PRECLINICAL RHEUMATOID ARTHRITIS

Markus MJ Nielen

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Birds flying high,
You know how I feel.
Sun in the sky,
You know how I feel.
Reeds driftin' on by,
You know how I feel.
It's a new dawn,
It's a new day,
It's a new life,
For me,
And I'm feeling good.

(L. Bricusse & A. Newley)

Contents

Chapter 1

General introduction

Rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease, characterised by chronic inflammation of the joints, resulting in pain, stiffness and the loss of function [1]. Women are more affected than men in a ratio 2:1. RA is the most common form of chronic inflammatory polyarthritis, affecting approximately 0.5 to 1% of the Northern European and North American population. Studies in Southern Europe report a lower prevalence of 0.3 to 0.7% [2]. In the Netherlands, the reported prevalence of RA varies between 1.0 and 1.3% [3]. The incidence of RA in the Netherlands is 0.2 to 0.4 per 1000, which is in line with other Northern European and North American countries [3, 4].

The aetiology of RA is unknown, but both genetic and environmental factors are thought to be important in the pathogenesis of RA (reviewed in [5]). The major histocompatibility complex (MHC) genes, which encode the human leukocyte antigens (HLA), are by far the most studied gene family in RA. HLA-DR4 and more in particular the shared epitope (SE) are associated with the development of RA [6, 7]. The strongest associations with RA are found for genes in the HLA region, but recently also other genes were found to be associated with the development of RA (reviewed in [8]), such as the protein tyrosine phosphatase non-receptor 22 (PTPN22) gene [9], the 6q23 region [10], the transducer and activator transcription 4 (STAT4) gene [11] and the genes TNF receptor associated factor 1 (TRAF1) and C5 [12].

However, twin studies showed that only approximately 60% of the development of RA can be explained by genetic factors [13]. Besides genetic factors, there are several environmental factors that might play a role in the pathogenesis of RA, such as smoking [14], infections [15] and hormonal factors (reviewed in [16]).

Early arthritis

In RA, joint damage is irreversible and associated with a loss of functional capacity in a later stage of the disease [17, 18]. Early recognition and treatment is important, because with the current treatment it is possible to prevent radiographic progression [19, 20]. In most studies the diagnosis of RA is not made on clinical parameters, but patients have to fulfil the 1987 classification

criteria for RA of the American College of Rheumatology (ACR) [21]. Unfortunately, these classification criteria have a low sensitivity in early arthritis [22]. Therefore studies of early arthritis cohorts focus mostly on the prediction of radiographic progression, rather than on the presence or absence of RA according to the classification criteria of RA.

Many studies have focused on the prediction of radiographic progression in early arthritis patients. Presence of autoantibodies is the strongest determinant of radiographic progression in early arthritis. Especially antibodies against citrullinated proteins or peptides (anti-CCP) [23-25] and rheumatoid factor (RF) [26, 27] are predictors of radiographic progression early in the disease. Besides autoantibodies, genetic parameters [28] and parameters of disease activity, such as erythrocyte sedimentation rate (ESR) [28, 29], C-reactive protein (CRP) [30], the disease activity score [31] and quality of life [31] at the onset of disease are known to predict radiographic progression. Finally, there is a potential role of biomarkers of bone metabolism to predict radiographic damage at disease onset. Biomarkers such as C-terminal crosslink of type I collagen (β-CTX), Receptor Activator of Nuclear Factor Kappa B (NFκB) ligand (RANKL) and osteoprotegerin (OPG) are associated with future radiographic damage and could predict progression at the onset of disease [32-34].

In 36-54% of the cases, patients have radiographic damage shortly after the onset of symptoms [28, 35, 36]. Therefore, despite the improved early recognition of RA patients, half of the patients already have radiographic damage at the first visit to the rheumatologist. Since this damage is irreversible but potentially avoidable by modern treatment [19, 20], it is important to detect RA earlier, preferably before joint damage occurs.

Preclinical rheumatoid arthritis

A major further step in the early recognition of RA would be to detect healthy persons at risk. To this end the preclinical phase of RA needs to be studied thoroughly. Little is known in this area as of yet. There are a number of possible methods to study preclinical RA. First, preclinical RA can be studied in a population with a high incidence of RA, such as Pima Indians [37] or unaffected first degree relatives from multicase RA families [38]. Second, specific cohorts can be used in which specimens were collected and blood samples of healthy people who developed RA later can be studied [39, 40].

Previous studies in preclinical RA patients mainly focused on autoimmunity and inflammation. RF and/or antikeratin antibodies were found in both high-risk [37, 38, 41] and healthy [39, 40, 42] populations before the start of the symptoms of RA. Autoantibody formation before the start of the symptoms was also described in several other autoimmune diseases, such as systemic lupus erythematosus [43] and insulin-dependent diabetes mellitus [44]. Results of preclinical inflammation studies in RA showed contradictory results [45, 46]. Aho et al did not find preclinical inflammation, measured by C-reactive protein (CRP) [45], which is in contrast with Masi et al who found a higher frequency of increased CRP levels in preclinical RA patients in comparison with healthy controls [46].
The limitation of these studies in preclinical RA patients was the fact that they were based on single serum samples. Therefore it was not possible to study the course of preclinical markers and to determine the moment of the appearance of these markers, which is necessary to make a reliable prediction of RA in healthy individuals or individuals at risk.
The studies described in this thesis are based on serial samples of blood donors who developed RA later. The studies were carried out with 79 RA patients and for each RA sample, 2 control samples were selected, matched for sex, age and time of blood donation to ensure identical storage conditions. Since the majority of the donors donate 2–4 times per year over periods of several years, a median of 13 serum samples per patient was available. These blood samples were collected, auto-antibodies were determined as well as genetic and clinical markers of the disease in order to predict factors of onset of RA and of radiographic damage in the future.

Thesis outline

The purpose of this thesis is to study the preclinical phase of RA patients with serological markers by using serial blood samples of blood donors who developed RA later and of matched controls. These markers were used to predict the development of RA in the preclinical phase of the disease. Outcome data of the later patients were also used. In addition, the value of autoantibodies to predict RA and future radiographic damage in early arthritis was studied with data from the Early Arthritis Clinic (EAC) at the Jan van Breemen Institute. This EAC includes patients with early oligo- and polyarthritis (symptom duration less than 2 years).

Seven studies were done in preclinical RA patients. The presence of autoantibodies prior to disease and the predictive value of the antibodies for RA in healthy individuals at risk is presented in ***chapter 2***.
Preclinical inflammation is investigated in two different studies. In the first study, CRP is measured and compared with healthy individuals, which is described in ***chapter 3***. Another inflammatory marker, sPLA2, is used in combination with CRP and autoantibodies to study the development of the acute phase response and autoantibodies in preclinical RA in ***chapter 4***.
In ***chapter 5*** measurements of total cholesterol, HDL cholesterol, triglycerides, apolipoprotein A-1, apolipoprotein B and lipoprotein (a) are used to study the lipid profile of preclinical RA patients.
In the Iowa Women's Health Study it was found that higher dietary intake of vitamin D (estimated by questionnaire) was associated with a lower risk of RA. We tested this possible association by directly measuring serum levels of 25-hydroxyvitamin D in preclinical RA (***chapter 6)***.
To study early bone changes, markers of bone metabolism and regulators of osteoclast activity in preclinical RA patients were measured and associated with radiographic progression after the onset of RA in ***chapter 7***.
The origin of RA is a combination of genetic and environmental factors. Therefore, genetic factors could be useful for the detection of RA in healthy individuals with an increased risk of RA. The association between autoantibodies (IgM-RF and anti-CCP) before the start of the symptoms of RA and genetic markers (HLA-DR4 and SE) is studied and the additional value of these genetic markers to predict RA in healthy persons is discussed in ***chapter 8***.
After the emergence of clinically apparent arthritis, progression of disease can be studied in early arthritis patients. The diagnostic and prognostic value of anti-citrullinated fibrinogen in early arthritis are compared with IgM-RF and the second generation anti-CCP test in ***chapter 9***.
A summary of the results, a general discussion and recommendations for future research are presented in ***chapter 10***.

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Chapter 2

Specific autoantibodies precede the symptoms of rheumatoid arthritis: A study of serial measurements in blood donors

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Abstract

Objective: Autoantibodies have been demonstrated in single serum samples from healthy subjects up to 10 years before they developed rheumatoid arthritis (RA). However, the time course for the development of antibodies before onset of clinical RA is unknown, nor is it known which antibody, or combinations of antibodies, might be most sensitive or specific for predicting future development of the disease. The present study was undertaken to investigate this.

Methods: Patients with RA who had been blood donors before the onset of disease symptoms were enrolled. Frozen serum samples from each donor were retrieved, together with 2 serum samples from controls matched for age, sex, and date of donation. All samples were tested for IgM rheumatoid factor (IgM-RF) and anti–cyclic citrullinated peptide (anti-CCP) antibodies.

Results: Seventy-nine patients with RA (62% female; mean age at onset of symptoms 51 years) were included. A median of 13 samples (range 1–51) per patient were available; the earliest samples had been collected a median of 7.5 years (range 0.1–14.5) before the onset of symptoms. Thirty-nine patients (49%) were positive for IgM-RF and/or anti-CCP on at least one occasion before the development of RA symptoms, a median of 4.5 years (range 0.1–13.8) before symptom onset. Of the 2,138 control samples, 1.1% were positive for IgM-RF, and 0.6% were positive for anti-CCP.

Conclusion: Approximately half of patients with RA have specific serologic abnormalities several years before the onset of symptoms. A finding of an elevated serum level of IgM-RF or anti-CCP in a healthy individual implies a high risk for the development of RA. We conclude that IgM-RF and anti-CCP testing with appropriately high specificity may assist in the early detection of RA in high-risk populations.

