The Extent of the ACPA Repertoire is Associated with Arthritis Development in Patients with Seropositive Arthralgia

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ABSTRACT

Objectives
To determine the fine specificity of anti–citrullinated protein antibodies (ACPA) in the early phase of arthritis development, the ACPA repertoire in arthralgia patients and the association with arthritis development were studied.

Methods
A total of 244 patients with arthralgia positive for anti–cyclic citrullinated peptide antibodies (aCCPs) and/or IgM rheumatoid factor (IgM–RF), without arthritis were included. Development of arthritis was defined as presence of one or more swollen joints at clinical examination during follow-up. Sera were tested at baseline for reactivity to five citrullinated peptides derived from fibrinogen (three), vimentin (one) and α–enolase (one) and five corresponding arginine peptides in an ELISA.

Results
In all, 69 patients (28%) developed arthritis in a median of 3 joints after a median follow-up of 11 (IQR 5–20) months. Reactivity to each peptide was significantly associated with arthritis development (p<0.001). The ACPA repertoire did not differ between patients who did or did not develop arthritis. Among aCCP–positive patients, patients recognising two or more additional citrullinated peptides developed arthritis more often (p=0.04). The number of recognised peptides was positively associated with the aCCP level (p<0.001). Crossreactivity between different peptides was minimal.

Conclusions
Arthritis development is not associated with recognition of a specific citrullinated peptide once joint complaints are present. The ACPA repertoire in some patients with arthralgia is expanded. High aCCP levels are associated with a qualitatively broad ACPA repertoire. Patients with an extended ACPA repertoire have a higher risk of developing arthritis.
INTRODUCTION

Rheumatoid arthritis (RA) is a systemic inflammatory disorder in which anti-citrullinated protein antibodies (ACPA) such as anti-cyclic citrullinated peptide antibodies (aCCPs) are thought to play a pathogenic role. These antibodies are highly specific for RA\(^1\) and can be detected several years before the onset of disease.\(^2,3\) Furthermore, ACPA are associated with RA severity and predict a more rapid progression of erosive disease.\(^4-7\)

Environmental and genetic factors predispose to the formation of ACPA. The association of RA with the well known genetic risk factor of the \(HLA-DRB1\) alleles encoding the shared epitope (SE) is restricted to ACPA-positive individuals.\(^8,9\) Additionally, it was shown that the citrullination of a vimentin-derived peptide increases the affinity for binding to the peptide binding groove of human leucocyte antigen (HLA)–DRB1 molecules with the SE, supposedly leading to improved presentation by antigen presenting cells.\(^10\)

ACPA by definition target citrullinated proteins. Citrullination is a calcium-dependent post-translational modification that comprises the substitution of peptidylarginine to peptidylcitrulline. Citrullinated proteins can be found in inflamed synovium\(^11\) and also in extra-articular tissues such as the lungs, in which proteins are citrullinated under the influence of smoking.\(^12\) Furthermore, in a patient with RA multiple citrullinated proteins can be targeted by ACPA.\(^13\) The different ACPA present in one patient form the ACPA repertoire.

Myriad research efforts have presented candidate proteins for the immunodominant epitope of ACPA,\(^11,14-19\) such as fibrinogen,\(^11\) \(\alpha\)-enolase\(^16\) and vimentin.\(^19\) However, most studies have been performed in patients with established RA. During the course of disease, epitope spreading might occur.\(^20\) Finding the immunodominant epitope in established RA is therefore troublesome. Few studies have reported the antibody repertoire of patients with early arthritis. Patients with established RA have higher frequencies of different ACPA isotypes as compared to patients with early undifferentiated arthritis.\(^21\) This difference in repertoire is also found between native North American patients with RA and their healthy, ACPA-positive relatives.\(^22\) Additionally, these healthy relatives recognised less citrullinated peptides than patients with RA, suggesting epitope spreading during progression to RA.

