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Features of the Synovium of Individuals at Risk of Developing Rheumatoid Arthritis

Implications for Understanding Preclinical Rheumatoid Arthritis

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Objective. **Findings from previous studies have suggested that subclinical inflammation of the synovium does not coincide with the appearance of rheumatoid arthritis (RA)–specific autoantibodies. This study was undertaken to examine the relationship between the presence of autoantibodies, changes in the synovium, and development of arthritis over time in a markedly larger, prospective study.**

Methods. **Fifty-five individuals who were IgM rheumatoid factor positive and/or anti–citrullinated protein antibody (ACPA) positive (detected by the anti–cyclic citrullinated peptide antibody test) and who were without any evidence of arthritis upon physical examination were included in the study. ACPAs were subsequently also detected using a multiplex chip-based assay. All individuals underwent magnetic resonance imaging and mini-arthroscopic synovial biopsy sampling of a knee joint at inclusion and were prospectively followed up. Proportional hazards regression analysis was performed to investigate whether changes in the synovium were associated with the onset of arthritis.**

Results. **Fifteen individuals (27%) developed arthritis after a median followup time of 13 months (interquartile range 6–27 months; range 1–47 months). No overt synovial inflammation was observed, but CD3+ T cell numbers in the biopsy tissue showed a borderline association with subsequent development of clinically manifest arthritis (hazard ratio 2.8, 95% confidence interval [95% CI] 0.9–9.1; $P = 0.088$). In addition, the presence of CD8+ T cells was associated with ACPA positivity (odds ratio [OR] 16.0, 95% CI 1.7–151.1) and with the total number of ACPAs present (OR 1.4, 95% CI 1.0–1.8).**

Conclusion. **These findings confirm and extend previous results showing the absence of clearcut synovial inflammation in individuals having systemic autoimmunity associated with RA. However, subtle infiltra-**

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Dr. Serre is listed on patents for the diagnostic use of citrullinated peptides; these patents are owned by bioMérieux, France. Dr. Klareskog has served as an expert witness on behalf of Pfizer. Dr. Tak owns stock or stock options in GlaxoSmithKline.

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tion by synovial T cells may precede the signs and symptoms of arthritis in preclinical RA.

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by inflammation of the synovial tissue. Certain genes, such as class II major histocompatibility complex (MHC) genes (1) and PTPN22 (2), increase the susceptibility to RA. In subjects with genetic susceptibility, environmental factors, including smoking and perhaps periodontitis, may lead to the development of autoantibodies, such as rheumatoid factor (RF) and anti–citrullinated protein antibodies (ACPAs) (3,4). These autoantibodies define individuals with systemic autoimmunity associated with RA (5). Although RA-specific autoantibodies can be present more than 10–15 years before joint inflammation becomes clinically manifest (6–8), only a minority of individuals with RA-specific autoantibodies actually proceed to develop clinically manifest RA.

We previously proposed that, whereas the initial immune response leading to the production of autoantibodies may take place at sites other than the synovium, a second "hit," due to either a minor trauma or a viral infection, may lead to citrullination of synovial proteins and subsequent epitope spreading (9). Consistent with the hypothesis that the initial changes may take place at sites other than the synovium, such as the lung (10,11), we found no evidence of overt synovial inflammation in the joints of 13 subjects at risk of developing RA (9). Because of the small sample size of that cross-sectional study, and in light of the importance of the implications for our understanding of the etiology of RA, we decided to validate and extend the results in a larger, prospective study. In addition, we aimed to investigate the ACPA fine specificity in association with synovial tissue inflammation.

SUBJECTS AND METHODS

Study subjects. Individuals who had arthralgia and/or a family history of RA, but without any evidence of arthritis upon thorough physical examination, and who were positive for IgM-RF and/or ACPAs (detected by the anti–cyclic citrullinated peptide [anti–CCP] antibody test) were included in the study between June 2005 and August 2010. These individuals were considered to be at risk of developing RA, a status characterized by the presence of systemic autoimmunity associated with RA (defined as phase c, according to the European League Against Rheumatism [EULAR] recommendations [5]), with or without environmental risk factors (defined as phase b, according to the EULAR recommendations [5]) and with or without symptoms without clinical arthritis (defined as phase d, according to the EULAR recommendations [5]). IgM-RF was measured using an IgM-RF enzyme-linked immunosorbent assay (ELISA) (upper limit of normal [ULN] 12.5 IU/ml) from Sanquin. This ELISA was used until December 2009, and thereafter, we used an IgM-RF ELISA from Hycor Biomedical (ULN 49 IU/ml). IgM-RF levels were categorized into negative, <ULN, low positive (≤3 times ULN), and high positive (>3 times ULN) (12). IgA-RF and IgG-RF were measured using Quanta Lite IgA-RF and IgG-RF ELISAs from Inova Diagnostics. Anti-CCP antibodies were measured using an anti–CCP-2 ELISA CCPlus kit (ULN 25 kAU/liter; Euro-Diagnostica).