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease of unknown origin, characterized by chronic joint inflammation leading to destruction of bone and cartilage, reduction of functional capacity, and increased mortality [1, 2]. Since structural joint damage is irreversible, early recognition and treatment are currently being emphasized, with the goal of halting progression of the disease [3–5]. However, in 36–54% of cases, patients attending arthritis clinics shortly after the onset of symptoms already have joint damage that is visible radiographically [6–8].

The pathogenesis of RA is poorly understood. There is evidence of a preclinical or asymptomatic phase of the disease. Histologic studies have demonstrated extensive synovitis in clinically uninflamed joints [9]. In addition, autoantibodies can be present before the disease becomes manifest [10–15]. The autoantibodies most frequently found in patients with RA are antibodies against IgG (IgM rheumatoid factor [IgM-RF]) and antibodies against citrullinated proteins. The latter were originally measured as antibodies against keratin or filaggrin and more recently as anti–cyclic citrullinated peptide (anti-CCP) [16,17]. In hospital-based groups of patients with early RA, the prevalence of RF is 50–66%, and the prevalence of anti-CCP is 41–48%; the prevalence rates of these antibodies in the absence of disease are reported to be 7–13% and 3–9%, respectively [17–19].

A number of reports have described the presence of autoantibodies in the blood of apparently healthy persons years before they developed RA. This was found not only in populations at increased risk for RA, such as multicase families [10,15] or Pima Indians [14], but also in the general populations of Finland [11,12] and Iceland [13]. Most of the results were based on findings in single serum samples and therefore provide no information about the course of autoantibody titers in the period before the onset of symptoms of RA or about which antibodies might be most sensitive and specific to predict the disease. In the present study, the pattern of autoantibodies in the preclinical phase of RA was investigated with serial measurements, using highly specific tests, of IgM-RF and anti-CCP in blood donors before they developed RA and in control blood donors who did not develop RA.

Patients and methods

Study subjects

Since 1984, the Sanquin Blood Bank Northwest Region (formerly Red Cross Blood Bank) in Amsterdam has stored (at -30°C) 1-ml aliquots of serum from donated blood. The majority of the donors, who are age ≤ 70 years, donate 2–4 times per year over periods of several years. The bank serves an area with approximately 2 million inhabitants. Within this area, most RA patients are registered at the Jan van Breemen Institute, a regional network of outpatient clinics.

In 1999, a letter was sent to all 5,000 registered patients with RA in the diagnosis registry of the Jan van Breemen Institute, asking whether they had donated blood at the Sanquin Blood Bank Northwest Region since 1984, but before the onset of their symptoms of RA. The diagnosis registry also includes cases in which the diagnosis is not entirely certain; therefore, the charts of the responding patients were reviewed for the fulfillment of the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) 1987 criteria for RA [20]. Of the 153 responding patients, 74 were excluded: all blood donation had occurred before 1984 in 35, 17 had donated at another blood bank, 18 did not fulfill the ACR criteria for RA, and medical records were unavailable for 4. The study was carried out with the 79 remaining patients. Thus, exclusions were due only to uncertainty about the diagnosis or unavailability of specimens. There were no familial relationships among the 79 patients.

The following data were collected from medical records: time of the start of symptoms, date of the diagnosis of RA according to the ACR criteria, IgM-RF status, erythrocyte sedimentation rate, use of disease-modifying antirheumatic drugs, and presence of bony erosions seen on radiographs. IgM-RF tests on samples provided before symptom onset (subject of the present study) and during followup after diagnosis were performed in the same way. Anti-CCP was not routinely tested in the clinic during the period the patients were followed up. Since the start of the early arthritis clinic in 1995, the time of the start of symptoms was also checked by a research nurse in 22 patients (28%).

The study was approved by the local Institutional Review Board.

Procedures

Serum aliquots of blood from each blood collection site had been stored in one stoppered container per day, each containing 50–100 vials and marked with a job number. A computer program was developed to trace all blood donations

from a particular RA patient in the period 1984–1999 and to locate a serum vial in a particular container. For each RA sample, 2 masked control samples from the same container were selected, matched for sex, age (± 3 years), and time of blood donation. This method of control selection was chosen to ensure identical storage conditions for the samples from patients and controls. The ability to find the correct serum samples was validated in pilot experiments. The quality of the selected samples was checked by measuring sodium concentration in 106 randomly selected control samples. The mean ± SD sodium concentration was 140.3 ± 4.8 mmoles/liter; this distribution is similar to that in freshly collected sera from a normal adult population, indicating that evaporation during storage had not occurred.

IgM-RF and anti-CCP antibodies were measured using in-house enzyme-linked immunosorbent assays and an ES 300 analyzer (Roche Diagnostics; Mannheim, Germany). Biotinylated rabbit IgG and, at the N-terminus, biotinylated citrullinated peptides (cyclic and with the amino acid sequence as described by Schellekens et al [18]) were bound to streptavidin-coated walls. After incubation with diluted serum, wells were developed using horseradish peroxidase–conjugated $F(ab)_2$ (Dako; Glostrup, Denmark) directed against human IgM and IgG. The day-to-day coefficients of variation in a weakly positive internal control serum sample were 4.8% and 4.2% for IgM-RF and anti-CCP, respectively (n = 50 days). IgM-RF was calibrated with a national reference serum containing 200 IU/ml [21]; no such standard serum is available for anti-CCP.

The criteria for recording a value as being positive were ≥30 IU/ml for IgM-RF and ≥50 arbitrary units/ml for anti-CCP, based on the findings in 2 cohorts. In cohort 1, which consisted of sera from 239 patients with RA that was in clinical remission, 55 patients with active RA from our outpatient clinic [22], and 187 control sera, the sensitivity with these criteria was 48.5% for IgM-RF and 49% for anti-CCP, with a specificity of 95.3% for IgM-RF and 98.9% for anti-CCP. Cohort 2 consisted of the baseline sera from patients with early arthritis. After 1 year, 258 patients had RA and 121 were classified as having undifferentiated polyarthritis (19). In that cohort, the sensitivity was 50.4% for IgM-RF and 42.6% for anti-CCP, with a specificity of 93.4% for IgM-RF and 97.5% for anti-CCP. The cutoff values were deduced from receiver operating characteristic curve analysis.

Statistical analysis

For each RA patient, antibody concentrations over time were plotted. In a first analysis, for blood donors who were positive at least once for IgM-RF and/or anti-CCP before the start of symptoms, the times of the first positive test result

for IgM-RF, the first positive test result for anti-CCP, and the first positive test result for IgM-RF and/or anti-CCP were determined. Patients were considered to be positive for these parameters from that time onward. Thus, the cumulative prevalence of positivity over the period before the onset of symptoms was measured. The frequency of positive test results and the median time between the first positive test results and the start of symptoms were calculated for the group of donors who developed RA.

In a second analysis, the time axis was divided into 15 1-year periods preceding the onset of symptoms. Period 1 covered the blood donations made within 1 year before the onset of symptoms, period 2, between 1 and 2 years, etc. For each period, the sensitivity and specificity of IgM-RF, anti-CCP, and IgM-RF and/or anti-CCP were measured by calculating the percentage of positive samples from blood donors who developed RA and the percentage of negative samples in the matched controls.

Next, the positive predictive value (PPV) and negative predictive value of the serologic tests for a period of 5 years before the onset of symptoms were calculated. The results also were used to estimate the 5-year risk of developing RA in 1) the general population and 2) a high-risk population of multicase families, using 5-year incidence figures. The estimates we used for overall risk of RA development within 5 years were 1 per 1,000 in the general Dutch population [23] and 39 per 1,000 in multicase families [10].

Finally, the statistical significance of the differences in various characteristics between the group of patients who were antibody positive and those who were antibody negative prior to symptom onset was determined. The groups were compared using Student's *t*-test, the Mann-Whitney U test, or the chisquare test, as appropriate.

Results

Patient characteristics

Seventy-nine patients (49 women, 30 men) were included; their mean age at the onset of symptoms was 51.4 years (table 2.1). At presentation in the clinic 1 year after the development of symptoms, one-third had erosive disease; this proportion had doubled at the last followup. A median of 13 serum samples per patient (range 1–51) were available, the first of which had been obtained a median of 7.5 years (range 0.1–14.5 years) before the onset of symptoms. A total of 1,188 patient sera and 2,358 matched control sera were tested (for 18

patient sera, only 1 control serum meeting the criteria was available). One hundred ten patient samples with 220 matched control samples were not used, because the patient samples were collected after the onset of symptoms.

Table 2.1: Characteristics of 79 blood donors who developed rheumatoid arthritis

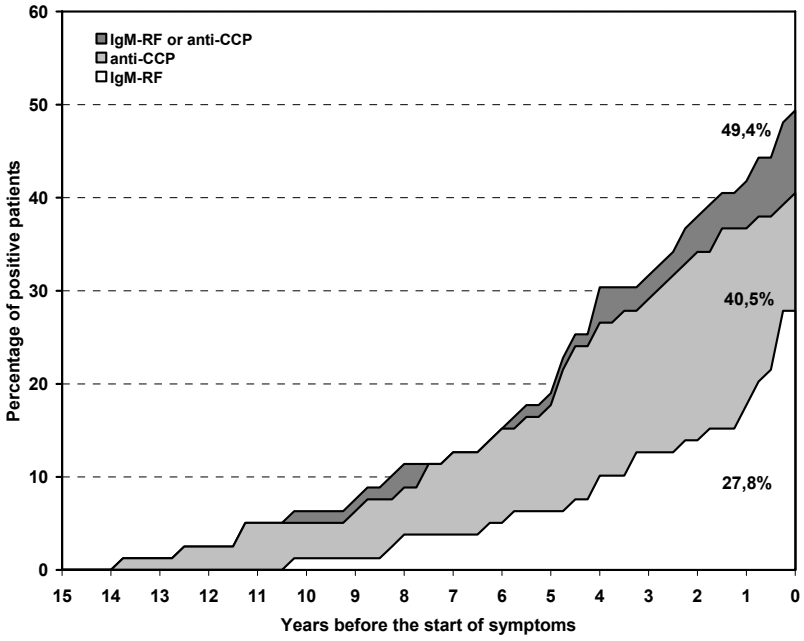
% women	62
Age at onset of symptoms, mean ± SD years	51.4 ± 11.1
Time between onset of symptoms and diagnosis, median (range) years	0.9 (0.1–6.5)
Radiographic erosions at first visit, no. of patients	25
Radiographic erosions at last followup, no. of patients	48
Duration of followup after onset of symptoms, median (range) years	5.6 (0.7–15.6)

Test results

Of the 2,138 control samples, 24 (1.1%) were positive for IgM-RF (specificity 98.9%), 12 (0.6%) for anti-CCP (specificity 99.4%), and 35 (1.6%) for IgM-RF and/or anti-CCP (specificity 98.4%). These specificities were constant over the period of 15 years.