To further elucidate the antibody response prior to the onset of arthritis, the ACPA repertoire and its association with arthritis development were analysed in a unique cohort of prospectively followed rheumatoid factor (IgM–RF)– and/or aCCP–positive arthralgia patients.
PATIENTS AND METHODS

Study population
Between August 2004 and August 2008, aCCP- and/or IgM-RF-positive patients with (a history of) arthralgia were recruited at our rheumatology outpatient clinics. The inclusion procedure was as described previously. In short, 244 patients without arthritis, with a positive aCCP and/or IgM-RF status were included. Absence of arthritis was confirmed by physical examination of 44 joints. Medical history, details of joint complaints and the number of tender joints were recorded. Patients with arthritis as revealed by chart review or baseline physical examination, a negative aCCP and IgM-RF status on second analysis, previous treatment with a disease-modifying antirheumatic drug or recent glucocorticoid treatment (<3 months) were excluded. Of the 244 study participants, 83 were also included in a randomised placebo-controlled trial studying the effects of intramuscular dexamethasone on arthritis development. Since dexamethasone did not delay or prevent arthritis these patients were considered suitable for the present analysis. However, they were excluded for the analysis of the number of recognised peptides after 1 year of follow-up.

During yearly follow-up visits, development of arthritis in any of 44 joints was independently confirmed by 2 investigators (WB or LAS and DS). Extra visits were planned if arthritis developed. Median follow-up was 36 months (range 18-60 months).

Laboratory investigations
aCCP and IgM-RF levels were determined at baseline by second-generation aCCP ELISA (Axis- Shield, Dundee, UK) and in-house ELISA, respectively, as described previously. The cut-off level for aCCP positivity was set at 5 arbitrary units/ml, according to the manufacturer's instructions. The cut-off level for IgM-RF positivity was set at 30 IU/ml determined on the basis of the analysis of receiver operating characteristic curves.
The extent of the ACPA repertoire is associated with arthritis development

Peptide-specific assays

Patient sera were tested at baseline and after 1 year of follow-up for reactivity to five citrullinated peptides derived from fibrinogen (cFib1 (β-chain amino acids 36–52): NEEGFFSACitGHRPLDKK,27 cFib2 (β-chain amino acids 60–74): CitPAPPPISGGGYCitACit,18 cFib3 (α-chain amino acids 36–50): GPCitVVECitHQSAKDS18, α-enolase (cEno (CEP-1): KIHACitEIFDSCitGNPTVE22,28) and vimentin (cVim: VYATCitSSAVCitLCitSSV27) and the five corresponding native arginine peptides in an ELISA. Peptides were constructed with an N-terminal biotin group with a spacer (SGSGC) in between. Streptavidin-coated microtitre plates (Streptawell High Bind; Roche, Mannheim, Germany) were coated with peptides (0.5 μg/ml in phosphate-buffered saline (PBS); NKI, Amsterdam, The Netherlands) and incubated subsequently with sera diluted 1:50 and 1:250 in ‘high performance ELISA’ (HPE) buffer (Sanquin, Amsterdam, The Netherlands). Antibodies were detected with horseradish peroxidase conjugated mouse monoclonal anti-human IgG (0.4 μg/ml, MH16, Sanquin) and then visualised with 3,3′,5,5′-tetramethylbenzidine (100 μg/ml) in 0.11 M acetate buffer pH 5.5 containing 0.003% H2O2 (Merck, Darmstadt, Germany). The reaction was stopped with 2 M H2SO4 and optical density (OD) was read at 450 nm. All incubation steps were performed for 1 h at room temperature, shaking. All washing steps were carried out with 0.02% Tween-20/PBS. Reactivity to citrullinated and native peptides was measured on the same plate. Reactivity was expressed as ∆OD between citrullinated and native peptides. Sera with ∆OD higher than the mean plus 2 SD of 40 healthy control sera in both dilutions were considered positive (supplementary figure 1). Sera that had an OD >1.8 for native reactivity in both dilutions were excluded (n=2) from further analysis (see supplementary methods for more details). Positive control sera were included on each plate. ELISA variation coefficients for cFib1, cFib2, cFib3, cEno and cVim plates were 13.4%, 9.5%, 12.9%, 13% and 18.5%, respectively.

Crossreactivity assays

Crossreactivity was analysed for citrullinated peptides derived from distinct proteins/protein subunits, that is, cFib1, cFib3, cEno and cVim. Five serum samples that showed reactivity to all peptides were selected. Sera were diluted in HPE buffer at decreasing concentrations (1:50 to 1:3200) and preincubated with 100 μg/ml blocking peptide (cFib1, cFib3, cEno or cVim) or HPE buffer only. Subsequent steps were carried out as described above. The remaining responses after inhibition were
calculated as percentage of the maximum reactivity, which by definition was set to 100% for samples preincubated with HPE buffer alone. More details regarding these assays are described in the supplementary material.

