## Clinical and molecular classification of very early arthritis patients

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## DIFFERENT STAGES OF RHEUMATOID ARTHRITIS: THE FEATURES OF THE SYNOVIUM IN THE PRECLINICAL PHASE

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# ABSTRACT

**Background**: The aetiology of rheumatoid arthritis (RA), a prototype immune-mediated inflammatory disorder, is poorly understood. It is currently unknown whether the disease process starts in the synovium, the primary target of RA, or at other sites in the body. Therefore, in a prospective study we examined the presence of synovitis in individuals with an increased risk of developing RA.

**Methods**: Thirteen individuals without evidence of arthritis, who were positive for IgM rheumatoid factor and/or anti-citrullinated protein antibodies, were included in the study. To evaluate synovial inflammatory changes, all participants underwent dynamic contrast-enhanced MRI and arthroscopic synovial biopsy sampling of a knee joint at inclusion. Results were compared with knee MRI data and synovial biopsy data of 6 and 10 healthy controls, respectively.

**Results**: MRI findings evaluated by measurement of maximal enhancement, rate of enhancement, synovial volume and enhancement shape curve distribution were similar in the autoantibody-positive individuals compared with the healthy controls. Consistent with these findings, all but one autoantibody-positive individuals showed very low scores for phenotypic markers, adhesion molecules, and vascularity, all in the same range as those in normal controls. The one individual with higher scores had patellofemoral joint space narrowing.

**Conclusion**: Subclinical inflammation of the synovium does not coincide with the appearance of serum autoantibodies during the pre-RA stage. Thus, systemic autoimmunity precedes the development of synovitis, suggesting that a 'second hit' is involved. This study supports the rationale for exploring preventive strategies aimed at interfering with the humoral immune response before synovial inflammation develops.

# INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease primarily affecting synovial tissue in multiple joints. Despite increasing insight into the inflammatory pathways, and environmental and genetic factors that play a role in the initiation and progression of RA, the aetiology of the disease is poorly understood.

The synovium is the primary target of this disease. Systematic evaluation of the features of synovial inflammation in patients with early RA (signs and symptoms <1 year and the same if early RA is defined as disease duration <3 months) has shown that cell infiltration as well as the expression of cytokines, chemokines, granzymes, adhesion molecules, and matrix metalloproteinases are on average similar to those observed in longstanding disease (>5 years duration), when controlling for disease activity and use of antirheumatic drugs.[1-4] Thus, early arthritis (as defined clinically) already represents chronic inflammation of the synovial tissue.[5] This notion is supported by the observation that radiological damage can be observed in very early stages of the disease.[6] Based on these findings we hypothesised that there might be a preclinical phase of RA, called pre-RA, characterised by inflammatory changes in the synovium before the onset of clinical signs and symptoms. This hypothesis is supported by the demonstration of synovial inflammation in clinically uninvolved knee joints from patients with established RA.[7] Furthermore, several animal models of RA have shown inflammatory synovial tissue changes in the latency phase of arthritis.[7] Of note, however, the preclinical phase in the different animal models is relatively short, as arthritis develops within a few weeks after induction. Prospective data on the features of the synovium during the preclinical phase of RA are as yet not available, since it has been difficult in the past to identify individuals at risk of developing RA.[8] Moreover, it is challenging to obtain synovial biopsies from individuals without arthritis.

Recent research has shown that IgM rheumatoid factor (IgM-RF) and anti-citrullinated protein antibodies (ACPA) can be detectable in the serum of RA patients up to 14 years before the first clinical signs and symptoms of arthritis become manifest.[9-11] These data show that immunological abnormalities precede the development of RA. In addition, the detection of these autoantibodies allows us for the first time to identify individuals who are at risk of developing RA. We have recently shown that 20% of autoantibody-positive individuals with arthralgia developed clinical signs and symptoms of arthritis after a median follow-up of 28 months.[12]

To provide more insight into the question whether the disease process starts in the synovial tissue, we examined the synovium of IgM-RF- and/or ACPA-positive individuals without arthritis by dynamic contrast-enhanced (DCE) MRI, which is a sensitive tool to demonstrate synovial inflammation.[13,14] In addition, we analysed arthroscopic synovial tissue samples from these same participants by immunohistochemistry, as histology is the gold standard for evaluating synovial inflammation.

