Patients were included when ExRA was diagnosed, before any new treatment was started. Anti-CCPs were detected with ELISA, rheumatoid factor was quantified using nephelometry and anti-nuclear antibodies (ANA) were investigated using indirect immune fluorescence.

Results: Anti-CCPs were detected in 77% of patients with ExRA versus 56% of controls without ExRA ($p=0.03$). Anti-CCP levels also tended to be higher in patients with ExRA ($p=0.09$). Rheumatoid factor was detected in 94% v 71% of patients and controls, respectively ($p=0.006$), and rheumatoid factor levels were higher in patients with ExRA (median interquartile range (IQR) 245 IU/ml (94–604) v 73 IU/ml (not detected–165); $p=0.001$). Levels and occurrence of ANA did not differ between patients with ExRA and controls. Patients with ExRA had higher swollen joint counts and C reactive protein levels, but no correlations were found between anti-CCP or rheumatoid factor levels and these measures within the ExRA group.

Conclusion: Rheumatoid factor is strongly associated with severe ExRA manifestations in patients with rheumatoid arthritis, and a similar but weaker association exists for anti-CCPs. This suggests a role for rheumatoid factor and anti-CCP in the pathogenesis of ExRA.

Rheumatoid arthritis is a systemic inflammatory disease, which in a substantial proportion of patients is associated with the development of extra-articular manifestations in addition to the characteristic joint involvement. These extra-articular rheumatoid arthritis (ExRA) manifestations can have a defining effect on disease outcome, including increased premature mortality compared with rheumatoid arthritis in general.^{1–4} Severe ExRA occurs both in patients recently diagnosed with rheumatoid arthritis, as well as in those with longstanding disease.² Suggested predictors of ExRA include clinical, serological and genetic factors.⁵ Patients who develop ExRA more often have had a positive test for rheumatoid factor⁶ or antinuclear antibodies (ANA).⁷ Particularly high levels of rheumatoid factor have been reported in patients with rheumatoid vasculitis,^{8–9} and circulating immune complexes have been implicated in the pathogenesis of vasculitis¹⁰ and other ExRA manifestations.¹¹

Antibodies to cyclic citrullinated peptides (anti-CCP) have been shown to be highly specific for rheumatoid arthritis.¹² Studies of early arthritis cohorts have shown that anti-CCPs are stronger predictors of disease persistence and structural joint damage,^{13–15} even stronger than rheumatoid factor.^{15–16} However, the role for anti-CCPs in ExRA has not been extensively evaluated. Antibodies to both citrullinated substrates^{17–18} and rheumatoid factor^{18–19} can be detected at an early stage, even before the clinical onset of arthritis. Rheumatoid factor and anti-CCP are strongly associated with each other in patients with rheumatoid arthritis,¹² although their relative importance in ExRA is not known. An improved understanding of the disease mechanisms involved in systemic aspects of rheumatoid arthritis, including the role of citrullinated peptides, may be useful in developing new treatment strategies. The objective of this study was to investigate anti-CCPs,

rheumatoid factor and ANA in patients with recently diagnosed, active ExRA.

PATIENTS AND METHODS

Patients

Patients with rheumatoid arthritis seen at the rheumatology outpatient clinics in Malmö were included in a prospective study of ExRA. Consecutive patients with recently diagnosed, severe extra-articular disease manifestations according to predefined criteria^{3–5} were enrolled. The extra-articular manifestations studied were clinically diagnosed pericarditis, pleuritis, Felty's syndrome, vasculitis-related polyneuropathy or mononeuropathy, scleritis, episcleritis, glomerulonephritis, major cutaneous vasculitis and vasculitis involving other organs, each supported by objective findings, and with other causes unlikely or excluded.^{3–5} Vasculitis-related polyneuropathy or mononeuropathy was diagnosed clinically and supported by electroneurography, with other potential causes excluded or unlikely. Major cutaneous vasculitis was defined as biopsy-proven necrotising or leucocytoclastic vasculitis, or vasculitis clinically diagnosed by a dermatologist due to findings of leg ulcers, purpura or peripheral gangrene. For each patient with ExRA, two controls without ExRA, individually matched with extra-articular patients for age, sex and disease duration, were selected from a community-based register of patients with rheumatoid arthritis in Malmö, Sweden,²⁰ or from a community-based early rheumatoid arthritis inception cohort.²¹ These controls had no current or previous evidence of severe