A cumulative graph of the percentages of patients who had at least 1 positive test result for IgM-RF, for anti-CCP, and for IgM-RF and/or anti-CCP before the start of symptoms is shown in figure 2.1. Twenty-two of 79 patients (27.8%) became IgM-RF positive, and 32 (40.5%) became anti-CCP positive prior to symptom onset; the median time from the first IgM-RF or anti-CCP positivity to development of symptoms was 2.0 years (range 0.3–10.3 years) and 4.8 years (range 0.1–13.8 years), respectively. Thirty-nine patients (49.4%) were positive for IgM-RF and/or anti-CCP at least once, a median of 4.5 years (range 0.1–13.8 years) before the onset of symptoms. (Chart review revealed that the proportion of IgM-RF–positive patients increased to 61% in the 2 years after the first visit to the clinic and to 67% after 6 years. Anti-CCP was not routinely tested in the clinic during the period the patients were followed up.)

Figure 2.1: Cumulative percentages of patients with 1 or more positive test results for IgM rheumatoid factor (IgM-RF), anti–cyclic citrullinated peptide (anti-CCP), and IgM-RF and/or anti-CCP before the onset of symptoms of rheumatoid arthritis



After the first positive IgM-RF test result, 98% of the subsequent samples from the same patient yielded positive results. Anti-CCP positivity persisted after the first positive test result in 69.2% of the samples from the same patient.
Individual examples of 4 different patterns of presymptomatic IgM-RF and anti-CCP concentrations (either 1 positive, both positive, or both negative) are presented in figure 2.2. Nine percent of the patients were positive only for IgM-RF and 21% only for anti-CCP before the start of symptoms. Nineteen percent were positive for both IgM-RF and anti-CCP, and 51% were negative for both tests.

The sensitivity of IgM-RF, anti-CCP, and IgM-RF and/or anti-CCP as measured per year over a period of 15 years before the onset of symptoms is shown in figure 2.3. There was a gradual increase of the sensitivity of the IgM-RF test over time, with a maximum of 28.5% positivity in the year before the start of

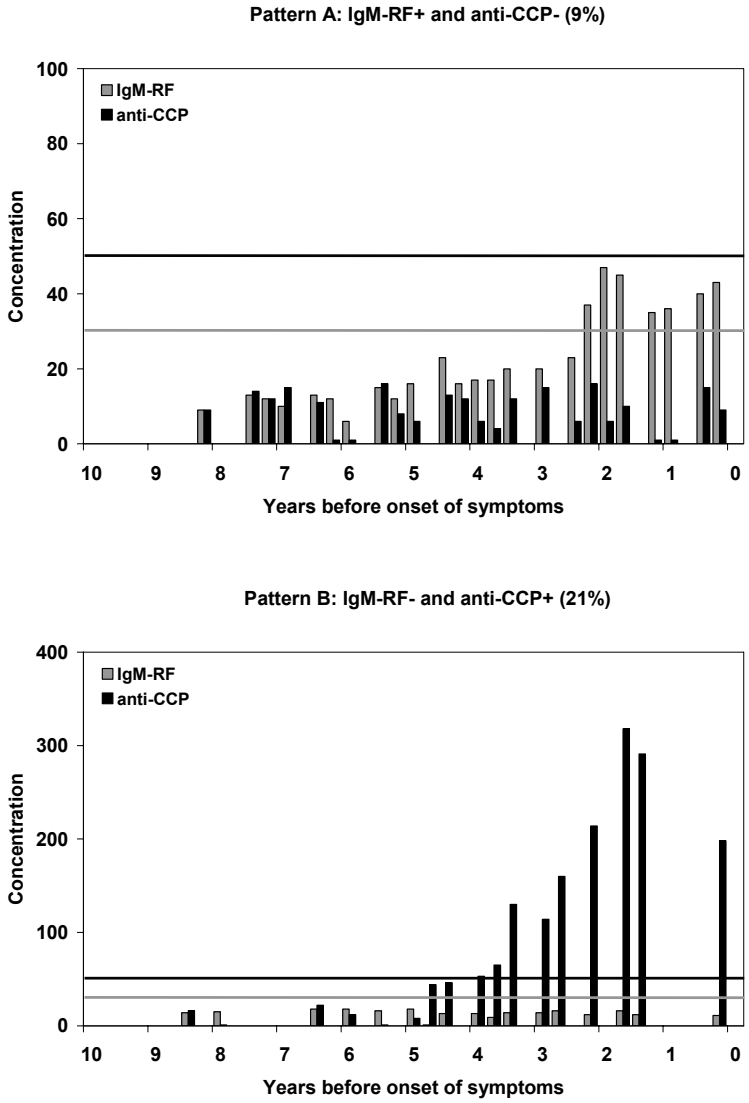
symptoms. Anti-CCP was more sensitive than IgM-RF in all time periods except the 9-year pre–symptom onset period, and became positive earlier (maximum 14 years versus 11 years before the start of symptoms). The highest sensitivity for anti-CCP was 31.7%, 2 years before the start of symptoms (a smaller peak occurred at 12 years before symptom onset). The combination criterion of IgM-RF and/or anti-CCP reached a maximum sensitivity of 42.3% in the year before the development of symptoms.

The diagnostic characteristics of the tests in the 5 years before the onset of symptoms are summarized in table 2.2. The 5-year PPV for anti-CCP in the blood donor population was 96.6%. In the general population, the PPV for anti-CCP was 5.3%, and in a high-risk population, i.e., consisting of persons from multicase families, it was 69.4%. If both IgM-RF and anti-CCP were positive, the PPV was 100%, but the sensitivity decreased to 13%. The risk of developing RA within 5 years (PPV) was higher with anti-CCP alone than with

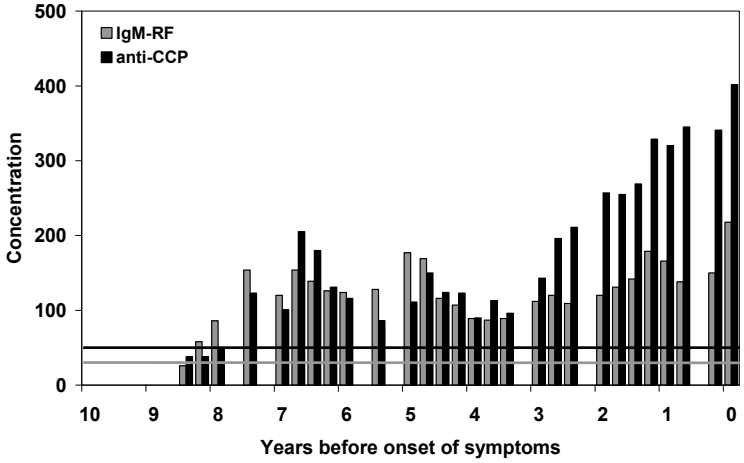
IgM-RF and/or anti-CCP, due to a higher number of controls who were positive for IgM-RF and/or anti-CCP than for anti-CCP alone; the lower PPV is the indirect consequence of the lower specificity of this test combination.

The clinical characteristics of patients with and those without a positive test result for IgM-RF and/or anti-CCP before the onset of symptoms are shown in table 2.3. The patients with positive serologic results before the onset of symptoms were significantly younger and had a higher frequency of radiographic erosions at last followup, compared with those in whom the results of tests for these antibodies were negative.

Figure 2.2: Patterns of IgM rheumatoid factor (IgM-RF) and anti-cyclic citrullinated peptide (Anti-CCP) before the onset of symptoms of rheumatoid arthritis. Titers of IgM-RF and anti-CCP are shown for the period of 10 years before the onset of symptoms. The criteria for positivity (indicated by horizontal lines) were ≥30 IU/ml for IgM-RF and ≥50 arbitrary units/ml for anti-CCP.