**HLA genotyping**

HLA-DQ typing was performed as described previously. HLA-DRB1 SE carrier status (one or two copies of the HLA-DRB1*0101, *0102, *0401, *0404, *0405, *0408, *0410 or *1001 alleles) was inferred from HLA-DQA1, and HLA-DQB1 haplotypes using strong linkage disequilibrium with HLA-DRB1 alleles in Caucasians. This HLA-DRB1 typing procedure correctly classified SE carriage in 86 out of 87 patients with established RA when validated with sequence-based high resolution typing (Sanquin). Independent confirmation in a second cohort of HLA-DRB1-typed and HLA-DQ-typed patients with RA showed correct classification in 165 out of 167 patients.

**Statistical analysis**

Data evaluation and statistical analysis were performed with SPSS V. 16.0 software (SPSS, Chicago, Illinois, USA). Categorical data were analysed by Chi² test; results were expressed as ORs with 95% CIs. Influence of number of recognised peptides on arthritis development was also analysed with Cox regression hazard analysis. Data with a Gaussian distribution were analysed by t test. Measures with a non-Gaussian distribution were analysed by Mann-Whitney or Kruskal-Wallis test where indicated. One-way tests were performed where appropriate. p Values ≤0.05 were considered significant.

**RESULTS**

**Arthritis development**

Baseline characteristics of the 244 patients examined are shown in table 1. Of these patients, 69 (28%) developed arthritis in 1 or more joints after a median follow-up of 11 (IQR 5–20) months. Their median tender joint count (for 53 joints) at the time of arthritis development was 5 (IQR 2–10), while the median swollen joint count (for 44 joints) was 3 (IQR 2–5).

As described previously for this patient cohort, a positive aCCP status was associated with arthritis development (p<0.001). IgM-RF positivity on its own was not associated with arthritis development (p=1.00) but increased the risk of
The extent of the ACPA repertoire is associated with arthritis development in combination with aCCP positivity (p=0.02) (data not shown).

### Tabel 2 Baseline characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. (total n=244)</th>
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<tbody>
<tr>
<td>Age in years, mean ± SD</td>
<td>47 ± 11</td>
</tr>
<tr>
<td>Female sex</td>
<td>181 (74%)</td>
</tr>
<tr>
<td>Arthralgia duration in months, median (IQR)</td>
<td>12 (8–36)</td>
</tr>
<tr>
<td>Number of reported painful joints, median (IQR)</td>
<td>2 (0–4)</td>
</tr>
<tr>
<td>Tender joint count (53 joints), median (IQR)</td>
<td>0 (0–1)</td>
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<tr>
<td>Antibody status</td>
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<tr>
<td>IgM-RF positive, aCCP negative</td>
<td>81 (33%)</td>
</tr>
<tr>
<td>aCCP positive, IgM-RF negative</td>
<td>89 (37%)</td>
</tr>
<tr>
<td>aCCP and IgM-RF positive</td>
<td>74 (30%)</td>
</tr>
<tr>
<td>Antibody levels</td>
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<tr>
<td>IgM-RF (IU/ml), median (IQR)*</td>
<td>76 (42–123)</td>
</tr>
<tr>
<td>aCCP (AU/ml), median (IQR)*</td>
<td>39 (12–145)</td>
</tr>
<tr>
<td>Shared epitope status</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>128 (52%)</td>
</tr>
</tbody>
</table>

*Patients who were positive only. aCCP, anti-cyclic citrullinated peptide antibody; AU/ml, arbitrary units/ml; IgM-RF, IgM rheumatoid factor.

### ACPA repertoire

In all, 9 (11%) aCCP-negative patients and 133 (82%) aCCP-positive patients showed reactivity to 1 or more citrullinated peptides. Of the nine aCCP-negative patients, eight (89%) recognised one peptide and one (11%) recognised three peptides. Among aCCP-positive patients, 28 (17.2%) recognised no additional peptides, 18 (11%) recognised 1, 19 (12%) recognised 2, 32 (20%) recognised 3, 41 (25%) recognised 4 and 23 (14%) recognised 5 additional peptides. Anti–CCP–positive patients that were also IgM–RF positive recognised more peptides compared to patients that were only aCCP positive (frequencies for recognising 0–5 peptides in IgM–RF–positive patients were 5%, 7%, 14%, 27%, 23% and 24%, respectively and in IgM–RF–negative patients 28%, 15%, 10%, 14%, 27% and 6%, respectively; p<0.001).