The study subjects were recruited either via the outpatient clinic of the Department of Clinical Immunology and Rheumatology at the Academic Medical Center, Amsterdam, via referral from the rheumatology outpatient clinic of Reade, Amsterdam, or via testing family members of RA patients in the outpatient clinic or at public fairs across The Netherlands. The study was performed according to the principles of the Declaration of Helsinki and was approved by the Institutional Review Board of the Academic Medical Center. All study subjects gave their written informed consent.

Study design. At baseline, demographic data and a serum sample were obtained from each study subject. Magnetic resonance imaging (MRI) of an arbitrary knee joint was performed within 1 week before mini-arthroscopic synovial biopsy sampling of the same knee joint was performed (9). Study subjects were followed up over time until arthritis onset or until January 1, 2012 (censored). There were yearly study visits. The development of arthritis, defined as a swollen joint, was the end point of this study. Individuals who developed arthritis were also evaluated by 2 independent investigators (MS and DG or MH and DG) at an interim visit, in order to confirm the presence of clinically apparent arthritis.

Clinical parameters. At every study visit, the following clinical and disease activity parameters were obtained: tender joint count in 68 joints, swollen joint count in 66 joints, duration of morning stiffness (in minutes), IgM-RF levels (in IU/ml), anti-CCP levels (in kAU/liter), erythrocyte sedimentation rate (in mm/hour), and serum levels of C-reactive protein (CRP) (in mg/liter).

Detection of the fine specificities of autoantibodies against citrullinated peptides. Serum samples were analyzed for the presence of ACPA-specific IgG, using a custom-made microarray based on the ImmunoCAP ISAC system (Phadia Austria). A full description of this system has been provided previously (13,14). Antibodies to the following citrullinated peptides and their native arginine-containing counterparts were determined: citrullinated α-enolase peptide 1 (CEP-1) (α-enolase 5–21), vimentin 2–17, vimentin 60–75, CCP-1 (filaggrin 307–324), fibrinogen β-chain 36–52, fibrinogen β-chain 60–74, fibrinogen β-chain 573 (fibrinogen β-chain 563–583), fibrinogen β-chain 591 (fibrinogen β-chain 580–600), fibrinogen α-chain 36–50, fibrinogen α-chain 621–635 (15).

The difference (in arbitrary units) between the citrullinated peptide and the arginine peptide was calculated. The cutoff level (in arbitrary units) defining positivity for each

ACPA specificity was determined on the basis of the 98th percentile of the values in 100 healthy controls.

MRI. Images of the knees joints were acquired on either a closed 1.5-Tesla MRI scanner (GE Signa Horizon Echospeed, LX9.0; General Electric Medical Systems) or an open 1-Tesla MRI scanner (Panorama Open; Philips); the latter scanner was used as a replacement for the former scanner. The minimum imaging protocol consisted of a sagittal STIR, an axial T2 with fat suppression, and a sagittal T1 before and after contrast injection. A musculoskeletal radiologist with 15 years of experience and a research fellow with 4 years of experience in musculoskeletal radiology scored all images. Both were blinded with regard to the study outcome (arthritis development) in each subject. The presence of synovitis and hydrops were scored in 4 compartments of the knee joint (medial, lateral, central, and suprapatellar; minimum score of 0 and maximum score of 3, for each compartment), as well as the presence (score of 1) or absence (score of 0) of bone marrow edema, erosions, and cartilage damage (in each of 6 locations: the patellofemoral joint [2 sites], medial compartment [2 sites], and lateral compartment [2 sites]).

Mini-arthroscopic synovial biopsy sampling. All study subjects underwent mini-arthroscopic synovial biopsy sampling of a knee joint at baseline (16). To correct for sampling error, 6–8 synovial tissue samples were collected for immunohistochemistry, as described previously (17–19). The synovial biopsy samples were snap-frozen en bloc in Tissue-Tek OCT compound (Miles) immediately after collection. Sections (5 μm each) were cut and mounted on StarFrost adhesive glass slides (Knittelgläser). Sealed slides were stored at −80°C until further used.

Immunohistochemistry. Synovial tissue sections were stained using mouse monoclonal antibodies against T cells (anti-CD3, clone SK7 [Becton Dickinson]; anti-CD4, clone SK3 [Becton Dickinson]; and anti-CD8, clone C8/144B [Dako]), B cells (anti-CD22, clone RFB4; Millipore), fibroblast-like synoviocytes (anti-CD55, clone 67; AbD Serotec), macrophages (anti-CD68, clone EBM11; Dako), plasma cells (anti-CD138, clone B-B4; Immunotech), blood vessels (anti–von Willebrand factor [anti-vWF)], clone F8/86; Dako), and citrullinated fibrinogen (anti–citrullinated fibrinogen, clone 20B2; ModiQuest Research).