# PATIENTS AND METHODS

## Study participants

Thirteen IgM-RF (serum level of >12.5 kU/L; determined by IgM-RF ELISA Sanquin, Amsterdam, the Netherlands) and/or ACPA (serum level of >25 kAU/L; determined by anti-CCP2 ELISA

Eurodiagnostica, Nijmegen, the Netherlands) positive individuals without arthritis (as determined by an experienced rheumatologist) were included. Twelve were individuals with arthralgia recruited from the Clinical Immunology & Rheumatology outpatient clinic of the Academic Medical Center (AMC) in Amsterdam of whom 8 patients were referred from the Rheumatology department of the Jan van Breemen Institute, Amsterdam, and one was a first-degree relative of an RA patient with arthralgia. Study participants were excluded if they had a history of arthritis, or if they had used disease-modifying antirheumatic drugs (DMARDs) or corticosteroids for inflammatory joint complaints.

The control group for MRI consisted of six healthy individuals without any current or previous joint complaints and a normal knee joint at clinical evaluation. The control group for synovial biopsy comprised 10 individuals who underwent knee arthroscopy because of unexplained knee pain. None of these subjects showed inflammatory or degenerative joint pathology upon physical examination, arthroscopy, or laboratory and radiological evaluation at inclusion or during 5 years of follow-up.[15]

Both autoantibody-positive individuals and controls gave written informed consent. This study was approved by the local Medical Ethical Committee. The study was conducted according to the principles expressed in the "Declaration of Helsinki".

## Study design

At inclusion we collected demographics and disease activity parameters and made X-rays of hands, feet and knee joints. Presence of the shared epitope was determined using sequence-based HLA-DRB1 typing. In addition, all autoantibody-positive individuals underwent DCE-MRI of the knee joint within 1 week before the arthroscopy. Follow-up consisted of yearly visits at which disease activity parameters and X-rays were collected. When a patient developed arthritis, an additional study visit was scheduled during which a second arthroscopy was performed and disease activity was assessed. Baseline MRI parameters and characterization of the cell infiltrate and vascularity in the synovium were compared between the autoantibody-positive individuals and the control groups.

## Disease activity parameters

At baseline we assessed disease activity by 68 tender and 66 swollen joint scores, patient's visual analog scale (VAS) for global disease activity (scale 0-100mm), VAS for pain (scale 0-100mm), erythrocyte sedimentation rate (ESR), and serum levels of C-reactive protein (CRP). X-rays of hands, feet and the knee joint that was selected for arthroscopy were obtained to study joint space narrowing and erosive changes.

## MRI

*MRI acquisition*

Images were acquired on a 1.5 T MRI scanner (GE Signa Horizon Echospeed, LX9.0, General Electric Medical Systems, Milwaukee, WI, USA) using a 3D T1-weighted gradient echo dynamic sequence that consisted of 20 consecutive images of 20 slices with a temporal resolution of 22 seconds (TR/TE/flip 8·1/3·5/30, slice thickness 4 mm, FOV 18 cm, 256*256 matrix, axial orientation). The total imaging time was 7 minutes and 19 seconds.

Autoantibody-positive individuals and healthy controls were placed supine with the knee joint centrally in the magnetic field in a dedicated extremity coil (quadrature detection). A 20-gauge needle infusion line was inserted in the right antecubital vein. Sixty seconds after the initiation of the dynamic protocol, a bolus of a Gd-DTPA contrast agent (0.1 mg/kg; Magnevist, Bayer Schering Pharma, Berlin, Germany) followed by a 15 mL saline chase was delivered at an injection rate of 5 mL/s using an automatic injection device (Spectris Solaris MR Injector, MEDRAD, Warrendale, PA, USA).

*MRI data analysis*

Images were processed using an in-house developed program running on MATLAB (MathWorks, Natick, MA, USA).[16] This program analyses the time-dependent signal intensity changes (TIC) of every voxel in an imaged 3D volume. Of these TIC, maximal enhancement, maximal slope of increase, and relative number of 7 different TIC shape types were calculated, as described previously.[14] Synovial volume was calculated as number of enhancing voxels multiplied by volume of each voxel.

