Longitudinal analysis of anti-citrullinated protein/peptide antibodies (anti-CP) during 5 year follow-up in early rheumatoid arthritis: anti-CP status is a stable phenotype that predicts worse disease activity and greater radiological progression

Extended report

Johan Rönnelid1,2, Marius C. Wick1,3, Jon Lampa3, Staffan Lindblad3, Birgitta Nordmark3, Lars Klareskog3, and Ronald F. van Vollenhoven3

1 Both authors contributed equally to this work

2 Unit of Clinical Immunology, Department of Oncology, Radiology and Clinical Immunology, Uppsala University/Akademiska Hospital, Uppsala, Sweden

3 Rheumatology Unit, Department of Medicine, Karolinska Institutet/Karolinska University Hospital, Stockholm, Sweden

Address correspondence to: Johan Rönnelid, MD PhD
Unit of Clinical Immunology
Rudbeck Laboratory C5
SE-75185 Uppsala
Sweden
Phone +46 (0) 18 6114182
Email: johan.ronnelid@klinimm.uu.se

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Abstract:
Objective: To study serum levels of anti-CP antibodies during up to 5 years’ follow-up of patients with early RA, and to relate serum levels to disease course and to therapies in clinical practice.

Methods: 279 early RA patients were followed with clinical investigations, radiographs and measurement of anti-CP antibodies at baseline and after three months, 1, 2, 3 and 5 years.

Results: 57.3% (160/279) of the patients were anti-CP positive at first visit (mean five months after first symptoms). During follow-up only 3.9% (11/279) of the patients changed their anti-CP status. Anti-CP levels fell significantly during the first year, and this drop correlated with the extent of sulphasalazine therapy but not with other drugs or clinical indices. Anti-CP positive and negative patients had similar disease activities at baseline, but during follow-up the anti-CP positive patients had worse clinical disease and greater radiological progression, despite at least equally intensive anti-rheumatic therapy.

Conclusions: Anti-CP antibody status is a stable phenotype during the first 5 years of RA, suggesting that events before rather than after onset of clinical manifestations of disease determine this phenotype. The presence of anti-CP antibodies at diagnosis predicts a less favorable disease course and greater radiological progression despite anti-rheumatic therapy, but subsequent changes in antibody levels do not reflect changes in disease activity. Taken together, these observations suggest that anti-CP positive RA is a distinct clinical and pathophysiological entity.
Introduction:

Rheumatoid arthritis (RA) as defined by classification criteria [1] probably represents various clinical phenotypes and pathophysiological entities, but identification of relevant subsets of RA has been limited to date. Rheumatoid factor (RF)-positivity has been shown to predict worse disease outcome [2, 3]. However, RF phenotyping is marred by two important limitations. Patients with other diseases can be RF positive, yielding a limited specificity for RA, and RF is not a stable phenotype, as patients who are initially RF-negative may become RF-positive with time.

The demonstration that, compared to RF, antibodies against citrullinated proteins or peptides (e.g. antibodies against cyclic citrullinated peptide [anti-CCP] antibodies) are more specific and similarly sensitive markers for RA [4, 5] has made anti-CP antibody status an obvious sub-phenotype in RA. This view is strengthened by the demonstration that anti-CP antibodies may occur years before clinical RA, and that the occurrence of anti-CP antibodies in individuals who later develop RA is most common close to onset of disease [6-9]. These findings have thus raised the question whether anti-CP immunity is causally related to the onset of clinical manifestations of disease, or whether it represents an epiphenomenon.

Most studies to date have focused on the initial qualitative anti-CP antibody status at study inclusion, and compared this status to clinical conditions at one or a few subsequent time points. In the present study we wanted to employ a quantitative and longitudinal approach with parallel investigations of antibody levels and clinical characteristics, including radiological data on multiple occasions. We particularly wanted to investigate whether the anti-CP phenotype was stable or fluctuated with
time and whether it predicted clinical and radiological disease course and response to pharmacological treatment.
Patients and methods:

Patients:

279 RA patients from a prospective cohort of early (< 12 months of disease duration) arthritis patients at Karolinska University Hospital were included between January 1995 and October 2000. All patients fulfilled the 1987 American College of Rheumatology classification criteria for RA [1]. Clinical evaluation included patients' global assessment of disease activity and pain scores on visual analogue scales (VAS), functional disability using the Swedish version of the Stanford Health Assessment Questionnaire (HAQ) [10], assessment of number of swollen and tender joints and of disease activity, C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), current medication including steroids, non-steroidal anti-inflammatory drugs and disease modifying anti-rheumatic drugs (DMARD). From the data obtained the disease activity score (DAS28) [11] was calculated. Clinical evaluation and serum sampling was performed at baseline, after three months, one, two, three and five years. For the calculation of cumulative DAS values and months-of-therapy with different DMARDs during the first two years, additional data were collected from 6, 9, 15, 18 and 21 months of follow-up. All participants had given informed consent and the study was ethically approved by the Northern Stockholm Ethical Committee.