Staining was performed using a 3-step immunoperoxidase method to detect bound anti-CD55, anti-vWF, anti-CD68, and anti-CD138 antibodies, as described previously (20). A 2-step immunoperoxidase method was used to detect bound anti-CD4, anti-CD8, and anti–citrullinated fibrinogen antibodies. For anti-CD3 and anti-CD22, we used a 2-step immunoperoxidase method with a secondary polymer–horseradish peroxidase–conjugated anti-mouse antibody (EnVision+ System; Dako). As a negative control, irrelevant isotype-matched immunoglobulins, instead of the primary antibody, were applied to the sections. The primary antibodies were incubated for 60 minutes (or overnight for CD4, CD8, CD55, vWF, and citrullinated fibrinogen antibodies). Aminoethylcarbazole was used as a chromogen (Vector Laboratories). Slides were counterstained with Gill's hematoxylin and mounted in Kaiser's glycerol gelatin (Merck). The intensity of the staining was scored by 2 independent observers (MH and BS or MH and NS), using semiquantitative analysis on a 5-point scale (score range 0–4, where 0 represents no expression and 4 represents the maximum expression in all tissue sections analyzed) (21). Samples from 5 RA patients were used as a positive control.

Analysis of CD68+ cell expression was performed separately in the intimal lining layer and synovial sublining. Since CD4 is expressed not only by T cells but also by macrophages ($CD4^{dim}$), only bright staining was scored as positive for CD4+ T cells. Scoring for the presence of citrullinated fibrinogen was done in a dichotomous manner (presence versus absence of staining). When scores between the 2 observers did not match, a definite score was obtained upon mutual agreement.

Statistical analysis. Continuous, normally distributed data are presented as the mean ± SD, and differences between study groups were analyzed using *t*-tests for unpaired samples. Non–normally distributed data are presented as the median (interquartile range [IQR]), and differences between study groups were analyzed using the Mann-Whitney U test. Categorical data are presented as the number (percent) of subjects, and differences between groups were analyzed using the chi-square test.

To investigate associations of synovial inflammation markers with arthritis onset, the expression levels were dichotomized, and Cox proportional hazards regression analysis was performed. Followup duration was defined as the time between inclusion in the cohort and the onset of clinically manifest arthritis, or between inclusion and January 1, 2012 (censored). First, variables were tested variable-by-variable. Variables showing an association at a significance level of $P < 0.2$ were arbitrarily selected for multivariable analysis (forward and backward selection procedures). Variables with meaningful statistical interactions were excluded up front.

To determine associations between the presence of ACPAs and synovial inflammation, odds ratios with 95% confidence intervals (95% CIs) were obtained using logistic regression analysis, with synovial inflammation as the dependent variable. Statistical analysis was performed using PASW Statistics version 18 (SPSS). *P* values less than 0.05 were considered significant.

RESULTS

Cohort description. Of the 55 individuals included in the study, 41 were single-positive for RF or anti-CCP (19 were IgM-RF positive only, 22 were anti-CCP positive only), and 14 individuals were double-positive. The subjects were followed up for a median duration of 27 months (IQR 14–47 months; range 1–75 months). Fifteen (27%) of the 55 individuals developed arthritis over time, at a median of 13 months (IQR 6–27 months; range 1–47 months) after inclusion. The clinical characteristics of the subjects and fulfillment of RA criteria at the moment of arthritis onset were described previously (22). Individuals who did not develop arthritis

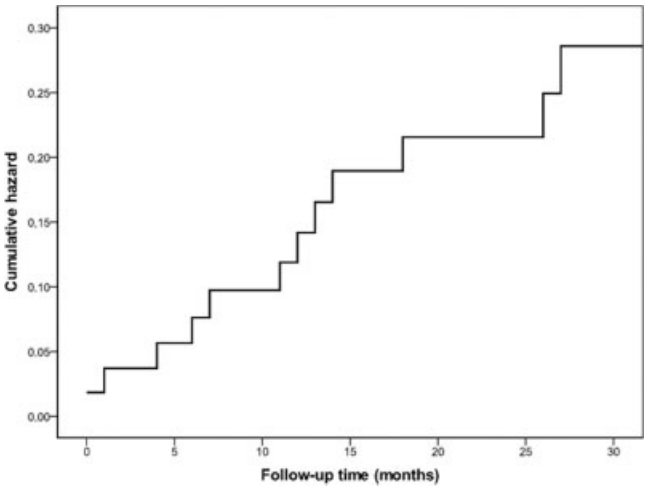

Figure 1. Cumulative hazard of arthritis onset over time in the study cohort of individuals at risk of developing rheumatoid arthritis.

were followed up for a median duration of 37 months (IQR 19–52 months; range 3–75 months). Figure 1 shows the cumulative hazard of arthritis development in this study. The baseline clinical characteristics of individuals who developed arthritis during followup were generally comparable to those of individuals who did not develop arthritis during followup (see Table 1).

To more extensively examine the risk profile for arthritis development in these autoantibody-positive individuals, we examined, in addition to IgM-RF, the IgA-RF and IgG-RF status of all subjects. We found that 38% of individuals in the cohort were IgA-RF positive and 32% were IgG-RF positive. In addition, of the 19 individuals who were IgM-RF positive and anti-CCP negative, 4 were also positive for either IgA-RF or IgG-RF, and 1 was also positive for both IgA-RF and IgG-RF. In the group of 15 individuals who developed arthritis, a higher percentage was positive for IgA-RF compared to the group of 40 individuals who did not develop arthritis.