## Arthroscopic synovial tissue biopsy

All individuals underwent arthroscopic synovial tissue biopsy sampling of a knee joint.[17] At least six synovial tissue biopsies were collected for immunohistochemistry, as described earlier [18,19] to minimise sampling error. The synovial biopsy samples were snap-frozen en bloc in Tissue-Tek OCT (Miles, Elkhart, IN, USA) immediately after collection. Cryostat sections (5 µm) were cut and mounted on Star Frost adhesive glass slides (Knittelgläser, Braunschweig, Germany). Sealed slides were stored at -80°C until use for immunohistochemistry.

## Immunohistochemistry

Synovial tissue sections were stained using the following monoclonal antibodies: anti-CD3 (SK7; Becton Dickinson, San Jose, CA, USA; T cells), anti-CD22 (CLB-B-ly/1,6B11; Sanquin, Amsterdam, the Netherlands; B cells), anti-CD55 (67; Serotec, Oxford, United Kingdom; fibroblast-like synoviocytes [FLS]), anti-CD68 (EBM11; Dako, Glostrup, Denmark; macrophages), anti-CD138 (B-B4; Immunotech, Marseille, France; plasma cells), anti-von Willebrand factor (vWF; F8/86; Dako; blood vessels), anti-E-selectin (BBIGE4C5D11; R&D Systems, Minneapolis, MN, USA), anti-ICAM-1 (MEM111; Sanbio, Uden, the Netherlands), and anti-VCAM-1 (IG11B1; Sanbio).

Staining of vWF was performed using a three-step immunoperoxidase method, as previously described.[20] Staining of cellular markers was done on a Dako Autostainer universal staining system using a two-step indirect immunoperoxidase technique applying the Dakocytomation Envision System kit, and then counterstained with haematoxylin. For staining of adhesion molecules and growth factors, biotinylated tyramine was used for amplification, as previously described.[21] As a negative control, irrelevant/isotype-matched immunoglobulins were applied to the sections instead of the primary antibody or the primary antibody was omitted.

After staining of the slides the sections were analysed by semi-quantitative analysis (SQA) by two independent observers (MGvdS and GPMvdS, or MGvdS and TJS), as previously described. [4] The expression of immunohistochemical markers was scored on a 5-point scale (range 0–4). A score of 0 represented minimal expression, while a score of 4 represented high expression. For evaluation of the expression of $CD68^+$ cells, SQA was performed for intimal macrophages

and synovial sublining macrophages separately. Minor differences between observers were resolved by mutual agreement.



### Statistical analysis

Mann-Whitney-U test was used to detect differences in MRI parameters between autoantibody positive individuals and controls; Chi-square test was used for the immunohistochemical analysis. *p*-Value of <0·05 was considered significant. Values were expressed as median (range) or number. SPSS V.16.0 software (SPSS, Chicago, IL, USA) was used for the analysis.

## RESULTS

### Study participants

Baseline characteristics of the autoantibody-positive individuals who were enrolled in this study are shown in Table 1. Serum levels of IgM-RF and ACPA were elevated in 8 and 10 of the autoantibody-positive individuals, respectively; 5 were positive for both autoantibodies. Titers of IgM-RF and ACPA in the positive individuals varied from 16 kU/L to 207 kU/L and from 35 kAU/L to 2,591 kAU/L, respectively. Three patients had one shared epitope allele, one patient had two shared epitope alleles, and the others were shared epitope-negative. In one patient no blood was available to determine shared epitope status. Although no clinical joint swelling was observed, most individuals had arthralgia of more than one joint. One person showed patellofemoral joint space narrowing of the knee joint, whereas all others had normal X-rays of hands, feet and the selected knee joint at inclusion. During follow-up of a median of 37 (range 25-45) months, four patients (31%) developed arthritis after a median period of 3 (1-6) months, consistent with our previous experience (after a median follow-up of 28 [19-39] months, 29/147 [20%] individuals with arthralgia developed arthritis)[12]. The presence of arthralgia at baseline was not related to the development of arthritis after follow up. When arthritis was clinically manifest only 1 patient fulfilled ACR 1987 RA criteria [22] while 3 patients fulfilled the ACR/EULAR 2010 RA criteria [23]. During follow up 2 patients fulfilled the ACR 1987 RA criteria while all patients fulfilled the ACR/EULAR 2010 RA criteria.