In aCCP–positive patients recognition of 2–5 additional peptides increased the risk for developing arthritis compared to recognition of 0–1 additional peptides with an OR (95% CI) of 2.1 (1.0 to 4.4) (**figure 1**). Cox regression hazard analysis, in which the time to conversion for each individual patient is taken into account, did not result in a significant association, but a strong trend with a hazard ratio (95% CI) of 1.7 (0.93 to 3.16) (p=0.08) was still present. Among patients who were aCCP positive, recognition of higher numbers of peptides was associated with higher aCCP levels (p<0.001) (**figure 1**). The number of recognised peptides remained
stable after 1-year follow-up in patients who developed arthritis as well as in patients who did not (data not shown).

Citrullinated Fib1 was recognised most frequently in the whole patient cohort (50% positive), followed by cFib2 (41%), cEno (37%), cVim (32%) and cFib3 (23%). There was no difference in antibody repertoire between patients who did or who did not develop arthritis and reactivity to all citrullinated peptides was significantly associated with arthritis development (figure 2). OR (95% CI) for cFib1, cFib2, cFib3, cEno and cVim were 4.3 (2.3 to 7.9), 4.0 (2.2 to 7.2), 2.5 (1.4 to 4.8), 2.7 (1.5 to 4.8) and 3.1 (1.7 to 5.6), respectively.

**Figure 1**
Association of the number of recognised peptides with arthritis development and anti-cyclic citrullinated peptide antibody (aCCP) levels. Depicted is the percentage of aCCP-positive patients that developed arthritis (bars) and the median aCCP level at baseline (triangles) for patients that recognised 0–5 peptides in addition to CCP. *p=0.04.

**Figure 2**
Association of reactivity to different peptides with arthritis development. Reactivity to different peptides is shown as percentage of positive patients within the group of subjects that did (black bars; n=69) or that did not (open bars; n=173) develop arthritis. *p<0.001, **p=0.001, ***p=0.003.
Specificity of citrulline-specific immune responses
Five patient sera positive for all citrullinated peptides were selected and their individual reactivity to cFib1, cFib3, cEno and cVim was tested after preincubation with each of these peptides. In each patient, reactivity to all peptides was almost completely inhibited after blocking with the homologous citrullinated peptide for which the reactivity was studied (supplementary figure 2). Inhibition by the other peptides was minimal. The mean percentages of remaining responses after inhibition are depicted in figure 3. In three patients with established RA comparable inhibition patterns were observed (data not shown).

Figure 3
Specificity of antibodies against different citrullinated peptides derived from fibrinogen, enolase and vimentin. Five sera from patients with arthralgia were tested for reactivity towards cFib1 (A), cFib3 (B), cEno (C) and cVim (D) after preincubation with 100 µg/ml cFib1, cFib3, cEno or cVim in liquid phase. Black bars represent reactivity to a peptide after preincubation with the homologous peptide, grey bars represent reactivity to a peptide after preincubation with another peptide. Depicted are the mean (±SEM) percentages of maximum reactivity at a serum dilution of 1:50 of five individual sera. Maximum reactivity was considered 100% for high performance ELISA buffer preincubated responses.

SE and ACPA
SE carriage was higher in the group of patients who developed arthritis than in the group of non-converters (65% vs 47%, p=0.01). For each peptide, the number of positive patients was higher in the group of SE-positive patients than in the group of SE-negative patients (table 2). Furthermore, the presence of the SE was associated with the number of recognised peptides (p<0.001). This association was also found within the subgroup of aCCP-positive patients (p<0.001) (figure 4).
Table 3: Association of the SE with reactivity to different citrullinated peptides