In addition, we investigated the ACPA repertoire and compared the fine specificity of the ACPAs present between individuals who did and those who did not develop arthritis. For 2 of the individuals (neither of whom developed arthritis), no samples were available to be included in these analyses. Moreover, for 2 other individuals (1 who developed arthritis, 1 who did not), we did not obtain results for the vimentins, filaggrin and fibrinogen β-chain specificities, due to technical reasons.

In the whole cohort, 60% of individuals were positive for any of the specific ACPAs analyzed. The median total number of ACPAs that were present in these individuals was 4 (IQR 3–7). Of the 19 individuals who were IgM-RF positive and anti-CCP negative, 3 were positive for a specific ACPA. Conversely, 4 of the 32 individuals positive for ACPAs by the anti–CCP-2 test were negative for a specific ACPA. Reactivity against the citrullinated peptides CEP-1, vimentin 60–75, and fibrinogen β 573, as well as reactivity against at least 1 of the citrullinated peptides evaluated, was significantly more frequent in individuals who developed arthritis compared to those who did not develop arthritis. Results are shown in Table 1. Importantly, the total number of ACPAs present was significantly higher in individuals who developed arthritis compared to those who did not develop arthritis.

Subtle synovial infiltration by T cells, but no overt synovial inflammation, preceding the development of arthritis. Complete synovial tissue samples from 6 individuals had to be excluded from the immunohistochemical analyses because they failed quality standards. Synovial tissue samples from 35–49 individuals could be included in the immunohistochemical analyses of expression of the various inflammation markers. In 6 individuals, MRI was not performed, because of logistic problems.

Semiquantitative scores for the expression of the inflammation markers in the synovium were low, as compared to the scores in the synovium of the 5 RA patients used as a positive control (results not shown), consistent with the findings in our previous study (9). In the autoantibody-positive individuals, the scores for the expression of CD3 and citrullinated fibrinogen were either 0 or 1. For the expression of CD4, CD8, and CD68, the scores were 0–3, categorized as 0 for negative and $\geq$1 for positive. CD55 expression was scored as 1 (low positive) or as 2 (high positive). Expression of vWF was scored in the range of 0–3, categorized as 1 (low) for scores $\leq$1 and 2 (high) for scores $\geq$2. Expression of CD22 and CD138 was observed in only 1 individual each (2 different subjects), and therefore CD22 and CD138 were not included in the statistical analyses.

Proportional hazards regression analysis was then performed. First, all variables were analyzed variable-by-variable (Table 2). Recognition of at least 1 of the citrullinated peptides analyzed showed a trend toward an association with arthritis development (hazard ratio [HR] 4.2, 95% CI 0.9–18.6, $P = 0.062$), and the total number of citrullinated peptides recognized was significantly associated with arthritis development (HR 1.2, 95% CI 1.0–1.4, $P = 0.012$). Of note, subjects were

Table 1. Comparison of the baseline demographic and clinical characteristics and positivity for autoantibodies against citrullinated peptides between individuals who developed arthritis and those who did not develop arthritis over the followup*

	No arthritis developed (n = 40)†‡	Arthritis developed (n = 15)‡	*P*
Sex, female	28 (70)	9 (60)	0.481
Age, mean ± SD years	44 ± 12	47 ± 8	0.471
Arthralgia	37 (93)	14 (93)	0.916
FDR with RA§	10 (29)	6 (40)	0.427
Arthralgia positive/FDR with RA	7 (20)	5 (33)	0.598
Arthralgia positive/FDR without RA	25 (71)	9 (60)	
Arthralgia negative/FDR with RA	3 (9)	1 (7)	
Arthralgia negative/FDR without RA	0 (0)	0 (0)	
IgM-RF			
Positive	24 (57)	9 (60)	0.847
Low positive level¶	15 (62)	4 (44)	0.350
High positive level¶	9 (38)	5 (56)	
Anti-CCP			
Positive	24 (60)	12 (80)	0.165
Level, median (IQR) kAU/liter¶	466 (70–1,230)	452 (103–2,519)	0.585
IgM-RF and anti-CCP double-positive	8 (20)	6 (40)	0.129
IgA-RF positive	11 (28)	6 (43)	0.314
IgG-RF positive	10 (26)	10 (71)	0.002
ESR, median (IQR) mm/hour	9 (2–20)	7 (5–15)	0.568
CRP, median (IQR) mg/liter	2.1 (1.0–4.3)	4.0 (1.5–10.0)	0.105
Morning stiffness, median (IQR) minutes	5 (0–30)	10 (5–30)	0.436
VAS score for pain, median (IQR) mm	32 (5–66)	48 (11–76)	0.321
VAS score for global disease activity, median (IQR) mm	38 (10–64)	53 (9–76)	0.253
TJC68, median (IQR)	2 (0–8)	2 (0–8)	0.715
SJC66, median (IQR)	0	0	1
Citrullinated peptide positivity			
Anti–CEP-1	9 (24)	9 (60)	0.012
Anti–vimentin 2–17	8 (22)	5 (36)	0.303
Anti–vimentin 60–75	8 (22)	9 (64)	0.004
Anti–CCP-1	8 (22)	6 (43)	0.129
Anti–fibrinogen β 36–52	10 (27)	7 (50)	0.120
Anti–fibrinogen β 60–74	14 (37)	7 (47)	0.510
Anti–fibrinogen β 573	8 (22)	8 (57)	0.015
Anti–fibrinogen β 591	10 (27)	5 (36)	0.543
Anti–fibrinogen α 36–50	1 (3)	1 (7)	0.487
Anti–fibrinogen α 621–635	6 (16)	6 (40)	0.058
At least 1 citrullinated peptide	20 (54)	12 (86)	0.037
Total number of ACPAs present, median (IQR)	1 (0–4)	4 (2–8)	0.017