Clinical data were available from all patients at inclusion, 97.1% at three months, 87.5% at one year, 86.4% at two years, 83.2% at three years and 54.1% at five years.
Antibodies to citrulline-containing peptides and rheumatoid factor (RF):

Anti-CCP antibodies were analyzed using the Immunoscan-RA Mark2 ELISA test (Eurodiagnostica, Malmö, Sweden). All samples yielding high values were further diluted to obtain definite values. A level above 25U/mL was considered as positive, yielding a specificity of 97% when 99 healthy individuals were analyzed. Of the initial 279 patients, sera were investigated at baseline, after three months and one year for 202 patients. The remaining 77 patients’ sera were also investigated at two, three and five years after inclusion.

RF was performed with nephelometry and recorded at the first visit as a qualitative (positive/negative) variable.

Radiographs:

A modification of the Larsen score that proved to be advantageous for long-term follow-up assessment of RA [12] was utilized. Identical radiographs of the hands and feet were scored blinded to treatment, in pairs (hands and feet), and in chronological sequence applying the Larsen method [13]. The scoring procedure was performed by an experienced investigator (MCW) and documented using the “X-Ray RheumaCoach”-software [14]. In each case, thirty-two joints were scored. The aggregate Larsen score was modified slightly by excluding grade 1, so that the scale became 0-4 [12]. Thus, the maximum possible score was 160. ∆Larsen score was calculated by subtracting baseline Larsen score values from the two year scores.
Statistics:
Comparisons between groups were performed using the Mann-Whitney’s U test for levels, or with the Chi square test for differences between proportions. The Wilcoxon signed rank test was used to investigate changes in individual patients between different time points. Spearman’s correlation coefficient test was used to analyze the correlation between levels of anti-CP and continuous clinical indices, or between changes in anti-CP levels and changes in the clinical indices detailed in table 1. In the latter analyses, differences and ratios in anti-CP between all time points for antibody analyses and baseline were compared to differences between various time points and baseline for the clinical variables listed in the patients’ section, resulting in 250 comparisons. ANOVA was used to dissect the relative prognostic importance of anti-CP status and RF status, where differences in clinical characteristics as compared to baseline were used as dependent variables. The Kruskal Wallis test was utilized to investigate the impact of medication on changes in anti-CP during the first year of investigation. No correction for mass significance has been performed except when detailed in the text. P-values <0.05 were considered significant.
Results:

Qualitative and quantitative anti-CP status at baseline

At inclusion, 57.3% (160/279) of the investigated patients had anti-CP antibodies >25 U/mL. The median anti-CP level among positive patients was 576kU/L (range 27-16995) and mean value was 1128kU/L (SD 2186).

Qualitative changes in anti-CP antibody status with time were rare. Only 3.9% (11/279) of the patients had an altered status (3 initially anti-CP negative patients became positive, and 8 of the initially anti-CP positive patients lost demonstrable antibodies at any occasion during follow-up). Most qualitative changes concerned marginally positive samples. There was no association between appearance and disappearance of anti-CP antibodies and clinical variables, including medication.

Baseline characteristics are detailed in table 1. Besides the earlier described association between anti-CP and RF, [4] the main difference was evident for age. The median age of anti-CP positive patients was 54.5 years, significantly different from 60.0 years for anti-CP negative patients. There was no difference at inclusion concerning other clinical or laboratory parameters. Furthermore, there was no difference in the proportion of patients initially obtaining DMARD therapy. Of the 279 patients, 125 were initially treated with sulphasalazine, 64 with methotrexate, 22 with auranofin, seven with antimalarials, three with gold sodium thiomalate, and 12 with other drugs. Two patients were initially treated with DMARD combinations, and 44 patients did not receive any DMARD therapy.
Calculation of cumulative use of DMARDs during the first two study years yielded 6696 individual months of therapy. Anti-CP positive patients had the highest percentage of treatment months with sulphasalazine alone (28.9% vs 23.7% of treatment months; p=0.05) or with a combination of two (10.8% vs. 6.6%; p=0.01) or three DMARDs (1.0% vs. 0%; p=0.01). Anti-CP negative patients were more often treated with auranofin alone (17.2% vs. 9.9%; p=0.01), and showed a non-significantly higher percentage of treatment months without any DMARD therapy (14.6% v. 11.6%). The groups did not differ concerning methotrexate therapy. These results suggest that anti-CP positive patients were recognized as having higher disease activity and demanded more effective anti-rheumatic treatment compared to anti-CP negative patients.