Abbreviations: ANA, anti-nuclear antibodies; anti-CCP, antibodies to cyclic citrullinated peptides; CCP, cyclic citrullinated peptides; CRP, C reactive protein; ExRA, extra-articular rheumatoid arthritis; HAQ, Health Assessment Questionnaire; IQR, interquartile range

extra-articular disease³⁻⁵ or rheumatoid nodules. All patients fulfilled the 1987 American College of Rheumatology criteria for rheumatoid arthritis.²² At inclusion, all patients were seen by the same doctor (CT), and examined according to a structured protocol. The Swedish validated version of the Stanford Health Assessment Questionnaire (HAQ)²³ was used to estimate the extent of disability. Blood was drawn, and physical examinations were carried out directly after ExRA was diagnosed and before any new treatment was started; serum samples were stored at -70°C until investigated. Data on previous occurrence of rheumatoid factor and ANA were collected from patient records. The study was approved by the research ethics committee at Lund University, Lund, Sweden, and all patients gave informed consent for the study.

Laboratory investigations

Anti-CCPs were detected with ELISA (Eurodiagnostica, Malmö, Sweden; second-generation test). Positive anti-CCP was defined as a serum concentration >50 U/ml. Sera with anti-CCP levels above the calibration curve were rerun after dilution to obtain actual quantitative values for all samples. Anti-CCP was detected in 1 of 99 controls by this technique at 1643 U/ml. Levels in two other controls were considered negative, but with detectable anti-CCPs of 30 and 42 U/ml, respectively.

Rheumatoid factor was detected and quantified using nephelometry (Beckman Image, Beckman Coulter, Fullerton, California, USA), which measures immunoglobulin (Ig)M rheumatoid factor.²⁴⁻²⁵ The analysis was standardised using the international standard National Institute for Biological Standards Control 64/002, and the cut-off was set to 20 IU/ml. Using this cut-off, 2 of 100 healthy control sera were rheumatoid factor positive, with rheumatoid factor levels of 20.4 and 21.6 IU/ml, respectively.

ANA were determined using indirect immunofluorescence on HEp-2 cells (Bio-Rad, Stockholm, Sweden) in a screening dilution of 1:200, with a secondary antibody to the γ chain of IgG (Dako A/S, Glostrup, Denmark). Using this procedure, ANA were detected in 5 of 100 (5%) control sera. ANA fluorescence patterns were determined as previously described.²⁶

Stored sera from all patients with ExRA and rheumatoid arthritis controls were investigated at a single time point for anti-CCP, rheumatoid factor and ANA. All ANA results were interpreted by the same investigator (JR).

Statistical analysis

The χ^2 test was used to study associations between ExRA, rheumatoid factor, anti-CCP and ANA. Swollen joint counts, Ritchie's index and HAQ scores were compared in patients with ExRA versus controls with rheumatoid arthritis using Student's *t* test. As levels of C reactive protein (CRP), rheumatoid factor and anti-CCP levels had non-normal distributions, the Mann-Whitney *U* test was used for comparisons between patients with ExRA and controls with rheumatoid arthritis, as well as for comparisons between patients with individual ExRA manifestations and controls. Spearman's rank correlation test was used to study correlations between anti-CCP or rheumatoid factor levels and clinical parameters in patients with ExRA and controls with rheumatoid arthritis separately.

RESULTS

In all, 35 patients with recently diagnosed ExRA were compared with 70 control patients without ExRA and with rheumatoid arthritis. The patients with ExRA fulfilled predefined criteria³⁻⁵ for one or more of the following manifestations: pericarditis ($n = 10$), pleuritis ($n = 10$), major cutaneous vasculitis ($n = 9$), Felty's syndrome ($n = 5$) or neuropathy ($n = 4$). Patients and controls were well matched for age, sex and duration of rheumatoid arthritis (table 1). Patients with ExRA had higher swollen joint counts ($p = 0.002$), CRP levels ($p < 0.001$) and HAQ disability scores ($p = 0.01$; table 1). Compared with controls with rheumatoid arthritis, fewer patients with ExRA were currently treated with disease-modifying anti-rheumatic drugs (table 1). Anti-CCPs were detected in 77% of patients with ExRA versus 56% of controls without ExRA ($p = 0.03$). Rheumatoid factor was present in 94% of patients with ExRA versus 71% of controls with rheumatoid arthritis ($p = 0.006$). ANA occurred at similar frequencies among patients with and without ExRA (table 2). The most frequent ANA pattern was homogeneous, occurring in 28 of 39 patients with positive ANA, without significant differences between patients with ExRA and controls. Compared with patients with rheumatoid arthritis, patients with ExRA were more likely to have been rheumatoid factor positive ($p < 0.001$) or ANA positive ($p = 0.08$) at any time before inclusion (table 2). Data on previous ANA tests were missing for three patients with ExRA and four controls with rheumatoid arthritis, and there was no previous rheumatoid factor test for one control with rheumatoid arthritis. A strong