* Except where indicated otherwise, values are the number (%) of subjects. Differences in categorical variables were analyzed using the chi-square test, and differences in continuous variables were analyzed using the Mann-Whitney U test. FDR = first-degree relative; RA = rheumatoid arthritis; ACPAs = anti–citrullinated protein antibodies; IQR = interquartile range; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; VAS = visual analog scale (range 0–100 mm); TJC68 = tender joint count in 68 joints; SJC66 = swollen joint count in 66 joints; anti–CEP-1 = anti–citrullinated α-enolase peptide 1.

† Seven individuals were at risk of arthritis development for a shorter period of time. Two of these patients were started on treatment with hydroxychloroquine for arthralgias, and 2 were started on anti–tumor necrosis factor therapy because of a diagnosis of ankylosing spondylitis (n = 1) or Crohn's disease (n = 1). Three of these patients were lost to followup after some time.

‡ For 2 of the individuals (who did not develop arthritis), no samples were available to be included in these analyses. For 2 additional individuals (1 who developed arthritis, 1 who did not), we did not obtain results for the vimentins, anti–cyclic citrullinated peptide 1 (anti–CCP-1; filaggrin 307–324), and fibrinogen β-chains, due to technical reasons.

§ Missing for 5 individuals (in whom no arthritis developed).

¶ Levels were determined only in individuals who were found positive. A low positive level of IgM rheumatoid factor (IgM-RF) was defined as $\leq$3 times the upper limit of normal (ULN), and a high positive level of IgM-RF was defined as >3 times the ULN.

Table 2. Univariate proportional hazards regression analysis assessing associations with the onset of arthritis in individuals at risk*

	HR (95% CI)	*P*
MRI factors		
Synovitis (per unit)	0.9 (0.6–1.4)	0.649
Hydrops (per unit)	1.1 (0.9–1.5)	0.277
Cartilage degeneration (per unit)	0.1 (0.0–9.4)	0.324
Bone marrow edema (per unit)	0.6 (0.2–2.2)	0.487
Erosions (per unit)	1.9 (0.2–15.2)	0.529
Synovial tissue factors		
CD3 (positive [n = 21] vs. negative [n = 28])†	2.8 (0.9–9.1)	0.088
CD4 (positive [n = 23] vs. negative [n = 12])	2.9 (0.4–23.6)	0.311
CD8 (positive [n = 19] vs. negative [n = 17])†	2.8 (0.7–10.5)	0.133
CD55 (low [n = 15] vs. high [n = 31])	2.2 (0.5–10.5)	0.275
CD68		
Sublining (positive [n = 1] vs. negative [n = 38])	0.0 (0.0–infinite)	0.628
Lining (positive [n = 5] vs. negative [n = 34])	0.5 (0.1–4.1)	0.549
von Willebrand factor (low [n = 17] vs. high [n = 21])	1.7 (0.5–5.7)	0.373
Citrullinated fibrinogen (positive [n = 28] vs. negative [n = 14])	1.4 (0.4–4.3)	0.591

* Synovial tissue factors were determined using immunohistochemical staining and visual inspection by microscopy. CD3, CD4, and CD8 are markers for T cells, CD55 is a marker for fibroblast-like synoviocytes, and CD68 is a marker for macrophages. Results are presented as the hazard ratio (HR) and 95% confidence interval (95% CI). MRI = magnetic resonance imaging.
† Variable included in multivariate analysis ($P < 0.2$).

selected based on being positive for RF and/or anti-CCP. No other clinical parameters (results not shown) or MRI parameters (Table 2) were associated with arthritis development.

With respect to immunohistochemical findings in the synovial tissue, there was no overt synovial inflammation in the subjects during preclinical RA. However, the presence of CD3+ T cells at baseline showed a trend toward an association with arthritis development after followup (HR 2.8, 95% CI 0.9–9.1; $P = 0.088$) (Table 2). No association between the expression of CD3 and the presence of arthralgia in the knee joint was observed ($P = 0.210$). A similar trend toward an association with arthritis development was seen for the expression of CD8 in the synovium (HR 2.8, 95% CI 0.7–10.5; $P = 0.133$). It appeared that, in particular, expression of both CD3 and CD8 (CD3+CD8+ T cells) explained the trend toward an association with arthritis development (for double-positive versus single-positive or double-negative, HR 2.9, 95% CI 0.9–9.4 [$P = 0.086$]; for double-positive versus double-negative, HR 6.3, 95% CI 0.8–53 [$P = 0.088$]). This may suggest that synovial CD8+ T cells are involved in the earliest stages of RA. Expression of other synovial tissue markers indicative of the presence of inflammatory cells and blood vessels was not associated with arthritis development, thus confirming and extending our previous results.