**Prognostic value of anti-CP status and quantitative anti-CP levels at baseline**

Whereas no difference in disease activity between anti-CP positive and negative patients was present at baseline, the groups started to diverge from each other already after three months. Anti-CP positive patients exhibited a higher disease activity measured by DAS28, a higher number of tender and swollen joints and had a higher disease activity (as assessed using the patient’s and physicians’ global assessment) as compared to anti-CP negative patients. All indices for clinical disease activity (presented in table 1) diminished during the follow-up period for the entire patient cohort, but the difference between anti-CP positive and negative patients became more obvious with time (figure 1). A similar picture to figure 1 was also obtained when the RA patients were split according to the occurrence of RF at baseline, with RF positive patients showing the most active disease at all timepoints (data not shown).
No correlations were evident between the levels of anti-CP antibodies at baseline and disease activity indices at baseline. However, the strength of the calculated correlations between baseline anti-CP and clinical indices increased steadily with time from baseline until five years, especially for physician’s assessment of disease activity and the swollen joint count where the correlations between baseline anti-CP and present clinical indices gradually increased from non-significant at baseline to highly significant after five years ($r=0.33$, $p<0.0001$ for physician’s assessment of disease activity, $r=0.31$, $p=0.0002$ for swollen joint count; data not shown). Comparisons between changes in the actual levels of anti-CP and changes in clinical measures created a number of weakly significant correlations, all of which (except for a drop in CRP between baseline and one year and a corresponding drop in anti-CP levels) were no longer apparent after Bonferroni correction (data not included).

**Anti-CP positivity predicts greater radiological progression.**

The mean baseline Larsen score for the 279 investigated patients was 7.9±9.1 (mean ±SD; median 4.5; inter quartile range 1.0-12.1) and increased to 13.7±12.0 (median 10.5; 4.0-20.3) after 1 year, and to 16.6±13.3 (median 14.0; 6.0-24.0) after two years of disease. When the anti-CP positive and negative groups were investigated separately, baseline Larsen scores were 7.5±8.8 (median 4.0, IQR 1.0-9.9) and 9.1±9.2 (median 5.0, IQR 1.8-13.8) respectively, and statistically not different. The two groups differed in the degree of radiographic progression during the next two years: anti-CP positive patients had a greater $\Delta$Larsen score between baseline and year 2 than anti-CP negative patients (9.7±7.1 vs. 6.9±5.7; $p=0.01$; Figure 2). Similar results were seen when the patients were separated by rheumatoid factor status at
baseline, with RF positive patients showing the greatest radiological progression (data not shown).

**Biphasic appearance of anti-CP antibody levels during five years of follow-up**

The levels of anti-CP antibodies declined rapidly between inclusion and one year. Later, these levels increased, showing a significant rise between three months, one and two years on the one hand and and five years on the other (figure 3).

**Relationship between DMARD therapy and changes in serum anti-CP antibody levels**

The decrease in anti-CP antibody levels during the first year was dose-dependently associated with the extent of sulphasalazine treatment during the same period (p = 0.009; figure 4a). No other DMARD showed such correlation, methotrexate-treated patients instead having a non-significant increase in anti-CP values as compared to baseline (figure 4b). There was no correlation between the use of peroral steroids or non-steroidal anti-inflammatory drugs and changes in anti-CP levels. The late increase in anti-CP did not correlate with changes in any clinical variables or medication during the same period.
Relationship between baseline anti-CP antibodies and RF

To distinguish the prognostic effects of baseline anti-CP antibodies from those of RF, we performed ANOVA with changes (between baseline on the one hand and 3 months, 1, 2, 3 and five years on the other) in the clinical indices in table 1 as dependent variables. RF could only be distinguished as an independent variable for the change in DAS between inclusion and 2 years (p=0.0041), whereas anti-CP was an independent variable for the change in swollen joint count between inclusion and one (p=0.048) and three years (p=0.035), respectively. The prognostic effect of baseline RF and baseline anti-CP could not be separated in any other comparison.