Table 1 Baseline characteristics for patients with extra-articular rheumatoid arthritis versus controls without extra-articular rheumatoid arthritis

	ExRA	Non-ExRA	p Value
n	35	70	
Sex			
Male	15	30	
Female	20	40	
Age (years; mean (SD))	65.5 (11.9)	65.5 (11.6)	
Duration (years; median (IQR))	9.6 (2.3–19.4)	8.6 (3.4–18.2)	
Swollen joints (mean (SD))	6.5 (5.7)	3.0 (3.4)	0.002
Ritchie index (mean (SD))	9.5 (7.7)	8.2 (7.6)	0.41
HAQ* (mean (SD))	1.19 (0.88)	0.75 (0.74)	0.01
CRP† (mg/l; median (IQR))	39.5 (11.5–54.5)	<9 (<9 –15)	<0.001
DMARDs, n (%)	18 (51)	57 (81)	0.001
Methotrexate, n (%)	8 (23)	27 (39)	0.11
TNF-inhibitors,‡ n (%)	1 (3)	7 (10)	0.19
Glucocorticosteroids, n (%)	13 (37)	19 (27)	0.29

CRP, C reactive protein; DMARDs, disease-modifying antirheumatic drugs; ExRA, extra-articular rheumatoid arthritis; HAQ, Health Assessment Questionnaire; IQR, interquartile range; TNF, tumour necrosis factor.

*Data available for 29 patients and 70 controls.

†Data available for 32 patients and 68 controls.

‡Infliximab, etanercept or adalimumab.

Table 2 Autoantibodies in patients with extra-articular rheumatoid arthritis versus controls without extra-articular rheumatoid arthritis

	ExRA	Non-ExRA	p Value
Anti-CCP positive (>50 U/ml)	27/35 (77%)	39/70 (56%)	0.03
RF positive (>20 U/ml)	33/35 (94%)	49/69 (71%)	0.006
ANA positive	13/35 (37%)	26/68 (38%)	0.91
Previously RF positive (ever)	30/35 (86%)	33/69 (52%)	<0.001
Previously ANA positive (ever)	17/32 (53%)	23/66 (35%)	0.08

ANA, antinuclear antibodies; CCP, cyclic citrullinated peptides; ExRA, extra-articular rheumatoid arthritis; RF, rheumatoid factor.

association was observed between anti-CCP and rheumatoid factor, with all anti-CCP-positive patients ($n = 66$) being rheumatoid factor positive, and 22 of 38 (58%) anti-CCP negative patients being rheumatoid factor negative ($p < 0.001$). Only 2 of 35 (6%) patients with ExRA were negative for both rheumatoid factor and anti-CCP, compared with 20 of 69 (29%) controls with rheumatoid arthritis ($p = 0.005$). All patients with vasculitis ($n = 9$) or Felty's syndrome ($n = 5$) were positive for both rheumatoid factor and anti-CCP (data not shown).

Current rheumatoid factor levels were significantly higher in patients with ExRA compared with those without ExRA (median (IQR) 245 IU/ml (94–604) ν 73 IU/ml (not detected–165); $p = 0.001$; fig 1). Compared with patients with rheumatoid arthritis and without ExRA manifestations, patients with major cutaneous vasculitis ($p = 0.002$ ν controls), Felty's syndrome ($p = 0.03$ ν controls) and pericarditis ($p = 0.02$ ν controls) had higher rheumatoid factor levels (table 3).

We found a trend towards higher anti-CCP levels in patients with ExRA (median 427 U/ml; IQR 76–1516) than in controls without ExRA (median 169 U/ml; IQR not detected–1133; $p = 0.09$; fig 2). Patients with Felty's syndrome ($p = 0.005$ ν controls) and major cutaneous vasculitis ($p = 0.02$ ν controls) had higher anti-CCP levels than those without ExRA (table 3). Anti-CCP levels did not differ significantly between patients with pericarditis, pleuritis or neuropathy on the one hand and controls with rheumatoid arthritis on the other, and rheumatoid factor levels were not significantly different from controls with rheumatoid arthritis for patients with neuropathy or pleuritis. However, these negative findings should be interpreted with caution because of the limited sample size.