Pattern C: IgM-RF+ and anti-CCP+ (19%)



Pattern D: IgM-RF- and anti-CCP- (51%)

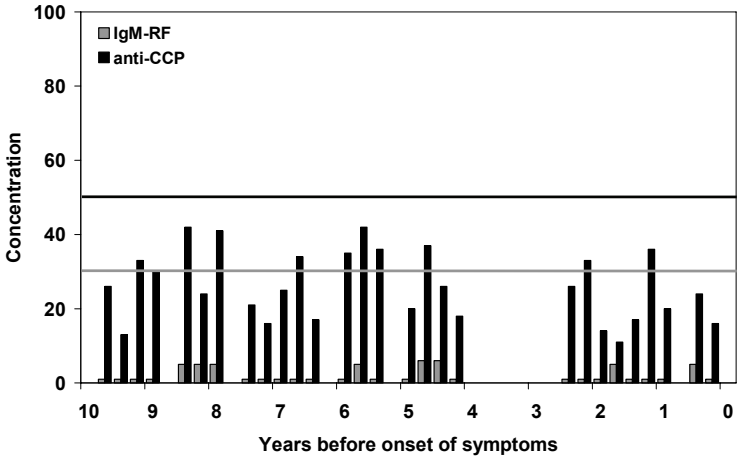


Figure 2.3: Sensitivity (percentage positivity) of IgM rheumatoid factor (IgM-RF), anti–cyclic citrullinated peptide (anti-CCP), and IgM-RF and/or anti-CCP in rheumatoid arthritis patients before the onset of symptoms

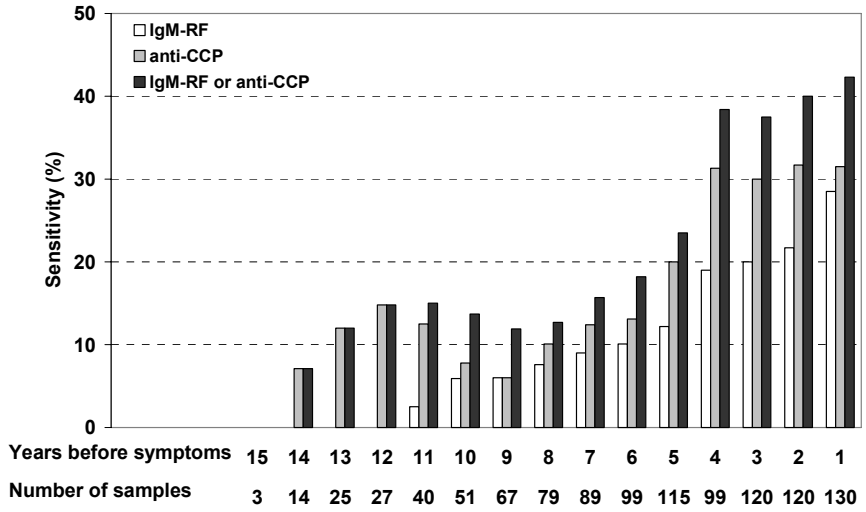


Table 2.2: Diagnostic value of IgM-RF and anti-CCP for RA*

	Blood donor population 0–5 years before symptom onset				Risk of developing RA within 5 years (PPV, %)	
	Sensitivity %	Specificity %	PPV %	NPV %	General population	High-risk population†
IgM-RF	20.5	98.6	88.2	71.1	1.5	37.7
Anti-CCP	28.9	99.5	96.6	73.5	5.3	69.4
IgM-RF or anti-CCP	36.5	98.1	90.6	75.4	1.9	43.8
IgM-RF and anti-CCP	13.0	100.0	100.0	75.4	100.0	100.0

* IgM-RF = IgM rheumatoid factor; anti-CCP = anti–cyclic citrullinated peptide; PPV = positive predictive value; NPV = negative predictive value.

† Defined as individuals who have ≥ 2 first-degree relatives with rheumatoid arthritis (RA) (5-year incidence of RA among such individuals has been reported to be 3.9% [10]).

Table 2.3: Differences between patients with positive serologic findings and those with negative serologic findings before the onset of rheumatoid arthritis symptoms*

	Positive serologic findings (n = 39)	Negative serologic findings (n = 40)	*P*
At first visit			
% women	59	65	0.58
Age at onset of symptoms, mean ± SD years	48.2 ± 12.2	54.5 ± 9.1	0.01
ESR, median (range) mm/hour	21 (1–105)	25 (3–106)	0.71
Radiographic erosions, no. of patients	14	9	0.11
At last followup†			
Radiographic erosions, no. of patients	30	20	0.01
No. of prescribed DMARDs, median (range)	2 (1–4)	2 (0–6)	0.73
Prednisone use, no. of patients	9	15	0.16

* ESR = erythrocyte sedimentation rate; DMARDs = disease-modifying antirheumatic drugs.
† Median duration of followup 5.6 years (range 0.7–15.6).

Discussion

Approximately half of 79 blood donors became positive for the autoantibodies IgM-RF and/or anti-CCP before the onset of clinical symptoms of RA. The median time from the first autoantibody positivity in a serum sample to development of symptoms was 4.5 years. Among the patients with antibodies, there was at least 1 positive anti-CCP test result a median of 2.8 years before IgM-RF positivity, but anti-CCP was slightly less likely to remain positive. Both tests were found to be highly specific in this study, which implies a high risk for the development of RA in healthy persons up to age 70 years (the upper age limit of the blood donors) who have elevated serum levels of 1 or both of these antibodies.

The group studied appears to be representative of the RA population, based on the age at onset of symptoms, percentage of patients with radiographic erosions at baseline, and frequency of IgM-RF at baseline. The percentage of women in the patient group is somewhat lower than expected, but it is not likely that this influenced the results, since the controls were matched for sex and age. Blood donors are not entirely representative of the general population, because many diseases and infections that increase the risk of a positive IgM-RF test result, such as hepatitis C, lead to exclusion as a donor. This selection might lead to a slightly higher specificity of the IgM-RF test in the studied population. The anti-

CCP test might also be influenced by infections such hepatitis C, although a study of antikeratin antibodies (a preliminary anti-CCP test) showed only 8% positivity in hepatitis C–infected patients [24].

Our results are in accordance with incidental earlier reports of RF or antikeratin antibodies in both high-risk [10,14,15] and healthy [11–13] populations. However, in the present study we were able to measure antibody levels in serially obtained samples, using 2 different tests that are highly specific for RA. The 44% frequency of positivity for RF in the Finnish population [11] corresponds to our findings, but that study yielded information from only one point in time. Autoantibody positivity prior to symptom development has also been found in other autoimmune diseases, e.g., systemic lupus erythematosus and insulin-dependent diabetes mellitus. Ten of 16 persons who developed systemic lupus erythematosus or mixed connective tissue disease were positive for antinuclear antibodies 0.7–4.5 years before the onset of symptoms [25]. Antibodies to glutamic acid decarboxylase, the primary immunologic marker in insulin-dependent diabetes mellitus, were present for up to 10 years in the prediabetic period in 82% of 28 women and in none of 100 controls [26]. This led to immunologic interventions to prevent the onset of diabetes in selected populations [27,28].

The results of this study shed more light on the time sequence of events in the pathogenesis of RA. An unknown trigger activates B lymphocytes to produce RA-specific antibodies several years before the appearance of a level of inflammation that is perceived as symptoms. In the majority of patients, seropositivity, once established, is stable. The production of antibodies in itself is not essential for clinical disease, since it occurred in only half of the patients before onset of symptoms. Still, these patients proved to be the more severely affected in terms of radiologic findings, and the prevalence of antibodies increased over time and was highest in the year before the symptoms appeared. Apparently, the conversion to RF seropositivity continued after the onset of symptoms and reached the commonly reported prevalence level of 67% after 6 years.

Apart from providing insight into the pathogenesis of RA, our findings indicate that it may be possible to predict RA development in high-risk populations, as long as appropriately high upper limits of normal are set for the tests used. For example, for multicase families with RA, we have estimated that the 5-year risk of developing RA in a family member with a positive anti-CCP result is 69.4%. We believe this figure is high enough to consider a clinical trial of a medical intervention to prevent the development of RA in these individuals. Because only a small proportion (2–3%) of all patients with RA are from multicase families [29],

another possible target group for intervention could be patients with arthralgia and positive serologic findings.
In conclusion, half of patients with RA have specific serologic abnormalities years before the development of symptoms. This finding should help guide our understanding of the early phases of RA and may contribute to earlier detection and more aggressive treatment of this disabling disease.

Acknowledgments

We gratefully acknowledge the personnel of the Sanquin Blood Bank Northwest Region for their help in obtaining the sera from patients and matched controls, and in particular, Nora Schram for writing the necessary computer programs and setting up the databases.

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Chapter 3

Increased levels of C-reactive protein in serum from blood donors before the onset of rheumatoid arthritis

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Abstract

Objective: We previously reported that approximately half of the patients with rheumatoid arthritis (RA) have specific serologic abnormalities (elevated serum concentrations of IgM rheumatoid factor and/or anti–cyclic citrullinated peptide antibodies) starting several years before the onset of symptoms. In this study, the presence of serologic signs of inflammation in patients with preclinical RA was investigated with serial measurements of C-reactive protein (CRP).

Methods: Seventy-nine patients (61% female; mean age at onset of symptoms 51 years) who had been blood donors before the onset of RA were identified. Frozen serum samples from each donor were retrieved, together with 1 sample from a control donor matched for age, sex, and date of donation. CRP was measured using a highly sensitive latex-enhanced assay. The dates of donation were categorized into 15 1-year periods preceding the onset of RA symptoms. For each period, the median CRP levels in the patient and control groups were compared using the Mann-Whitney U test. The course of CRP concentrations over time in the patient group was estimated with random coefficient analysis.

Results: A median of 13 samples (range 1–51) per patient were available; the earliest donation was made a median of 7.5 years (range 0.4–14.5 years) before the onset of symptoms. A total of 1,078 patient samples and 1,071 control samples were tested. For all 1-year periods, the median CRP concentration was increased in the patient group compared with the control group, but this difference was statistically significant only for the periods 0–1 year, 1–2 years, and 4–5 years before the onset of symptoms. The CRP concentration increased significantly over time in patients with preclinical RA; levels were slightly higher in the group of patients who had serologic abnormalities before the onset of symptoms than in those without such serologic abnormalities.

Conclusion: After observing specific serologic abnormalities 5 years before the onset of RA symptoms, we now report increased levels of CRP in blood donors in whom RA later developed; these increases were most common within the 2 years before the onset of symptoms. The preclinical increase in CRP levels was observed both in donors with and in those without serologic abnormalities.