Taken together, these results confirm that there was no evident synovitis in individuals at risk of developing RA during the preclinical phases (phases c and d, according to the EULAR recommendations [5]). However, we found an indication for subtle infiltration of T cells in the synovium that preceded the development of clinically manifest arthritis.

Association of arthritis development with synovial T cell infiltration combined with the presence of ACPAs. After exclusion of potentially meaningful statistical interactions in the univariate proportional hazards regression analysis (results not shown), those variables that showed an association with arthritis development at the level of $P < 0.2$ were tested in multivariable analyses. Table 3 shows the results of the multivariate proportional hazards regression analyses.

The combination of ACPA positivity (defined as recognition of ≥1 citrullinated peptide versus 0 citrullinated peptides) and expression of CD3 in the synovium resulted in an increased association of CD3 with arthritis development (HR 3.2, 95% CI 1.0–10.5; $P = 0.053$), as compared to that in the univariate model with only CD3 expression included (designated model 4 in Table 3). The combination of CD8 expression in the synovium and either a status of ACPA positivity or CD3 expression or both resulted in the absence of a significant association with the development of arthritis (results not shown).

To investigate whether there is an association of onset of arthritis with subgroups of ACPA and CD3 positive or negative status, we performed proportional hazards regression analyses based on these subgroups

Table 3. Multivariate proportional hazards regression analysis assessing associations with the onset of arthritis in individuals at risk*

Variables in model	HR (95% CI)	*P*
Model 1: ACPAs (recognition of ≥1 citrullinated peptide vs. 0 citrullinated peptides)	4.2 (0.9–18.6)	0.062
Model 2: CD3 (positive vs. negative)	2.8 (0.9–9.1)	0.088
Model 3: CD8 (positive vs. negative)	2.8 (0.7–10.5)	0.133
Model 4: ACPAs (recognition of ≥1 citrullinated peptide vs. 0 citrullinated peptides) and CD3 (positive vs. negative)	4.2 (0.9–19.2); 3.2 (1.0–10.5)	0.053

* CD3 and CD8 are markers for synovial T cells. Results are presented as the hazard ratio (HR) and 95% confidence interval (95% CI). ACPAs= anti–citrullinated protein antibodies.

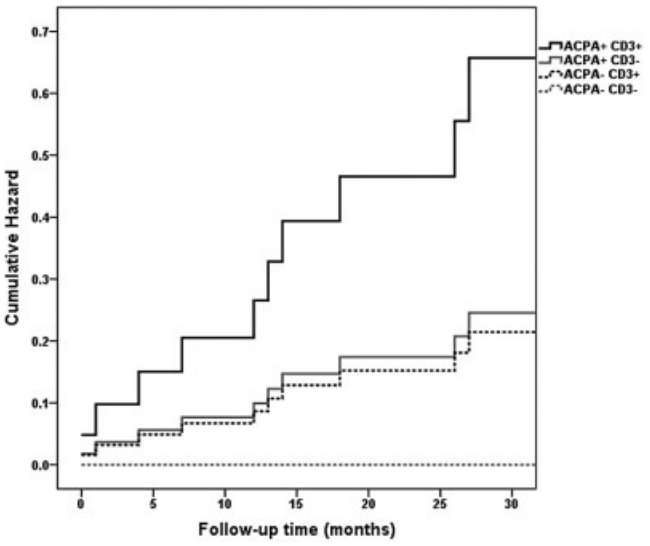

Figure 2. Proportional hazards regression analyses of the cumulative hazard of arthritis development over the followup period in subsets of individuals with or without anti–citrullinated protein antibodies (ACPAs) and with or without CD3 expression in the synovium.

(see Figure 2 for the cumulative hazard plots). No statistically significant associations with the development of arthritis were observed (results not shown), which may be due to the lack of statistical power in this relatively small sample size. Thus, as expected, positivity for both ACPAs and CD3 expression in the synovium was significantly associated with arthritis development, when compared to the absence of positivity for either or both of these factors (double-positive versus single-positive or double-negative, HR 3.8, 95% CI 1.3–11.3; $P = 0.018$).