Figure 1 Rheumatoid factor (RF) levels were higher in patients with extra-articular rheumatoid arthritis (ExRA) compared with controls with rheumatoid arthritis (RA) without extra-articular manifestations. Lines indicate medians.

Rheumatoid factor levels correlated with CRP ($r = 0.52$, $p < 0.001$) among controls with rheumatoid arthritis, but not among patients with ExRA ($r = -0.22$, $p = 0.22$). Higher rheumatoid factor levels, on the other hand, tended to be associated with higher HAQ scores among patients with ExRA ($r = 0.29$, $p = 0.12$) and controls with rheumatoid arthritis ($r = 0.28$, $p = 0.02$). No significant correlation was seen between rheumatoid factor levels and number of swollen joints or Ritchie's index among patients with ExRA or controls with rheumatoid arthritis. Similarly, CRP correlated positively with anti-CCP levels in controls with rheumatoid arthritis ($r = 0.36$; $p = 0.003$), but negatively ($r = -0.39$; $p = 0.03$) among patients with ExRA. No correlations were seen between anti-CCP levels and HAQ score, swollen joints or Ritchie's index.

DISCUSSION

In this case-control study, rheumatoid factor and anti-CCP were associated with current ExRA manifestations. Rheumatoid factor levels, in particular, were significantly higher in patients with ExRA compared with controls with rheumatoid arthritis and without extra-articular manifestations, with very high rheumatoid factor levels in patients with vasculitis, Felty's syndrome and pericarditis. Anti-CCP and rheumatoid factor were strongly associated, and few patients with ExRA were negative for both tests. This is in accordance with other reports of the presence of anti-CCP and rheumatoid factor as indicators of disease severity in rheumatoid arthritis, and suggests a particular role for these autoantibodies in the pathogenesis of ExRA. No association was found between current ExRA and the presence of ANA.

Interestingly, all the investigated patients with Felty's syndrome had high levels of anti-CCP. Felty's syndrome was shown to be strongly associated with the *HLA-DRB1*0401* allele,^{27–28} which is also a marker of severe rheumatoid arthritis in general.²⁹ *HLA-DRB1*0401* is suggested to have a particular importance for T cell-mediated immune responses to citrullinated peptides.³⁰ Taken together, this indicates that rheumatoid arthritis-specific activation of T and B cells by citrullinated peptides may be important in Felty's syndrome and other systemic manifestations of rheumatoid arthritis. Patients with vasculitis also had significantly higher levels of anti-CCP compared with controls with rheumatoid arthritis.

Suggested disease mechanisms in severe ExRA include systemic endothelial activation,³¹ and expansion of abnormal T cells with an immunosenescent phenotype and natural killer cell-like capabilities.^{32–33} Rheumatoid factor and anti-CCP may be markers of other abnormalities of the immune system, or as previously suggested for rheumatoid factor,^{10–14} involved in the formation of pathogenic immune complexes in severe ExRA. It should be noted that whereas nephelometry detects rheumatoid factor measures, mainly IgM rheumatoid factor, the anti-CCP ELISA detects only IgG. Studies on other isotypes for both anti-CCP and rheumatoid factor in patients with rheumatoid arthritis may be of interest, particularly as previous studies

Table 3 Rheumatoid factor and anti-cyclic citrullinated peptides levels in patients with extra-articular rheumatoid arthritis, individual extra-articular rheumatoid arthritis manifestations and controls with rheumatoid arthritis

	n	RF (IU/ml) Median	IQR	Anti-CCP (U/ml) Median	IQR
ExRA	35	245	94–604	427	76–1516
Major cutaneous vasculitis	9	413	150–1450	1171	458–1420
Felty's syndrome	5	299	117–1043	8087	1999–14686
Neuropathy	4	158	43–1026	663	129–1087
Pericarditis	10	275	83–359	339	64–1769
Pleuritis	10	155	35–494	87	ND–271
Controls with RA	70	73	ND–165	169	ND–1133

CCP, cyclic citrullinated peptides; ExRA, extra-articular rheumatoid arthritis; IQR, interquartile range; ND, not detected; RA, rheumatoid arthritis; RF, rheumatoid factor.

suggested an association between IgA rheumatoid factor and ExRA.³⁵ Furthermore, the role of complement activation in this context deserves additional attention.