Introduction

The pathogenesis of rheumatoid arthritis (RA) is poorly understood, but there is evidence for an immunologic disturbance preceding clinical RA. This process is associated with the occurrence of antibodies against immunoglobulins (rheumatoid factor [RF]) [1–7] and citrullinated peptides (mostly measured as anti–cyclic citrullinated peptide [anti-CCP]) [6,7]. Recently, the presence of 1 or both of these antibodies was demonstrated in half of the serum samples obtained from a group of 79 blood donors in whom RA later developed. Seroconversion to anti-CCP and IgM-RF positivity, respectively, occurred a median of 5 years and 2 years before symptom onset [6]. In a comparable study of blood donors in whom RA later developed, Rantapää- Dahlqvist et al reported sensitivities of 20% and 34%, respectively, for detecting IgM-RF and anti-CCP more than 1 year before the onset of symptoms [7].

Because the immunologic derangement preceding overt RA is often present for several years, the existence of subclinical inflammation during this period can be assumed. There have been conflicting reports on this issue [8,9]. In Finland, Aho et al found that the prerheumatic immunologic process was not associated with inflammation, as measured by the C-reactive protein (CRP) concentration in serum samples obtained from 124 RA patients up to 20 years before the onset of disease [8]. In contrast, Masi et al reported a higher frequency of increased CRP concentrations in serum samples obtained from 18 male RA patients 3–20 years before the onset of symptoms compared with the concentrations in samples from 72 matched controls [9]. However, these studies used single serum samples and therefore provide no information about the increase in CRP concentrations during the period before the onset of RA symptoms.

In this study, the presence of inflammation in the preclinical phase of RA was investigated with serial measurements of CRP in serum samples obtained from the blood of donors before RA developed and in samples from control blood donors.

Patients and methods

Patients

Since 1984, the Sanquin Blood Bank Northwest Region in Amsterdam, The Netherlands, has stored (at -30°C) 1-ml aliquots of serum from donated blood. The majority of the donors, who are age ≤ 70 years, donate 2–4 times per year over long periods of time. The bank serves an area with approximately 2 million inhabitants. Within this area, most RA patients are registered at the Jan van Breemen Institute, a regional network of outpatient clinics. Seventy-nine patients with RA according to the 1987 American College of Rheumatology (formerly, the American Rheumatism Association) criteria [10] were identified as having donated blood before the onset of symptoms, as described previously [6]. The following data were collected from the medical records: time of the start of symptoms and smoking status at the first visit. The study was approved by the local Institutional Review Board.

Procedures

Patient sera obtained during the period from 1984 to 1999 were collected as previously described [6]. Briefly, for each RA sample, 1 masked control sample from the same container was selected, matched for sex, age (±3 years), and time of blood donation. This method of control selection was chosen to ensure identical storage conditions for the samples from patients and controls.

CRP was measured using a highly sensitive latexenhanced assay supplied by Roche Diagnostics (Almere, The Netherlands) on a Hitachi 911 analyzer (Roche Diagnostics), according to the manufacturer's instructions. This test measures the CRP concentration very sensitively, in the range of 0.1–20 mg/liter.

Statistical analysis

In a first analysis, the time axis (dates of blood donations) was divided into 15 1-year periods preceding the onset of symptoms. For each period, the median CRP concentrations in the patient and control groups were compared using the Mann-Whitney U test.

In a second analysis, all of the patient and control samples collected 0–10 years before the onset of symptoms were used to estimate the increase in CRP concentrations in the patient group over time, compared with that in the control group. Samples obtained 10–15 years before the onset of symptoms were not used in this analysis, because of their small number. The increase in CRP levels over time was estimated with random coefficient analysis [11]. Basically, with this

longitudinal regression technique, both the intercept and the increase over time are allowed to differ between patients. The increase over time was modeled either linearly or (if necessary) quadratically. In the patient group, the time course of the increase in CRP levels was corrected for age, sex, and smoking status at the time of the diagnosis of RA, because smoking is associated with elevated CRP concentrations [12]. Because the distribution of CRP concentrations was skewed to the right, the natural log of CRP concentrations was used in all analyses.

To study whether the degree of inflammation was different between the group of RA patients who had serologic abnormalities and those who did not have such abnormalities before the onset of symptoms, the increase in CRP levels over time was compared in a third analysis. Patients who were positive for IgM-RF or anti-CCP at least once before the onset of symptoms were counted as having serologic abnormalities [6].

In addition, all patient samples were used in a time-lag analysis to determine whether increased CRP levels preceded the development of antibodies (IgM-RF/anti-CCP) in the preclinical phase of RA or vice versa. Concentrations of IgM-RF and anti-CCP were associated with CRP levels measured at the same time point and 1, 2, and 3 years before, and 1, 2, and 3 years after. By comparing the magnitude of the different regression coefficients with each other, one can determine whether or not a time lag is present in the relationship under investigation [11]. The magnitudes of the regression coefficients were investigated with random coefficient analysis, with correction for age and sex. In all analyses, the natural log of IgM-RF, anti-CCP, and CRP concentrations was used because of the non-normal distribution of these variables. Random coefficient analyses were performed with the statistical program MLwiN (Multilevel Models Project, Institute of Education, University of London, London, UK).

Results

Patient characteristics

Seventy-nine patients with RA (61% women; mean age at symptom onset 51 years) were included. A median of 13 serum samples (range 1–51) per patient were available, the earliest of which had been collected a median of 7.5 years (range 0.4–14.5 years) before the onset of symptoms. In total, 1,078 patient sera

and 1,071 matched control sera were tested (for 7 patient sera, no control serum matching the criteria was available).

Test results

For all 1-year periods, the median CRP concentration was higher in the patient group compared with the control group, but this difference was statistically significant only for the periods 0–1 year, 1–2 years, and 4–5 years before the onset of symptoms (table 3.1). Due to multiple testing, the calculated *P* values must be interpreted cautiously.

Table 3.1: C-reactive protein levels before the onset of symptoms in patients with rheumatoid arthritis and controls*

Time period before onset of symptoms	No. of patient samples	C-reactive protein level, mg/liter		*P*
		Patients	Controls	
0–1 year	130	2.2 (1.0–3.9)	1.0 (0.5–2.8)	0.001
1–2 years	120	1.9 (1.0–3.2)	1.5 (0.7–2.6)	0.035
2–3 years	120	1.7 (0.7–2.7)	1.4 (0.5–2.6)	0.492
3–4 years	99	1.6 (0.8–3.2)	1.3 (0.6–3.2)	0.125
4–5 years	115	1.7 (0.6–2.8)	1.0 (0.6–2.0)	0.012

* Values are the median (interquartile range).

The CRP levels before the onset of symptoms in patients with RA and controls are shown in igure 1. Whereas the mean CRP level in the control group remained stable over time (the slight increase was not statistically significant), the mean CRP level in the patient group showed a statistically significant increase over time, with the highest level observed at the time of symptom onset. The progression of CRP concentrations over time within the patient group was not confounded by smoking status (data not shown).

Figure 3.1: C-reactive protein levels before the onset of symptoms in patients with rheumatoid arthritis (RA) and controls

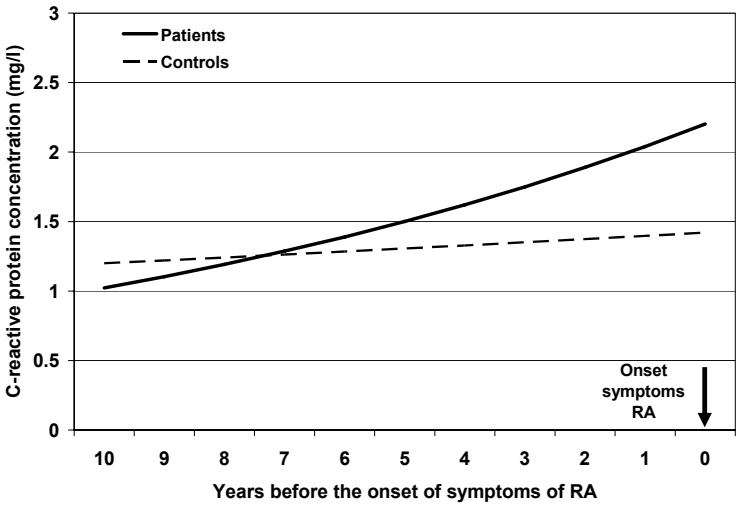
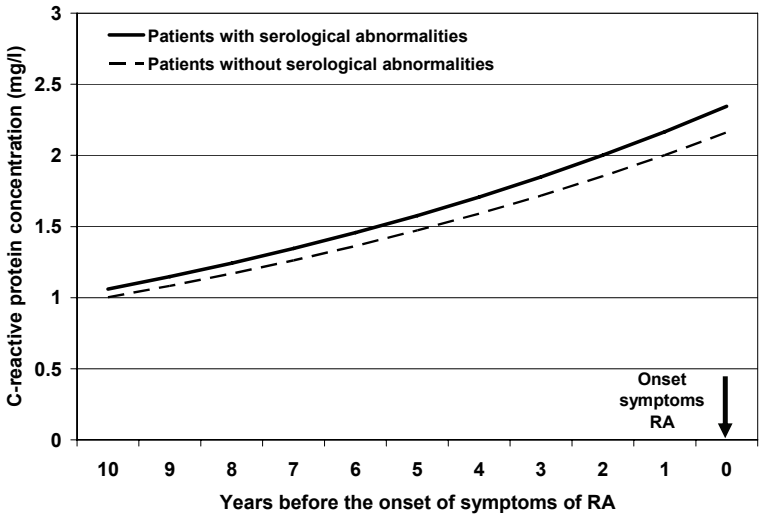


Figure 3.2: C-reactive protein levels in patients who were positive for IgM rheumatoid factor or anti–cyclic citrullinated protein at least once before the onset of rheumatoid arthritis (RA) symptoms (serologic abnormalities) and in those without serologic abnormalities before the onset of symptoms



The CRP levels before the onset of RA symptoms in patients with and those without serologic abnormalities are shown in figure 3.2. Both groups of patients had a statistically significant increase in the CRP concentration over time, with the highest levels observed at the time of symptom onset. The group of patients who had serologic abnormalities before the onset of symptoms had slightly higher mean CRP levels at all time points, compared with the patients who did not have serologic abnormalities before symptom onset ($P < 0.05$).
Time-lag analyses were used to determine whether increased CRP levels preceded the development of antibodies (IgM-RF/anti-CCP) or vice versa. No trend was apparent (data not shown). Therefore, these analyses could not clarify the sequence of increased CRP levels and the development of antibodies in the preclinical phase of RA.