### DCE-MRI reveals normal synovium in autoantibody-positive individuals who are at risk of developing RA

There were no clear-cut differences between autoantibody-positive individuals and controls in descriptive DCE-MRI parameters, TIC curve shape expression and synovial volume. All parameters at baseline were in the same range in both groups (Table 2), even in individuals who developed arthritis over time (data not shown).

### Immunohistochemical analysis shows that the features of synovial biopsy samples are similar between autoantibody-positive individuals and controls

Of the 13 enrolled autoantibody-positive individuals, 1 was excluded from the synovial tissue analysis, because of insufficient quality of tissue sections according to the strict quality control system based on the absence of an intimal lining layer.

**Table 1.** Baseline characteristics of the autoantibody-positive subjects (n = 13).

| **Characteristic** | |
|---|---|
| Age (yrs) | 43 (22-57) |
| Female, n | 10 |
| **Disease activity parameters** | |
| ESR (mm/hr) | 8.0 (3-18) |
| CRP (mg/L) | 1.6 (1.0-10.0) |
| Patient global VAS (mm) | 40 (0-94) |
| Patient VAS pain (mm) | 45 (1-98) |
| Morning stiffness (min) | 5 (0-30) |
| TJC-68 (n) | 2 (0-10) |
| SJC-66 (n) | 0 |
| **Autoantibody status** | |
| IgM-RF positive, n | 8 |
| ACPA positive, n | 10 |
| IgM-RF and ACPA positive, n | 5 |
| ACPA titre (kAU/L)# | 537 (35-2,591) |
| IgM-RF titre (kU/L)# | 56 (16-207) |

All values are expressed as median (min-max). ESR = erythrocyte sedimentation rate, CRP = C-reactive protein, VAS = visual analogue scale, TJC= tender joint count, SJC= swollen joint count, IgM-RF = IgM rheumatoid factor, ACPA = anti-citrullinated protein antibodies. #Only of positive individuals.

**Table 2.** Dynamic contrast-enhanced TIC shapes in IgM rheumatoid factor (IgM-RF)-positive and/or anti-anti-citrullinated peptide antibody (ACPA)-positive individuals without arthritis and healthy controls.

| | **IgM-RF- and/or ACPA-positive individuals** | **Healthy controls** | ***p*-Value** |
|---|---|---|---|
| Type 2 TIC (%) | 75.0 (52.0-90.0) | 74.0 (61.0-89.0) | 0.79 |
| Type 3 TIC (%) | 2.3 (1.2-11.9) | 3.1 (1.3-4.7) | 0.79 |
| Type 4 TIC (%) | 5.4 (1.9-20.2) | 8.1 (2.6-8.9) | 0.43 |
| Type 5 TIC (%) | 8.1 (3.5-14.0) | 7.4 (4.5-15.4) | 0.73 |
| Volume synovium (mL) | 23 (13-57) | 20 (4-40) | 0.38 |
| Maximal enhancement | 0.7 (0.5-1.1) | 0.6 (0.5-0.8) | 0.22 |
| Rate of enhancement | 11.6 (7.7-21.5) | 10.6 (9.2-13.1) | 0.51 |

All values are expressed as median (min-max). Mann-Whitney U test was used to compare both groups. *p*-Value <0.05 was considered significant. TIC = time intensity curve.

All but one autoantibody-positive individual showed low scores for T cells, B cells, plasma cells, macrophages, adhesion molecules and vWF, all similar to those observed in the controls (Figure 1 and Table 3). Scores for $CD55^+$ FLS were lower in autoantibody positive individuals compared to healthy individuals, which can presumably be explained by chance (Table 3). The

one autoantibody-positive individual with increased scores showed patellofemoral joint space narrowing on X-ray of the knee joint, but did not fulfil ACR criteria for knee osteoarthritis.[24] Previous work has shown that the synovium is inflamed in patients with osteoarthritis.[25] This patient did not develop arthritis during follow up.