Our results differ from those of de Rycke *et al*,³⁶ who found no association between anti-CCP and ExRA manifestations. The main explanation for this is probably differences in patient selection. Whereas de Rycke *et al* included patients with rheumatoid nodules ($n = 33$) or vasculitis ($n = 7$) at any time during their disease course, we focused on patients with current, severe ExRA manifestations. Further studies are necessary to evaluate variations over time in anti-CCP and rheumatoid factor levels in patients with severe ExRA.

Overall, our data imply that high rheumatoid factor levels may be more important than anti-CCP in active ExRA. Rheumatoid factor levels have been reported to be suppressed by successful treatment with methotrexate³⁷ and infliximab,³⁸ whereas anti-CCP levels may be more stable and unrelated to changes in acute-phase reactants during treatment,³⁹ although others have reported a parallel decrease in rheumatoid factor and anti-CCP after anti-tumour necrosis factor treatment.^{38–40, 41}

Rheumatoid factor is a predictor of poor survival in the general population⁴² and in patients with rheumatoid arthritis.^{43–45} On the other hand, a positive anti-CCP test was not predictive of mortality in a recent study on a large group of patients with rheumatoid arthritis from Tampere, Finland, by Sihvonen *et al*.⁴⁵ However, anti-CCP levels (>174 U/ml) were associated with a borderline increased risk of death during the follow-up period.⁴⁵

Figure 2 Antibodies to cyclic citrullinated peptides (anti-CCP) levels tended to be higher in patients with extra-articular rheumatoid arthritis (ExRA) compared with controls with rheumatoid arthritis (RA) without extra-articular manifestations. Horizontal lines represent median values.

We have recently shown that serum rheumatoid factor levels, but not anti-CCP, correlate with levels of synovial fluid-derived immune complexes and immune complex-induced tumour necrosis factor α levels in patients with established rheumatoid arthritis.⁴⁶ This suggests that rheumatoid factor might evolve as a response to immune complexes,^{47, 48} and enhance the precipitation of immune complexes in rheumatoid arthritis,^{49, 50} but that anti-CCP might be linked to the pathogenesis of rheumatoid arthritis by other mechanisms.

As there were major differences in disease activity measures between patients with ExRA and controls with rheumatoid arthritis, the differences in rheumatoid factor and anti-CCP levels may to some extent reflect disease activity. Further studies comparing patients with ExRA and controls with rheumatoid arthritis and active disease may be needed to deal with this issue. Rheumatoid factor has been shown to reflect disease activity in rheumatoid arthritis.⁵¹ In this study, rheumatoid factor correlated with CRP in patients with non-ExRA, but not in patients with ExRA. No association was found between rheumatoid factor or anti-CCP levels and disease activity measures in patients with ExRA. Overall, rheumatoid factor and anti-CCP may be more strongly associated with long-term outcomes such as structural joint damage, disability and systemic involvement, rather than with cross-sectional measures of joint disease.⁵²

The strengths of this study include the prospective design, with inclusion of patients with active, untreated ExRA, evaluated at a single centre by the same observer. The limitations are mainly related to the sample size. Although this is one of the larger studies on severe ExRA published, the number of patients in each category of ExRA manifestations is limited. This restricts the power to detect important differences between various types of systemic involvement. Also, our patients were mainly Caucasians of north European origin, and it is unknown to what extent these results can be extrapolated to other ethnic groups.

In conclusion, we have shown that anti-CCP and rheumatoid factor are associated with ExRA manifestations, and that in particular high levels of rheumatoid factor are often found in severe ExRA. This suggests a role for these antibodies in severe ExRA. Whereas other recent studies have suggested that the prognostic importance of anti-CCP is at least equal to that of rheumatoid factor,^{15, 16} our data imply a primary association between ExRA and rheumatoid factor. Several studies have tied the occurrence of rheumatoid factor to immune complexes in rheumatoid arthritis,^{10, 32} chronic infection^{53, 54} and in healthy people,⁴⁸ but no studies have been published on the association between anti-CCP or other citrulline-associated antibodies on the one hand and immune complexes on the other. Further studies should examine the relative role of different auto-antibodies and circulating immune complexes in the pathogenesis of systemic manifestations in rheumatoid arthritis.

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