Discussion

In the group of healthy blood donors in whom RA later developed, the median serum CRP level was higher than that in the control group for all 15 1-year periods; however, the difference was statistically significant only for the periods 0–1 year, 1–2 years, and 4–5 years before the onset of symptoms. The CRP concentration increased significantly over time in the subjects with preclinical RA, with slightly higher levels in the group of patients who had serologic abnormalities before symptom onset than in the group of patients without serologic abnormalities.
The median CRP levels in the patient and control groups were compared within the 15 1-year periods. In each of these periods, samples from a different set of patients and sometimes more than 1 sample from the same patient were included. Because multiple high or low CRP levels in some patients might influence the median CRP concentration in the whole group, multiple linear regression analysis was also performed, with correction for the intercept and development over time for each patient. In addition, the relationship between the CRP level and time was corrected for age and sex. The results may have been confounded by differences in smoking habits between patients and controls (12), because we have only limited information on smoking habits in the patient group. Current smoking status at the time of the diagnosis of RA did not appear to be related to CRP levels before the onset of symptoms.
The present results are consistent with those in the study by Masi et al (9), who reported that 4 of 18 male RA patients had increased (> 8 mg/liter) CRP levels

before the onset of symptoms. In contrast, Aho et al did not observe increased CRP levels in serum obtained from RA patients up to 20 years before the onset of RA compared with healthy controls (8). Possible explanations for the discrepancies could be the small number of measured sera and the long period of time between collection of blood samples and the onset of symptoms in these earlier studies. Another argument in favor of the existence of preclinical inflammation is the finding of synovitis in clinically uninvolved knee joints of patients with early RA (13).

Patients who had serologic abnormalities before the onset of symptoms had slightly higher CRP concentrations compared with patients without serologic abnormalities, which indicates an intensified inflammatory process in patients with IgM-RF and/or anti-CCP positivity before the onset of symptoms. However, both groups showed a very similar pattern of increase in the CRP concentration over time, which does not indicate a pivotal role for autoantibodies in the development of inflammation during the preclinical phase of RA. Rather, the somewhat higher CRP concentrations observed in patients with serologic abnormalities suggest that the production of autoantibodies is a phenomenon secondary to an increased level of inflammation in these patients. Even the appearance of IgM-RF or anti-CCP years earlier may have been a consequence of an inflammatory stimulus that was either too brief or too weak to be detected with the presently used technique and frequency of samples. Unfortunately, time-lag analyses could not clarify whether increased CRP levels preceded the development of antibodies in the preclinical phase of RA, or vice versa.

Although the differences between patients and controls were statistically significant in the 2 years before the onset of RA symptoms, it is uncertain whether these differences are also clinically relevant. An example of the clinical relevance of even small changes in the CRP concentration, albeit in a different disease, is given in a report of the influence of CRP levels on the risk of cardiovascular diseases in a healthy North American population (14). In that study, it was shown that a difference of <1 mg/liter in CRP levels could lead to an increased risk of cardiovascular disease. Therefore, the small difference in CRP concentrations observed in the present study may well be a significant factor in the development of later symptomatic inflammation. It must be noted that the findings of this study can be used only in a population of patients and are not suitable for decision-making in individual patient care.

In conclusion, after previously observing specific serologic abnormalities in RA patients 5 years before the onset of symptoms, we now report increased levels of CRP in blood donors in whom RA later developed; these increased levels were most common within the 2 years before the onset of symptoms. This

increase in CRP levels was demonstrated both in subjects with and in those without serologic abnormalities.

Acknowledgments

Margret de Koning is greatly appreciated for her help in locating sera from the patients and matched controls. Nora Schram wrote the necessary computer programs and set up the databases. We thank Maarten Boers for helpful discussions concerning the time sequence of the increase in antibody and CRP levels.

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Chapter 4

Simultaneous development of acute phase response and autoantibodies in preclinical rheumatoid arthritis

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ANNALS OF THE RHEUMATIC DISEASES Vol. 65, No. 4, April 2006, pp 535–537

Abstract

Objective: To investigate the temporal relationship between onset of inflammation (as measured by secretory phospholipase A2 (sPLA2) and C reactive protein (CRP)) and the presence of autoantibodies (IgM rheumatoid factor (IgM RF) and antibodies against citrullinated peptides (anti-CCP)) in the preclinical phase of rheumatoid arthritis (RA).

Methods: For 79 patients with RA who had been blood donors before the onset of disease, a median of 13 serum samples per patient was available. sPLA2 was measured in patient and matched control samples and related to previous CRP, IgM RF, and anti-CCP measurements. The temporal relationship between the increased markers of inflammation and autoantibodies was analysed with time lag analysis.

Results: IgM RF and anti-CCP concentrations were significantly associated ($p<0.001$) with concentrations of sPLA2, CRP, and the combination of sPLA2 and CRP at the same time point. However, we found no stronger association between the two autoantibody tests and the three inflammation measures 1, 2, and 3 years before or after a time point than for measurements at the same time, in the whole group or in subgroups of IgM RF and anti-CCP positive patients.

Conclusion: Both the acute phase response and autoantibody formation often develop years before the first symptoms of RA occur, and these phenomena are probably closely connected in time.

Introduction

The preclinical phase of rheumatoid arthritis (RA) is characterised by the presence of specific autoantibodies, such as IgM rheumatoid factor (IgM RF) [1–3] and antibodies against citrullinated peptides (anti-CCP) [2, 3]. One half of blood donors who later developed RA were found to be positive for IgM RF and/or anti-CCP at least once before the onset of RA [3]. C reactive protein (CRP) levels, as a marker of the acute phase response, increase over time in patients with preclinical RA, with the highest values at the start of the symptoms. However, it remains unclear whether increased inflammation, measured by CRP, occurs before, after or simultaneously with the development of antibodies (IgM RF and/or anti-CCP) in the preclinical phase of RA [4].

This issue is relevant to an understanding of the pathogenesis of RA: appearance of autoantibodies before a rise in inflammation markers might suggest an antibody driven inflammatory response. Conversely, detection of inflammation before the increase in autoantibodies would provide evidence that antibody formation only occurs after a detectable level of inflammation has been reached. In addition, evidence for a temporary concentration peak of a marker of inflammation before autoantibody formation would lend support to the possibility of an infectious process preceding the development of RA.

Therefore, in the present study secretory phospholipase A2 (sPLA2)—another sensitive marker of the acute phase response—was measured to investigate the temporal relationship between the onset of inflammation and the presence of autoantibodies in the preclinical phase of RA. In addition, this relationship was also tested using CRP and the combination of sPLA2 and CRP.

Patients and methods

Study subjects

Since 1984, the Sanquin Blood Bank North West Region in Amsterdam, The Netherlands, has stored serum from donated blood at -30°C. We identified 79 patients with RA registered at the Jan van Breemen Institute who donated blood, in general 2–4 times a year, before the onset of the symptoms, as described previously [3]. For each RA sample, one control sample was selected, matched for sex, age, and time of blood donation. sPLA2 was measured with an in-house

enzyme linked immunosorbent assay (ELISA) [5]. CRP, IgM RF, and anti-CCP had been determined previously [3, 4]. The study was approved by the local Institutional Review Board (ethics committee: Slotervaart Hospital, Jan van Breemen Institute and BovenIJ Hospital, Amsterdam, The Netherlands).

Statistical analysis

In a first analysis, the progression of the sPLA2 concentration over time in the patient and control groups, corrected for age, sex, and CRP, was estimated with random coefficient analysis. This longitudinal regression technique was used because each patient had a different number of measurements at different points in time.

In a second analysis, all patient samples were used to study the temporal relationship between the increased markers of inflammation and autoantibodies in preclinical RA with time lag analyses [6]. Concentrations of IgM RF and anti-CCP, on the one hand, were associated with concentrations of (a) sPLA2; (b) CRP; and (c) the combination of sPLA2 and CRP (Z score ln_sPLA2 + Z score ln_CRP), on the other hand, at the same time point as well as at 1, 2, and 3 years before, and 1, 2, and 3 years after that time. By comparing the magnitude of the different regression coefficients, one can determine if a time lag is present. The regression coefficients were calculated by random coefficient analysis and corrected for age and sex.

Finally, the same time lag analyses were repeated in two subgroups: (a) the relationship between the increase of IgM RF and the inflammation markers over time was studied in all samples of the patients who were positive for IgM RF at least once before the onset of the symptoms; and (b) the relation between anti-CCP and the inflammation markers was analysed in all serum samples of the patients who were positive for anti-CCP at least once before the start of the symptoms.

In all analyses, the natural logs of sPLA2, CRP, IgM RF, and anti-CCP were used, because of the non-normal distribution of these variables. Random coefficient analyses were performed with MLwiN (Multilevel Models Project; Institute of Education, University of London, London, UK), a statistical program for multilevel analyses.