Synovial expression of citrullinated fibrinogen in individuals at risk of developing arthritis. The role of synovial citrullinated peptides in the pathogenesis of RA is unknown and has never been studied in individuals at risk of developing the disease (23). Therefore, we examined the synovial expression of citrullinated fibrinogen, one of the major candidate autoantigens in RA. Of the autoantibody-positive individuals, 33% were positive for citrullinated fibrinogen, as compared to 80% of the 5 RA patients used as a positive control. In comparison to the RA patients, the expression level of citrullinated fibrinogen was very low in all autoantibody-positive individuals (results not shown). Expression of citrullinated fibrinogen in the synovium was observed not only in individuals who developed arthritis after followup (42%), but also in those who did not develop arthritis (30%) ($P = 0.462$). The presence of citrullinated fibrinogen was not predictive of arthritis onset. Moreover, expression of citrullinated fibrinogen was not dependent on the anti-CCP status, being observed in 39% of anti-CCP–positive individuals and 21% of anti-CCP–negative individuals ($P = 0.247$).

Association of citrullinated peptide recognition with the presence of synovial CD8+ T cells. Since we observed a borderline association between the presence of synovial T cells and arthritis development, and since recognition of citrullinated peptides was higher in individuals who developed arthritis compared to those who did not develop arthritis, we investigated whether the

Table 4. Association between ACPAs and expression of CD3, CD4, CD8, and citrullinated fibrinogen in the synovium of individuals at risk of developing rheumatoid arthritis*

	Odds ratio (95% confidence interval)			
Peptide positivity	CD3	CD4	CD8	Citrullinated fibrinogen
Anti–CEP-1	2.9 (0.8–10.1)	2.7 (0.6–13.0)	1.3 (0.3–5.1)	1.0 (0.3–4.1)
Anti–vimentin 2–17	0.7 (0.2–2.7)	2.3 (0.4–13.4)	6.2 (1.1–36.2)	1.3 (0.3–5.6)
Anti–vimentin 60–75	2.2 (0.6–8.0)	1.6 (0.3–8.1)	3.4 (0.8–14.8)	3.2 (0.8–12.8)
Anti–CCP-1	0.9 (0.2–3.4)	2.8 (0.5–16.2)	3.9 (0.8–18.6)	1.4 (0.4–5.8)
Anti–fibrinogen β 36–52	1.0 (0.3–3.4)	11.0 (1.2–102.0)	6.2 (1.3–30.2)	2.6 (0.7–10.1)
Anti–fibrinogen β 60–74	1.1 (0.3–3.7)	2.7 (0.6–13.0)	6.0 (1.3–26.7)	1.9 (0.5–7.1)
Anti–fibrinogen β 573	2.8 (0.7–10.5)	1.6 (0.3–8.1)	2.7 (0.6–11.7)	1.9 (0.5–7.6)
Anti–fibrinogen β 591	1.2 (0.3–4.2)	7.5 (0.8–69.7)	2.1 (0.5–9.3)	1.0 (0.2–4.4)
Anti–fibrinogen α 36–50	NA	NA	NA	NA
Anti–fibrinogen α 621–635	0.9 (0.2–3.6)	4.4 (0.5–42.0)	1.9 (0.4–9.9)	0.9 (0.2–4.4)
Recognition of at least 1 citrullinated peptide	0.8 (0.2–2.7)	3.5 (0.7–17.7)	16.0 (1.7–151.1)	2.4 (0.5–11.0)
Total number of ACPAs present	1.1 (0.9–1.3)	1.3 (1.0–1.8)	1.4 (1.0–1.8)	1.1 (0.9–1.4)

* CD3, CD4, and CD8 are markers for synovial T cells. ACPAs = anti–citrullinated protein antibodies; anti–CEP-1 = anti–citrullinated α-enolase peptide 1; anti–CCP-1 = anti–cyclic citrullinated peptide 1 (anti–filaggrin 307–324); NA = not applicable due to extreme skewing of the data.

presence of synovial T cells was associated with reactivity against (specific) citrullinated peptides. These results are shown in Table 4.

We found that the presence of CD8+ T cells was associated with reactivity against citrullinated vimentin 2–17 and citrullinated fibrinogen β-chains 36–52 and 60–74. In addition, the presence of CD8+ T cells was associated with the recognition of at least 1 citrullinated peptide and with the total number of citrullinated peptides recognized.

The presence of CD4+ T cells was associated with reactivity against citrullinated fibrinogen β-chain 36–52. Interestingly, the ACPAs that were more frequently present in individuals who had developed arthritis in comparison with those who had not developed arthritis were different from the ACPAs that were associated with the presence of CD4+ or CD8+ T cells. The presence of synovial CD3+ T cells and citrullinated fibrinogen was not associated with reactivity against citrullinated peptides.

DISCUSSION

We evaluated the synovium by MRI and immunohistochemical analyses before the onset of clinical signs and symptoms of arthritis in autoantibody-positive individuals who were at risk of developing RA. After adjustment for differences in followup duration, we observed that the presence of inflammatory cells or blood vessels in the synovial tissue was not associated with the development of arthritis. Consistent with these findings, MRI showed no indication of synovitis, confirming and extending our previous findings (9). However, there was a trend toward increased synovial T cell numbers in subjects who subsequently developed arthritis compared to those who had not (yet) developed arthritis; this effect was stronger when combined with ACPA status.