The immunohistochemical findings in the four patients who developed arthritis were in the same range as those in the other autoantibody-positive individuals, who did not develop arthritis during follow-up, as well as in the normal controls (data not shown).

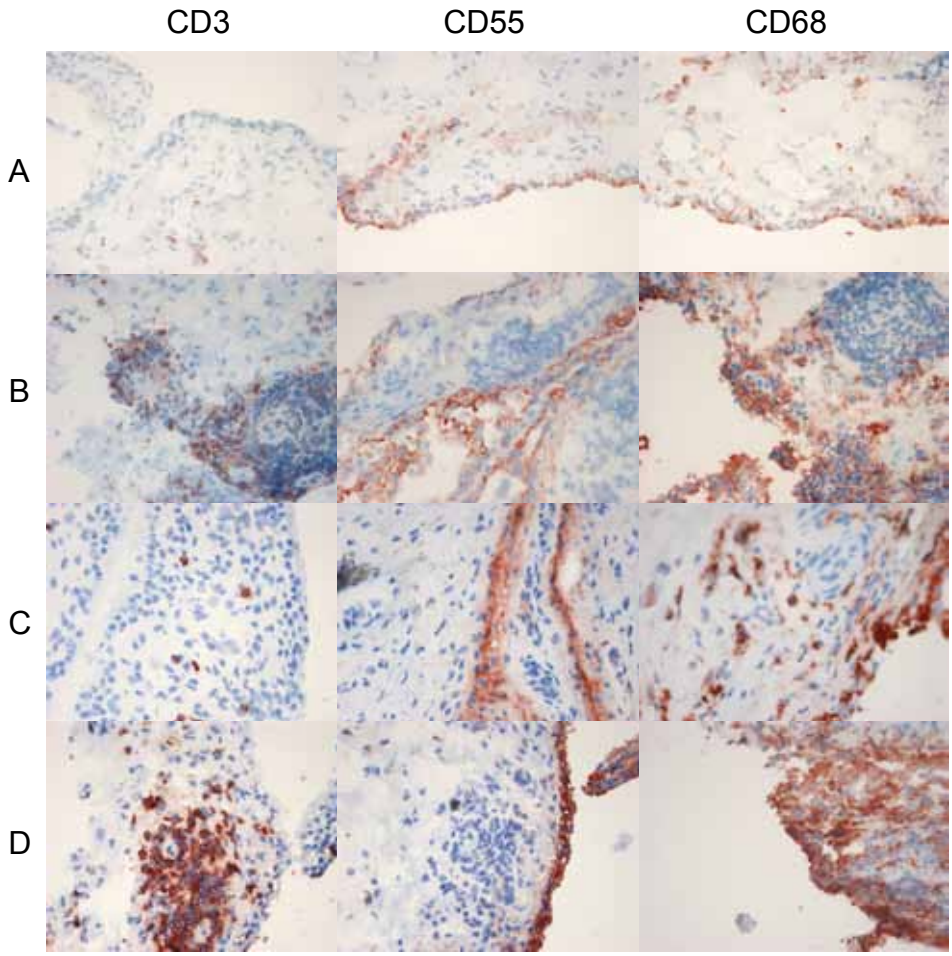


**Figure 1. Synovial tissue expression of CD3+ T cells, CD55$^+$ fibroblast-like synoviocytes, and CD68$^+$ macrophages.** A: autoantibody-positive individual, B: autoantibody-positive individuals with patellar-femoral joint space narrowing, C: healthy control (negative control), D: patient with active arthritis (positive control).