Results

Seventy nine patients (50 (62%) female; mean age at onset of symptoms 51 years) who had been blood donors before the onset of RA were identified. A median of 13 serum samples per patient (range 1–51) was available; the median time between the first donation and the onset of the symptoms was 7.5 years (range 0.1–14.5). In total, 1078 patient sera and 1071 matched control serum samples were tested.

Figure 4.1: Secretory phospholipase A2 (sPLA2) levels before the onset of symptoms in the preclinical phase of patients with RA and in controls

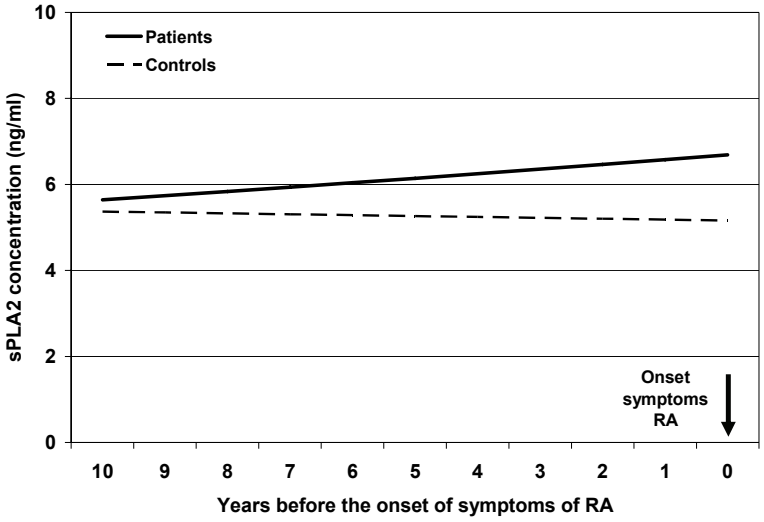

Figure 4.1 shows the sPLA2 levels of the patients and controls before the onset of symptoms, corrected for age, sex, and CRP. The mean sPLA2 level of the patient group increased significantly over time (p=0.005) with the highest values at the onset of the symptoms, whereas the mean sPLA2 level of the controls remained stable (p=0.50).
Table 4.1 shows the results of the time lag analyses in the group of patients with RA. The concentrations of IgM RF and anti-CCP were significantly associated

(p<0.001) with the concentrations of sPLA2, CRP, and the combination of sPLA2 and CRP at the same point in time. In the group as a whole, the association between the two autoantibody tests and the three inflammation parameters as measured 1, 2, and 3 years before as well as 1, 2, and 3 years after a point in time was no stronger than the association based on measurements at that same point in time.

Table 4.1: Association between autoantibodies and parameters of inflammation (CRP, sPLA2 and the combination of CRP and sPLA2) at different points in time

	B (95% CI)		
	CRP	sPLA2	CRP + sPLA2
IgM-RF			
Inflammation 3 years earlier	0.06 (-0.01-0.12)	0.07 (0.04–0.10)	0.18 (0.07-0.29)
Inflammation 2 years earlier	0.07 (0.01-0.13)	0.05 (0.02–0.08)	0.16 (0.06-0.25)
Inflammation 1 year earlier	0.12 (0.06-0.18)	0.05 (0.01–0.08)	0.19 (0.10-0.29)
Inflammation at same point in time	***0.09 (0.04-0.13)***	***0.07 (0.04–0.09)***	***0.20 (0.13-0.27)***
Inflammation 1 year later	0.06 (0.00-0.12)	0.04 (0.02–0.07)	0.15 (0.06-0.24)
Inflammation 2 years later	0.11 (0.05-0.16)	0.02 (-0.01–0.04)	0.14 (0.05-0.23)
Inflammation 3 years later	0.05 (-0.01-0.12)	0.02 (-0.01–0.05)	0.09 (-0.01-0.20)
Anti-CCP			
Inflammation 3 years earlier	0.05 (0.00-0.10)	-0.02 (-0.05–0.01)	0.00 (-0.09-0.09)
Inflammation 2 years earlier	0.03 (-0.03-0.08)	-0.01 (-0.03–0.02)	0.01 (-0.07-0.09)
Inflammation 1 year earlier	0.08 (0.03-0.13)	0.00 (-0.03-0.03)	0.08 (-0.01-0.16)
Inflammation at same point in time	***0.05 (0.02-0.09)***	***0.05 (0.02-0.07)***	***0.13 (0.07-0.19)***
Inflammation 1 year later	0.00 (-0.05-0.05)	0.01 (-0.02-0.03)	0.01 (-0.07-0.04)
Inflammation 2 years later	0.08 (0.03-0.14)	0.02 (0.00-0.05)	0.13 (0.04-0.21)
Inflammation 3 years later	0.06 (-0.01-0.13)	0.01 (-0.02-0.05)	0.09 (-0.01-0.19)

Also, in the subgroups of IgM RF positive and the anti-CCP positive patients there was no stronger association between the antibody tests and the inflammatory measures 1, 2, and 3 years before and after a point in time in comparison with the association based on measurements at that same time (data not shown).

Discussion

Serumlevels of sPLA2 were increased in the preclinical phase of patients with RA in comparison with levels of healthy controls, in a manner similar to CRP, as

published previously [4]. Both measures of inflammation were used to study the temporal relationship between the increased markers of inflammation and autoantibodies in preclinical RA. The sophisticated technique of time lag analysis, did not detect a stronger association between the antibody tests and the inflammatory measures up to 3 years before or after a point in time in comparison with measurements at the same time.

In a previous study we concluded that it remained unclear whether increased inflammation, measured by CRP, occurs before, after or simultaneously with the development of autoantibodies in preclinical RA [4]. sPLA2 is also increased in preclinical RA, but the patterns over time of sPLA2 and CRP differ between individual patients. Therefore, both measures of inflammation were analysed separately and combined in a new time lag analysis in an attempt to unravel the sequence of increased inflammation and raised autoantibody concentrations in the preclinical phase of RA. Because again we did not find a time lag, it is unlikely that a time lag can be found by adding other inflammatory measures to the analysis, and the possibility of a simultaneous occurrence of these phenomena becomes more likely.

A possible explanation for missing an actually existing time lag might be that the available blood samples, although numerous, were taken too far apart in time to be able to detect a short time lag. A possibly temporary sPLA2 or CRP peak indicative of an infectious process involved in causing the RA might also have been missed because the samples were obtained too far apart. The opposite conclusion, that because there is no evidence for a time lag, the phenomena of inflammation and autoantibody formation must be intimately coupled, is likely to be true but cannot be established beyond doubt on the basis of the present data. Support for the possibility of simultaneous development comes from the observed link between local antibody production and level of inflammation in the rheumatoid synovium [7]. In the somewhat different situation of B cell depletion in established RA, the relapse of active arthritis after recovery from this depletion coincides with increasing CRP levels, which are preceded by a rise in autoantibody levels [8].

In conclusion, both the acute phase response and autoantibody formation often develop years before the first symptoms of RA occur, and these phenomena are probably closely connected in time.

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Chapter 5

Lipids and inflammation: serial measurements of the lipid profile of blood donors who later developed rheumatoid arthritis

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Abstract

Background: Rheumatoid arthritis is characterised by inflammation and an increased cardiovascular risk. It was recently shown that active early rheumatoid arthritis is associated with dyslipidaemia, which may partially explain the enhanced cardiovascular risk. However, it is unknown when this dyslipidaemia starts.

Objective: To investigate the progression of the lipid profile over time and the influence of inflammatory parameters on this lipid profile, in people who later developed rheumatoid arthritis.

Methods: Levels of total cholesterol, high-density lipoprotein cholesterol (HDLc), triglycerides, apolipoprotein AI (apo AI), apolipoprotein B (apo B) and lipoprotein(a) (Lp(a)) were determined in 1078 stored, deepfrozen, serial blood bank samples, collected between 1984 and 1999, of 79 blood donors who later developed rheumatoid arthritis. These samples were compared with 1071 control samples of unselected blood donors, matched for age, sex and storage time.

Results: Samples of patients who later developed rheumatoid arthritis showed, on average, 4% higher total cholesterol, 9% lower HDLc, 17% higher triglyceride and 6% higher apo B levels than matched controls ($p \leq 0.05$). The magnitude of the differences in lipid levels between groups, explained by C reactive protein (CRP), was limited. For example, only 3.6% of the difference in HDLc levels between the groups was explained by the CRP concentrations.

Conclusion: Patients who later develop rheumatoid arthritis have a considerably more atherogenic lipid profile than matched blood donors at least 10 years before onset of symptoms. As inflammation only marginally explains the differences between the two groups, a modulating effect of lipids on inflammatory processes is hypothesised.

Introduction

Rheumatoid arthritis, an inflammatory joint disease, is associated with increased cardiovascular morbidity and mortality, as shown by several inception cohort studies [1–6]. The cause of this enhanced cardiovascular risk is unknown, but inflammation is thought to play an important part [7], which is in line with the accumulating evidence that inflammation has a prominent role in the development of atherosclerosis [8, 9]. The relevance of inflammation in the development of cardiovascular disease is shown by associations between future cardiovascular events and raised C reactive protein (CRP) levels [10–13]. The underlying pathophysiological mechanism has not yet been completely elucidated and several possibilities have been suggested. Acute-phase proteins might: (1) deteriorate ‘‘fatty streaks’’ into (instable) plaques [9], (2) destabilise plaques and cause plaque ruptures [12], (3) give complement activation [13] or (4) facilitate deterioration of the lipid profile [14].

Dyslipidaemia may be responsible for the increased cardiovascular risk in patients with rheumatoid arthritis. Several investigators have shown that active rheumatoid arthritis is associated with an unfavourable lipid profile—that is, a decreased total cholesterol and relatively lower high-density lipoprotein cholesterol (HDLc) levels [14–16]. The result is a less favourable atherogenic index, suggesting a relationship between inflammation and dyslipidaemia.