<table>
<thead>
<tr>
<th></th>
<th>SE negative, n=115</th>
<th>SE positive, n=127</th>
<th>OR (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cFib1</td>
<td>43 (37%)</td>
<td>77 (61%)</td>
<td>2.5 (1.5 to 4.3)</td>
</tr>
<tr>
<td>cFib2</td>
<td>28 (24%)</td>
<td>72 (57%)</td>
<td>4.1 (2.3 to 7.1)</td>
</tr>
<tr>
<td>cFib3</td>
<td>17 (15%)</td>
<td>39 (31%)</td>
<td>2.5 (1.4 to 4.8)</td>
</tr>
<tr>
<td>cEno</td>
<td>22 (19%)</td>
<td>67 (53%)%</td>
<td>4.7 (2.6 to 8.4)</td>
</tr>
<tr>
<td>cVim</td>
<td>17 (15%)</td>
<td>60 (47%)%</td>
<td>5.1 (2.7 to 9.6)</td>
</tr>
</tbody>
</table>

SE, shared epitope

Figure 4

Association of the shared epitope (SE) with the number of recognised peptides. The percentage of patients who were SE positive is shown for patients that recognise 0–5 different peptides. White bars represent all patients; black bars represent patients positive for anti-cyclic citrullinated peptide antibodies only.

Discussion

In the present study, the ACPA repertoire of seropositive arthralgia patients and the association with arthritis development were investigated. Most aCCP-positive arthralgia patients and few aCCP-negative, IgM–RF-positive patients showed reactivity to one or more citrullinated peptides. Overall, the ACPA repertoire in the former patient group was already expanded considerably. A total of 115 patients (71%) recognised more than 1 additional citrullinated peptide and these patients had an increased risk for developing arthritis as compared to patients who recognised 0–1 additional peptides.

No difference was seen in the ACPA repertoire between patients who did and who did not develop arthritis after a median follow-up of 36 months and thus an immunodominant epitope could not be deduced from this study. Furthermore, the number of recognised peptides remained stable over a period of 1-year follow-up, indicating that epitope spreading either occurs before onset of joint complaints or progression is more slowly thereafter. Therefore, the ACPA repertoire earlier in the
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course of disease needs to be studied, preferably over a course of several years before the development of joint issues. Recent data from a limited number of blood bank donors who later developed RA showed epitope spreading prior to onset of clinical RA.\(^{32}\)

The number of recognised peptides was associated with arthritis development, suggesting that a more expanded immune response reflects further progression towards clinical disease. Therefore, patients recognising more peptides would be expected to develop arthritis earlier. Cox regression hazard analysis revealed a strong trend towards earlier arthritis development in patients who recognised more peptides, which supports our assumption. Longer follow-up is needed to verify these data.

Additionally, the number of recognised peptides was associated with the aCCP level, a parameter that has been established as a risk factor for the development of arthritis.\(^{21}\) This association could be the result of crossreactivity of antibodies with different peptides when these antibodies are present in large amounts. However, we found only limited crossreactivity between different peptides. This indicates that the major part of the antibody response to different peptides is specific. Thus, quantitative and qualitative expansion of the antibody repertoire may occur at the same time. Alternatively, because aCCP antibodies are probably measured with an assay consisting of multiple peptides,\(^{33}\) the presence of antibodies to different epitopes could result in higher aCCP levels.

Along with the aCCP level, the number of recognised peptides was associated with the presence of the SE. This is in line with previous reports that show a relation between the SE and the magnitude of the aCCP response\(^{8,34}\) and implies that quantitative and qualitative expansion of the antibody response is influenced by the SE. However, contrary to what was found in prior studies,\(^{27}\) presence of the SE was associated with reactivity to all epitopes. Our findings indicate that the SE predisposes to the development of antibodies to citrullinated peptides in general, rather than influences the fine specificity of the response. However, we did not study the influence of the number or type of SE alleles or the influence of smoking on reactivity to different citrullinated peptides. These factors might be associated with the fine specificity of the response.\(^{35,36}\)

The number of recognised peptides was higher in aCCP-positive patients who were also positive for IgM–RF than in patients who were aCCP positive only. Since the former group has a higher risk for developing arthritis, this further supports the hypothesis that patients with arthralgia presenting with a broader ACPA repertoire
are the individuals that have progressed most towards clinical disease and can be considered high-risk patients. There is likely a complex interaction between all aforementioned risk factors, which may predispose some associations to confounding, effect modification or mediation. Elucidating the individual contribution of each of these risk factors to the pathophysiological mechanism of RA would greatly improve our insight into its pathogenesis. However, due to a relatively low number of participants, the present study is not suited to answer this question. Nevertheless, one can argue that within the ‘cycle of RA’ epitope spreading contributes to the maintenance of this perpetual cycle. We hypothesise that, after citrullination of proteins is triggered, presentation by antigen presenting cells bearing SE containing HLA molecules ensues, leading to the production of ACPA. A secondary trigger causes inflammatory cells in the synovium to switch to an active phenotype, resulting in a cascade of inflammatory events. This process is accompanied by the citrullination of different proteins, due to the local activation of peptidylarginine deiminase (PAD). Degraded and citrullinated proteins form multiple neoantigens and epitope spreading occurs. Citrullinated proteins, ACPA and perhaps IgM-RF can form immune complexes that further stimulate inflammation, maintaining chronic disease.