When instead the 102 RF negative patients (including 12 anti-CP positive patients) were investigated separately, anti-CP antibody status at inclusion showed a predictive value in parallel to, but weaker than, the findings in figure 1 for the total cohort, especially concerning the swollen joint count (data not included).
Discussion:

We have investigated at what time points after diagnosis any clinical differences between anti-CP positive and negative patients would be manifest. By serial quantitative measurements of anti-CP and parallel clinical and radiological investigations we wanted to explore the significance of anti-CP levels and to examine whether changes in antibody levels would predict, parallel or succeed changes in clinical variables, or relate to preceding or synchronous anti-rheumatic therapy.

We provide data concerning the anti-CP antibody status over time in newly diagnosed RA, showing that very few patients change this status over a 1-5 year period. The study also confirms previous reports of the prognostic value of these antibodies (see e.g. [15-19]). Finally, novel data are presented that demonstrate that changes in levels of anti-CP antibodies do not reflect disease activity, but may instead depend on pharmacological therapy.

In our study, we observed no difference in clinical parameters besides age between anti-CP positive and negative patients at the time of diagnosis. Thereafter, clinical variables showed a gradually increasing difference between the anti-CP positive and negative patients, although it should be kept in mind that clinical data only had been obtained from 54% of the patients after five years. In all comparisons for which the subgroups differed, anti-CP positive patients had the least favorable outcome. Likewise, patients who presented with anti-CP antibodies at baseline showed a significantly larger increase in radiological damage during the first two years of disease. Put in other words, the effect of anti-rheumatic treatment on both the clinical course and on radiographic progression was less impressive for the anti-CP positive patients than for the anti-CP negative ones. The anti-CP positive patients were
significantly younger than the anti-CP negative patients. Since higher age at onset of RA is associated with more severe disease outcome [20] we believe that this age difference cannot explain the observed differences in clinical presentation.

The small number of qualitative changes in anti-CP status is of interest from a pathogenic perspective. There is now firm evidence that anti-CP antibodies can develop years before onset of disease and that they occur prior to onset of clinical symptoms in a significant proportion of RA patients [6-9]. The fact that only few patients develop anti-CP antibodies after onset of disease is a strong indication that anti-CP immunity is associated with events causing RA, rather than being a consequence of already existing disease. The stability of the anti-CP phenotype has also been observed in a recent study [21]. Pathogenetic investigations of anti-CP immunity should therefore concentrate on events occurring before disease onset.

We determined no relationship between changes in antibody levels and changes in disease activity. However, correlation to quantitative anti-CP levels at baseline increased steadily over time for physicians’ assessment of disease activity and swollen/tender joint counts, being highest at five years. Similar data were produced for radiological progression over the first two years. These data imply that the prognostic importance of quantitative anti-CP levels at baseline might increase with time and indicate that follow-up studies in more long-standing RA are warranted. They also indicate that the immunological events leading to quantitatively different anti-CP antibody levels at the time of development of overt RA are associated with the subsequent development of disease with varying inflammatory responses and disease severity over years to come.
Use of sulphasalazine, but not other DMARDs, was dose-dependently associated with decreased anti-CP serum levels during the first year. Sulphasalazine and its metabolite 5-aminosalicylic acid have both been shown to suppress secretion of IgG, IgA and IgM in vitro [22]. Sulphasalazine therapy has also been shown to decrease IgG and IgA production in vivo [23, 24]. Taken together, it appears that sulphasalazine is able to down-regulate antibody responses in RA. The fact that sulphasalazine, but not other DMARDs, is associated with a decrease in anti-CP antibody levels indicates that sulphasalazine may have more profound effects on humoral immunity than do other DMARDs.

In conclusion, the stability of anti-CP antibody status and the failure of efficient treatment to eliminate these antibodies from serum strengthen the notion that anti-CP positive RA may differ from anti-CP negative RA in several important aspects, and that these two subgroups thus should be evaluated separately in future studies of both etiology and therapy.
Acknowledgements:

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Ethics approval:

All participants had given informed consent to participate in the study that had been ethically approved by the Northern Stockholm Ethical Committee.
Competing interests: none declared.

References:


Figure legends:

Figure 1. Line graphs depicting changes in mean clinical indices detailed in table 1 for anti-CP positive (anti-CP+) and anti-CP negative (anti-CP-) patients treated separately. Statistical comparisons were performed between anti-citrulline positive and negative patients at the indicated time points, where * denotes $p<0.05$, ** denotes $p<0.01$ and *** denotes $p<0.001$. No asterisk implies that no significant difference was determined.