Thus, no overt subclinical synovitis is generally present more than 1 month before the onset of arthritis in individuals who are at risk of developing RA. This suggests that infiltration of the synovial tissue by inflammatory cells would be a process that occurs relatively late in the pathogenesis of RA, beginning close to the onset of clinically manifest disease. In contrast, systemic changes may be observed years before the onset of arthritis, as shown by the detection of RA-specific autoantibodies (IgM-RF and ACPAs) (6–8), increasing levels of CRP toward the onset of arthritis (24), and increased levels of monocyte chemotactic protein 1 in individuals who developed RA later (25). To gain new insights into the earliest phase of RA pathogenesis, it will therefore be important to study other compartments of the immune system, in addition to the synovium. For this reason, the first studies analyzing lymph node tissue in different phases of RA are under way (26,27).

However, our results suggest that subtle infiltration of the synovium by T cells might precede the onset of clinically manifest arthritis. The exact role of T cells in the pathogenesis of RA is still not completely clear (28,29). The presence of abundant synovial T cells in established RA and the association with certain class II MHC genes clearly support the involvement of T cells in disease pathogenesis. We recently detected highly expanded T cell clones in the synovium of patients with early RA as compared to that of patients with longstanding RA (30), suggesting that T cell involvement has a role in the early phase of the disease.

In the current study, it appeared that, in particular, expression of both CD3 and CD8 was associated with arthritis development. Activated CD8+ T cells may have cytotoxic activity, as evidenced by the production of granzymes and perforin, leading to cell death of (pathogenic) cells, and can produce proinflammatory cytokines such as interferon-γ and tumor necrosis factor. We previously found that soluble granzyme B levels are an independent predictor of erosive disease in RF-positive RA (31). The possible role of synovial CD8+ T cells during the preclinical stage of RA pathogenesis is, however, still unclear, and our findings will first need to be confirmed in an independent cohort. Interestingly, we found that the presence of CD8+ T cells (and, to a lesser extent, CD4+ T cells) is associated with the presence of specific ACPAs, as well as with the total number of ACPAs present. Based on these results, it can be hypothesized that these synovial T cells may be directed against specific citrullinated peptides, which should be a topic of further investigation.

Another interesting observation is that expression of citrullinated fibrinogen in the synovial tissue was not associated with the development of arthritis or with the ACPA status. Since the monoclonal antibody that we used was generated by selection against modified citrullinated fibrinogen, it may also bind to other citrullinated peptides. However, previous work has shown that the presence of citrullinated proteins is not specific for RA synovial tissue (32,33), and citrullinated peptides can also be found in inflamed tissue or cancer tissue outside the synovium (32,34). Moreover, citrullinated proteins have been detected in healthy synovial tissue (32). Our data suggest that initial ACPA formation is not necessarily directed against joint-specific peptides, but rather

against citrullinated peptides in other compartments of the body. Citrullination of peptides in the lung, possibly as a result of smoking, suggests that the lung may be an early site of RA-related autoimmunity (3). Interestingly, in a comparable cohort of autoantibody-positive individuals at risk of RA, airway abnormalities similar to those found in patients with RA were observed, but the frequency of airway abnormalities was significantly higher in autoantibody-positive subjects compared with autoantibody-negative controls (10).

A possible limitation of our study is that synovial inflammation was examined only in the knee joints, whereas the disease usually presents in the small joints of the hands or feet. Obviously, it is difficult to obtain sufficient synovial tissue from nonarthritic small joints to allow reliable analysis. However, in 44% of the cases, synovial biopsy sampling was performed in a symptomatic, painful knee joint (without swelling), and it can therefore be expected that if the pain was due to synovial inflammation in that joint, we would have detected this by MRI and synovial biopsy. Moreover, 25% of the individuals in the cohort underwent ultrasonography or MRI of the hands as well, in the context of regular patient care, and there was also no sign of synovitis in the small joints in these subjects (results not shown).

In conclusion, in this prospective, relatively large cohort study of autoantibody-positive individuals at risk of developing RA, we confirmed that there was no clearcut synovial inflammation before the development of clinically apparent arthritis. It is possible, however, that subtle infiltration of the synovium by T cells might precede the onset of arthritis, but this needs further validation. In addition, we observed a clear association between the presence of synovial CD8+ T cells and the presence of antibodies against citrullinated peptides. The question of whether these T cells are directed against specific citrullinated peptides needs to be addressed in the future. We propose a model in which systemic autoimmunity may exist years before the onset of RA. Apparently, a second hit (for instance, a trauma or an infection leading to expression of citrullinated antigens) in the synovium is needed for arthritis development. This could subsequently lead to expansion of the ACPA repertoire (35) and progression toward chronic synovial inflammation.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Tak had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. de Hair, van de Sande, Klareskog, Gerlag, van Baarsen, Tak.

Acquisition of data. de Hair, van de Sande, Ramwadhdoebe, Hansson, van der Leij, Maas, van Schaardenburg, Klareskog, Gerlag.

Analysis and interpretation of data. de Hair, van de Sande, Landewé, Serre, Gerlag, van Baarsen, Tak.

ADDITIONAL DISCLOSURES

Authors Gerlag and Tak are employees of GlaxoSmithKline.

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