**Table 3.** Semi-quantitative scores (0-4) for phenotypic and vascular markers as well as adhesion molecules in synovial tissue of IgM rheumatoid factor (IgM-RF)-positive and/or anti-citrullinated peptide antibody (ACPA)-positive individuals without arthritis and healthy controls.

| | IgM-RF- and/or ACPA-positive individuals | Healthy controls | *p*-Value |
|---|---|---|---|
| $CD68^+$ intimal macrophages | 1.0 (0.0-3.0) | 1.0 (0.0-3.0) | 0.08 |
| $CD68^+$ sublining macrophages | 1.0 (0.0-4.0) | 1.5 (0-3.5) | 0.29 |
| $CD3^+$ T cells | 1.0 (0.0-4.0) | 0.5 (0.0-3.0) | 0.40 |
| $CD22^+$ B cells | 0.0 (0.0-4.0) | 0.0 (0.0-2.0) | 0.35 |
| $CD138^+$ plasma cells | 0.0 (0.0-4.0) | 0.0 (0.0-2.0) | 0.36 |
| $CD55^+$ fibroblast-like synoviocytes | 1.5 (1.0-2.0) | 2 (0.5-3.0) | 0.04 |
| vWF | 1.0 (0.0-2.5) | 2.0 (0.0-3.5) | 0.21 |
| E-selectin | 1.0 (0.0-2.0) | 2.0 (0.0-3.0) | 0.09 |
| ICAM | 1.0 (0.0-4.0) | 1.0 (0.0-4.0) | 0.24 |
| VCAM | 1.0 (0.0-4.0) | 1.0 (0.0-2.0) | 0.36 |

Values are shown as median (min-max). Chi-square test was used to compare both groups. *p*-Value of <0·05 was considered significant (bold). vWF = von Willebrand factor. No difference was observed between the healthy controls and the autoantibody-positive subjects, including the four autoantibody-positive individuals who developed arthritis after follow-up.

# DISCUSSION

The aetiology of RA is still largely elusive, although significant progress has been made in understanding the pathogenetic mechanisms in established disease. Interestingly, recent work has shown that autoantibody formation may precede the development of clinical signs and symptoms of RA by several years.[9,10] First, this observation provides important insights into the aetiological role of the humoral response in RA. Second, the measurement of RF and ACPA allows the identification of pre-RA patients before the development of arthritis [12], which was previously impossible.[8] About one quarter of IgM-RF- and/or ACPA-positive individuals with arthralgia develop arthritis after a median follow-up of 28 months.[12]

To determine whether the primary target of RA, the synovium, is already involved during the earliest phases preceding clinical signs and symptoms of RA, we performed MRI and synovial biopsy in IgM-RF- and/or ACPA-positive individuals without a past history of arthritis who were prospectively followed. The results presented here show for the first time that the synovium is not abnormal during this stage, even in those who develop arthritis during follow-up. Thus, systemic autoimmunity appears to precede the development of synovial inflammation in individuals who are at risk of developing RA.

A limitation of this study is the relatively small number of subjects evaluated. Obviously, the study design is challenging, as it is difficult to perform arthroscopy in individuals without arthritis strictly for research purposes. However, the results are strikingly consistent: we observed normal synovium by both MRI and immunohistochemistry in all subjects at baseline, including those who developed RA, except for the one patient who showed joint space narrowing on X-ray of the knee joint without osteoarthritis diagnosis. Moreover, in previous work we have been able

to demonstrate significantly increased synovial inflammation in a study of comparable size, when comparing synovial biopsy samples from clinically uninvolved joints of patients with established RA to those from normal controls.[7] Another limitation is that we were able to perform arthroscopies from the knee joints only in this study. It is technically not possible to obtain sufficient synovial tissue for reliable analysis from clinically uninvolved small joints. However, it appears unlikely that examination of synovial tissue samples obtained from other joints would have resulted in different findings, since: 1. RA is a systemic disease and, accordingly, we have previously shown that there is a strong correlation between the features of paired synovial biopsies from large and small joints in RA patients [17], 2. six of the subjects in the present study had in fact arthralgia of the knee joint, 3. seven of the subjects also underwent ultrasound examination of the hand in the context of routine patient care, which did not demonstrate synovitis of the small joints (data not shown), and 4. in a larger cohort it was recently shown that most autoantibody-positive individuals with arthralgia have normal ultrasound examination of the small joints of the hands.[26]

It should also be noted that by prospectively selecting all individuals with an increased risk of developing RA (based on their autoantibody status and complaints of arthralgia), we have included individuals who might never develop clinically manifest arthritis. To date, it has not been possible to identify those individuals in whom systemic autoimmunity will proceed to evident RA in all cases. By applying this prospective study design and increasing participant numbers we hope to identify future biomarkers that can identify individuals who will go on to develop clinically manifest RA with an even higher likelihood.