Apparently, this process has advanced considerably in some patients with seropositive arthralgia. These patients have a higher risk for the development of arthritis as reflected by the increased risk in aCCP-positive patients who recognise more than one citrullinated peptide. In a collagen-induced arthritis mouse model, arthritogenicity is substantially higher when a cocktail of monoclonal antibodies recognising different epitopes of collagen is used. Also, in humans, reactivity directed towards more than one epitope could be essential for the development of chronic disease. Treatment of high-risk patients, preferably in a clinical trial to study arthritis prevention, might be justifiable. Whether the ACPA repertoire should be used to identify these high-risk patients is a matter of debate. The test is at present not suitable for routine use and parameters such as aCCP titre, IgM–RF status, C reactive protein, family history, tender joint count and morning stiffness might be more appropriate to use in prediction models to identify patients at high risk. The present patient cohort will be used to address this question in further research.

In conclusion, the ACPA repertoire of some seropositive arthralgia patients is already expanded. Patients with a broader ACPA repertoire have a higher risk of
The extent of the ACPA repertoire is associated with arthritis development developing arthritis. This suggests that among patients with arthralgia, a subgroup has a subclinical inflammation that has progressed considerably and that these patients might be regarded as having a high risk of developing arthritis. Whether development of clinical disease is preventable remains an intriguing question that will hopefully be answered in future trials, for which these patients seem ideal candidates.

ACKNOWLEDGEMENTS

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Supplementary Figure 1
Reactivity to peptides of healthy controls versus arthralgia patients. Reactivity to citrullinated peptides was measured by an ELISA in which reactivity to both the citrullinated and native peptides was tested. Sera were diluted 1:50 and 1:250. Reactivity to citrullinated peptides was expressed as ΔOD between citrullinated and native peptides. Sera with ΔOD more than the mean plus 2SD of 40 healthy control sera in both dilutions were considered positive. Depicted are the ΔOD values of healthy control sera (HC; open dots, n=40) and arthralgia patients (Art; closed dots, n=244) of reactivity to cFib1, cFib2, cFib3, cEno and cVim of sera diluted 1:250 (A) and 1:50 (B). The dotted lines represent the cutoff value.
The extent of the ACPA repertoire is associated with arthritis development.

Supplementary Figure 2
Specificity of antibodies in individual patients. Five arthralgia patient sera were tested for reactivity towards cFib1, cFib3, cEno and cVim after pre-incubation with HPE buffer (open bars), 100 µg/ml cFib1 (grey bars), cFib3 (striped bars), cEno (black bars) or cVim (grey, striped bars) in liquid phase. Each panel shows the OD values of an individual patient for the reactivity towards different peptides.
METHODS

Peptide specific assays
Final peptide reactivity was expressed as ΔOD between citrullinated and native peptides. Sera with ΔOD higher than the mean plus 2SD of 40 healthy control sera in 1:50 and 1:250 serum dilutions were considered positive. Positive reaction to native peptide was defined as OD higher than the mean plus 2SD of 40 healthy control sera in 1:50 and 1:250 serum dilutions (supplementary figure 1). Sera with an OD higher than 1.8 for the native peptide in both serum dilutions were excluded from further analysis (n=2). The number of sera positive for the native peptides and the degree of reactivity to both native and citrullinated peptide is shown in supplementary table 1. The numbers of patients that were positive for the native peptide according to aCCP status are given in supplementary table 2. There was no difference in the reactivity to the native peptides between aCCP negative and aCCP positive patients.