Figure 2. Line graph representing changes in mean Larsen score ± SEM among anti-CP negative (open circles) and anti-CP positive (closed circles) patients separately.

Figure 3. Line graphs showing percentage change in median anti-CP antibody levels among the anti-CP positive patients among the subgroup of 77 RA patients for whom serum samples were obtained at baseline and after three months, one, two, three and five years, respectively. Paired statistical comparisons were performed between baseline and the indicated time points (asterisks above the graph; all differences denoting decreased ratios over time) and between various timepoints and five years (asterisks below the graph, all differences denoting increased ratios over time), where * denotes $p<0.05$ and *** denotes $p<0.001$. No asterisk implies that no significant difference was determined. The same highly significant drop in anti-CP levels was also found between baseline and three months and one year respectively when the whole group of 279 patients were investigated.

Figure 4. Changes in anti-CP antibody levels during the first year as a function of pharmacological therapy. On the abscissa patients are grouped according to treatment
with (a) sulphasalazine and (b) methotrexate therapy. “Never” indicates that the patient had not had the drug prescribed at any time point during the period, and “partly” that the patient had the drug prescribed at least for three months but not for the whole year. “Entire period” indicates that the drug was prescribed at the baseline visit with continuous medication at three, six and nine month visits. On the ordinate the ratio between the anti-citrulline levels at one year and baseline is depicted. A few patients increased more than 100% during the study period; these have been omitted from the figure but are included in the statistical calculations. Horizontal bars indicate median ratios for the corresponding groups. Only anti-citrulline positive patients have been included.
Table 1 Baseline characteristics of the 279 included RA patients. Figures are given as median values and as proportions. Differences between median values are analyzed using the Mann-Whitney U test, whereas differences between proportions are analyzed using Chi square test. NS = not significant.

<table>
<thead>
<tr>
<th></th>
<th>All patients</th>
<th>anti-citrulline positive patients</th>
<th>Anti-citrulline negative patients</th>
<th>Difference between groups (p)</th>
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<td>60</td>
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<td>Sex, % females</td>
<td>70.2% (196/279)</td>
<td>71.9% (115/160)</td>
<td>68.1% (81/119)</td>
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<td>147/159</td>
<td>28/118</td>
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<td>CRP</td>
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<td>16</td>
<td>13</td>
<td>NS</td>
</tr>
<tr>
<td>ESR</td>
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<td>23.5</td>
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<td>Number of swollen joints</td>
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<td>--------------------------</td>
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<td>Number of tender joints</td>
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<tr>
<td>Number of patients starting DMARD therapy</td>
<td>235/279</td>
<td>129/160</td>
<td>106/119</td>
<td>NS</td>
</tr>
</tbody>
</table>
Figure 1

- **CRP mg/dL**: The graph shows a decrease in CRP levels over time, with anti-CP+ showing a steeper decline compared to anti-CP-.
- **ESR**: ESR levels also show a decrease over time, with a significant drop in the first year for both groups.
- **Pain VAS**: Pain levels decrease over time, with a significant reduction in the first year for both groups.
- **Global VAS**: Similar to pain VAS, global VAS scores decrease over time, with a significant reduction in the first year for both groups.
- **HAQ**: HAQ scores decrease over time, with a significant reduction in the first year for both groups.
- **Physicians' assessment**: Physicians' assessment scores show a significant improvement over time, with a marked improvement in the first year for both groups.
- **Swollen joint count**: The number of swollen joints decreases over time, with a significant reduction in the first year for both groups.
- **Tender joint count**: Tender joint count also decreases over time, with a significant reduction in the first year for both groups.
- **DAS28**: DAS28 scores decrease over time, with a significant reduction in the first year for both groups.
Figure 3

The graph shows the median anti-CP ratio compared to baseline over time after study inclusion. The x-axis represents the time after study inclusion in months (0, 3 mo, 1 yr, 2 yrs, 3 yrs, 5 yrs), and the y-axis represents the median anti-CP ratio. The graph indicates a significant decrease in the anti-CP ratio from baseline at 3 months, followed by a gradual increase over the subsequent years. The asterisks (*) and triple asterisks (***, **) indicate statistical significance, with **asterisks** indicating a p-value less than 0.05 and **double asterisks** indicating a p-value less than 0.01.
Figure 4a

Antiviral, % change during the first study year

Sulphasalazine therapy during the first study year

P=0.0090
Figure 4b

Methotrexate therapy during the first study year

Anti-CCP, % change during the first study year

P=NS (0.057)