Genetic, stochastic and environmental factors may all play a role in the activation of the innate and adaptive immune system involved in the earliest, preclinical phase of RA, called pre-RA (Figure 2). During this stage, which may last several years, increased levels of CRP may be detected in the peripheral blood together with RF and/or ACPA.[27,28] Our data suggest that the initial immune response leading to the production of autoantibodies takes place at sites other than the synovium. One candidate site is the lung, where various agents, including cigarette smoke, can trigger inflammation resulting in citrullination of specific peptides.[29] Combined with a loss of tolerance to these specific citrullinated peptides, this may result in ACPA formation.[29,30] Of note, Klareskog and colleagues have shown that smoking is an independent risk factor for the development of ACPA-positive RA.[31]

What determines the transition from the pre-RA phase to chronic synovitis? Obviously, this will be one of the major research questions for the near future.[32] Possibly a 'second hit' is necessary to induce citrullination of proteins in the synovium. This could for example be a minor

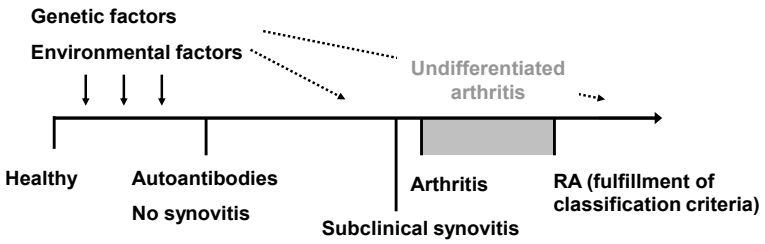


**Figure 2. The timeline of autoantibody-positive rheumatoid arthritis: the different stages of disease.** Autoantibody formation may precede the development of clinical signs and symptoms of RA by several years. The presence of subclinical synovitis may probably last several weeks rather than months.

trauma or a viral infection. We previously found that citrullination may occur in the synovium in any form of arthritis.[33] Conceivably, in the presence of pre-existing immunity against citrullinated antigens, this might lead to epitope spreading and autonomous progression of synovitis. ACPA immunoglobulin isotypes and epitopes recognised by ACPA have indeed been shown to evolve over time.[11,34,35]

Consistent with this hypothesis, ACPA reactivity against joint specific epitopes has only been observed in RA patients, but not in ACPA-positive relatives of RA patients without arthritis.[11]

Based on studies in animal models of RA, presymptomatic synovitis may precede the development of clinical signs and symptoms of arthritis by several weeks.(7) As we found in the present study that the synovium was normal in subjects, who developed arthritis after a median follow-up of 3 months, we postulate that the phase of subclinical synovitis in RA is in the range of weeks rather than months (Figure 2).

In conclusion, subclinical inflammation of the synovium does not coincide with the appearance of serum IgM-RF or ACPA antibodies during the pre-RA stage. Thus, systemic autoimmunity may precede the development of (subclinical) synovitis by several months to years and we therefore suggest to explore preventive strategies aimed at interfering with the humoral immune response before synovial inflammation develops. We hypothesise that in the presence of circulating ACPA, a 'second hit' may be required leading to citrullination of peptides in the synovium with subsequent broadening of the humoral response against citrullinated antigens in the joint and autonomous disease progression, as a result, in ACPA-positive RA.

## ABBREVIATIONS

| | |
|---|---|
| ACPA | anti-citrullinated protein antibodies |
| AMC | Academic Medical Center |
| CRP | C-reactive protein |
| DCE-MRI | dynamic contrast-enhanced MRI |
| DMARDs | disease-modifying antirheumatic drugs |
| ESR | erythrocyte sedimentation rate |
| FLS | fibroblast-like synoviocytes |
| IgM-RF | IgM rheumatoid factor |
| RA | rheumatoid arthritis |
| SQA | semi-quantitative analysis |
| TIC | time-dependent signal intensity changes |
| VAS | visual analogue scale |
| vWF | von Willebrand factor |

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