Cross reactivity assays
The inhibitory potential of citrullinated peptides (cFib1, cFib3, cEno and cVim) in liquid phase was tested in 3 sera from RA patients fulfilling ACR criteria. Sera were diluted in HPE buffer at a dilution which resulted in a half maximum response as determined with dose response curves. Sera were incubated with increasing concentrations of blocking peptide (either cFib1, cFib3, cEno or cVim) for one hour at room temperature. Pre-incubated sera were then incubated for 30 minutes on plates coated with citrullinated peptides. All subsequent steps were performed as described for the peptide specific ELISA's. Reactivity to each of the peptides was dose dependently inhibited by the homologous peptide in the RA patient sera, in which maximum inhibition was achieved with a minimal concentration of 50 μg/ml blocking peptide. For subsequent experiments with sera of arthralgia patients a concentration of 100 μg/ml blocking peptides was chosen.
The extent of the ACPA repertoire is associated with arthritis development

TABLES

Supplementary Table 1: Patients positive for native peptide and the degree of reactivity

<table>
<thead>
<tr>
<th>Patients</th>
<th>Nr positive for native peptide</th>
<th>Median OD</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Native*</td>
<td>Citrullinated†</td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>1:50‡</td>
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<td></td>
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<tr>
<td>cFib1 (n = 242)</td>
<td>27</td>
<td>0.75</td>
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<td>cFib2 (n = 242)</td>
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<td>0.18</td>
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<td>68</td>
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<td>37</td>
<td>0.68</td>
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<td>0.90</td>
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<td>cVim (n = 242)</td>
<td>35</td>
<td>0.90</td>
<td>0.21</td>
<td>0.71</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>Negative patients §</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cFib1 (n = 122)</td>
<td>20</td>
<td>0.91</td>
<td>0.29</td>
<td>1.01</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>cFib2 (n = 142)</td>
<td>28</td>
<td>0.63</td>
<td>0.21</td>
<td>0.64</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>cFib3 (n = 186)</td>
<td>58</td>
<td>0.41</td>
<td>0.11</td>
<td>0.32</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>cEno (n = 153)</td>
<td>26</td>
<td>0.74</td>
<td>0.22</td>
<td>0.71</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>cVim (n = 165)</td>
<td>31</td>
<td>0.87</td>
<td>0.21</td>
<td>0.69</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>Positive patients **</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cFib1 (n = 120)</td>
<td>7</td>
<td>0.61</td>
<td>0.13</td>
<td>2.30</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td>cFib2 (n = 100)</td>
<td>4</td>
<td>0.63</td>
<td>0.15</td>
<td>2.40</td>
<td>1.28</td>
<td></td>
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<tr>
<td>cFib3 (n = 56)</td>
<td>10</td>
<td>0.29</td>
<td>0.08</td>
<td>1.14</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>cEno (n = 89)</td>
<td>11</td>
<td>0.56</td>
<td>0.14</td>
<td>1.71</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>cVim (n = 77)</td>
<td>4</td>
<td>1.08</td>
<td>0.22</td>
<td>1.97</td>
<td>0.64</td>
<td></td>
</tr>
</tbody>
</table>

* reactivity directed to native peptide, † reactivity directed to citrullinated peptide, ‡ serum dilution, § ∆OD < mean + 2SD of 40 healthy control sera, **∆OD ≥ mean + 2SD of 40 healthy control sera, nr: number, OD: optical density, n = 242

Supplementary Table 2: Number of patients positive for native peptide according to aCCP status

<table>
<thead>
<tr>
<th>aCCP status</th>
<th>Negative n = 81</th>
<th>Positive n = 161</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>cFib1</td>
<td>10 (12%)</td>
<td>17 (11%)</td>
<td>ns</td>
</tr>
<tr>
<td>cFib2</td>
<td>11 (14%)</td>
<td>21 (13%)</td>
<td>ns</td>
</tr>
<tr>
<td>cFib3</td>
<td>22 (27%)</td>
<td>46 (29%)</td>
<td>ns</td>
</tr>
<tr>
<td>cEno</td>
<td>9 (11%)</td>
<td>28 (17%)</td>
<td>ns</td>
</tr>
<tr>
<td>cVim</td>
<td>9 (11%)</td>
<td>26 (16%)</td>
<td>ns</td>
</tr>
</tbody>
</table>

* p > 0.05 = not significant (ns)
REFERENCES


Chapter 3

...pathophysiologic mechanism for humoral anti-type II collagen immunity in arthritis. *Arthritis Rheum* 2006;54:1759–71.