Other lipoproteins have been suggested to play an important part in the development of atherosclerosis—that is, lipoprotein(a) (Lp(a)), apolipoprotein AI (apo AI) and apolipoprotein B (apo B). A few studies have investigated Lp(a) levels in patients with rheumatoid arthritis, and the results are contradictory. Several earlier studies showed considerably lower Lp(a) levels in patients with rheumatoid arthritis and a marked association with acute-phase response [17]. A later study found neither considerably raised levels of Lp(a) in patients with rheumatoid arthritis nor a notable relationship between Lp(a) and the acute-phase response [18]. Evidence is growing that both apo AI and apo B are useful predictors for future cardiovascular risk. Apo AI may protect against cardiovascular disease, whereas apo B may increase cardiovascular risk [19–21]. Apo AI is the major apolipoprotein in HDLc and has various structural and functional roles in HDLc metabolism. Apo B is the main apolipoprotein of chylomicrons, very low-density, low-density and intermediate-density lipoproteins, and seems to be a better predictor for cardiovascular disease than

low-density lipoprotein cholesterol [22]. So far, data regarding apolipoproteins in rheumatoid arthritis are sparse and contradictory.
Recently, we showed that in a large group of blood donors, the presence of a preclinical phase preceding the actual clinical phase of rheumatoid arthritis is characterised by serological changes—that is, raised levels of immunoglobulin M rheumatoid factor (IgM-RF) and anti-cyclic citrullinated peptides (anti-CCP) [23]. In addition, we also found raised CRP levels in the samples of the blood donors who later developed rheumatoid arthritis compared with samples with random control donors [24]. Furthermore, the presence of dyslipidaemia in early active rheumatoid arthritis raises the question of whether or not this phenomenon starts in the preclinical phase of rheumatoid arthritis.
This study was undertaken to investigate the lipid profile over time and its relationship with inflammation and serological markers, in patients who later developed rheumatoid arthritis.

Methods

Participants

The Sanquin Blood Bank Northwest Region, formerly the Red Cross Blood Bank, in Amsterdam, The Netherlands, serves an area with about 2 million inhabitants. The blood bank stores 1 ml aliquots of serum at -30°C, from each donation since 1984. On average, donors donate blood 2–4 times a year over a long-term period. The maximum age of donors is 70 years. Within the area in which the blood bank collects blood from donors, most of the patients with rheumatoid arthritis are registered in the Jan van Breemen Institute, a regional network of rheumatology outpatient clinics. After receiving approval from the local institutional review board, all registered patients with rheumatoid arthritis were sent a letter asking them if they had been a blood donor before the symptoms of rheumatoid arthritis started. When the reply was positive, patients were asked to sign informed consent, permitting the use of stored blood samples for the present study. Ultimately, we identified 79 non-related patients with rheumatoid arthritis, satisfying the 1987 American College of Rheumatology criteria for rheumatoid arthritis [25]. Chart review yielded the following data: time of the start of symptoms, time of diagnosis of rheumatoid arthritis, IgM-RF at the time of diagnosis and during follow-up, and the presence of bony erosions on radiographs.

Procedures

From 1984 to 1999, patient samples were collected longitudinally from each consecutive blood donation. This was done during the entire period in which the patients were blood donors. For each sample of every patient with rheumatoid arthritis, one control sample from a random blood donor matched for sex, age (SD 3 years) and time of blood donation
was selected as described earlier.23 24 This method of control selection was chosen to ensure identical storage conditions for the materials from patients and controls.

Laboratory measurements

Serum total cholesterol (<5.0 mmol/l) and triglycerides (<2.2 mmol/l) were analysed by an enzymatic method using the appropriate assays supplied by Roche Diagnostics (Almere, The Netherlands), on a Hitachi 911 analyzer (Roche Diagnostics), according to the instructions of the manufacturer. Polyethylene glycol modified enzymes were used to assess HDLc levels (in men, >0.9 mmol/l; in women, >1.1 mmol/l). Apo AI (in men, 1.04–2.02 g/l; in women, 1.08–2.25 g/;), apo B (in men, 0.66–1.33 g/l; in women, 0.60–1.17 g/l) and Lp(a) (<30 mg/dl) were analysed by an immunoturbidimetric method, using appropriate assays (Roche Diagnostics). CRP (<10 mg/l) was measured using a latex-enhanced highly sensitive assay (Roche Diagnostics). Secretory phospholipase A2 (sPLA2; <5 ng/ml) was measured by means of a highly sensitive ELISA (Sanquin Research at CLB, Amsterdam, The Netherlands). IgM-RF and anti-CCP antibodies were measured using inhouse ELISAs on an ES 300 analyzer (Roche Diagnostics) [23, 24]. IgM-RF was calibrated with a national reference serum containing 200 IU/ml. No reference serum is available for anti-CCP. The applied criteria for positive tests were >30 IU/ml for IgM-RF and >50 AU/ml for anti-CCP.

Statistics

We divided the data into 1-year periods before the start of the symptoms and we used the samples from 10 to 0 years before the start of the symptoms to estimate the time course of the different lipids. Samples from 15–10 years before the start of the symptoms were omitted because of the small number of available samples. The time course was estimated using a random coefficient multilevel regression analysis—that is, a longitudinal regression technique that allows both starting levels and progression over time to differ between subjects [26]. Multilevel regression analysis can be seen as a longitudinal linear regression analysis, which combines many cross-sectional linear regression models into

one model of one variable over time. In our study, we looked at the lipid levels over time and investigated the influence of being a future patient or a control and the influence of inflammation parameters on the progression of the various lipid levels over time. Multilevel regression analysis quantifies these influences, or rather associations, between the lipid levels and these two sets of variables, and tests within for significance.

In addition to this increase of statistical power by combining the cross-sectional data of various time points into one association, the multilevel method enables the user to correct for confounding caused by multiple testing within subjects.

The differences in triglyceride and Lp(a) levels between patients and controls were reported as the differences in geometric means, as these variables were non-normally distributed. Subsequently, the natural logarithm of these levels was used for the analyses.

Preceding the time course analysis, it was investigated whether the various lipid levels were constant over time or if there were increases or decreases in certain 1-year periods.

The first step of modelling the data was carried out to determine differences between patients and controls in the lipid levels over time. These differences were reported as absolute values and as percentages of the mean lipid levels as measured in the control groups. The possibility of interaction between time and group was also investigated in this model. In the second step of this analysis, CRP, IgM-RF and anti-CCP were added to the model to investigate whether the difference between patients and controls could be explained by CRP, IgMRF or anti-CCP. The differences in the various lipid concentrations between patients and controls, which could be explained by CRP, IgM-RF or anti-CCP, were reported as percentages of the observed total differences between the two groups. We also added another acute-phase reactant to the model—that is, sPLA2—to investigate whether this would have additional value.

In another analysis, the longitudinal relationships between the lipids and CRP, IgM-RF and anti-CCP were analysed for patients and controls combined into one group. Firstly, a model investigating variables measured at the same time was used. Secondly, a ‘‘time-lag’’ –model, in which the relationship between the lipid concentrations of a particular 1-year period with inflammation or serological markers of a previous 1-year period, was investigated [26].

The random coefficient analyses were carried out using the statistical program MLwiN.27 A p value <0.05 was considered significant.

Results

Patient characteristics

We included 79 patients with rheumatoid arthritis, 61% were women, with a mean age of 51 years at the onset of symptoms. The range of available serum samples was 1–51, with a median of 13 samples per patient. The first sample was taken at a median of 7.5 (range 0.1–14.5) years before the onset of symptoms. For serum samples from seven patients, no matched control sera were available, resulting in a total of 1078 serum samples of patients and 1071 from controls.

Lipid levels

Figure 5.1 shows the development of lipid levels over time as measured in blood samples of the patients and controls. An investigation of the total cholesterol, HDLc, triglyceride, apo AI, apo B and Lp(a) levels, using the 1-year periods separately, yielded no trend breach in the progression of lipid levels over time; in other words, the increase or decrease in lipid levels was constant over time. This justified time course estimations in which the 1-year periods were investigated as one whole period.

The first step modelling the data yielded significant differences in lipid levels between patients and controls. Mean total cholesterol levels were higher in the patients than in the controls, on average 0.21 mmol/l ($p=0.05$). Triglyceride and Apo B levels were also higher in patients than in controls, on average 0.26 mmol/l ($p<0.001$) and 0.06 mg/l ($p=0.02$), respectively. The HDLc levels were lower in patients, on average 0.08 mmol/l ($p<0.001$). Expressed as percentages of the calculated mean of the controls, we found that the total cholesterol levels in patients were on average 3.8% higher, triglyceride levels were 16.8% higher, apo B levels were 5.8% higher and HDLc levels were 9.0% lower. We found no effect modification between time and division into groups, indicating that the variance of the lipid levels over time was similar in both groups.

The magnitude of the observed difference in lipids between patients and controls, as explained by CRP, was 4.0% for total cholesterol, 0.4% for triglyceride, 3.6% for HDLc and 2.0% for apo B levels. The influence of IgM-RF and anti-CCP on the differences in total cholesterol, triglyceride, HDLc and apo B levels was also minor, ranging from ,1% to a few per cent. In addition, the other inflammation marker sPL-A2 had a similar effect on the differences in lipids between the two groups and it had no additional value above CRP (data not

shown). Table 5.1 shows the differences in lipid levels between patients and controls, after adjustment for the influence of CRP, IgM-RF and anti-CCP.

Figure 5.1: Development of lipid levels over time in patients and controls. Values in patients who later developed rheumatoid arthritis are represented by solid lines and the values in controls are dashed lines

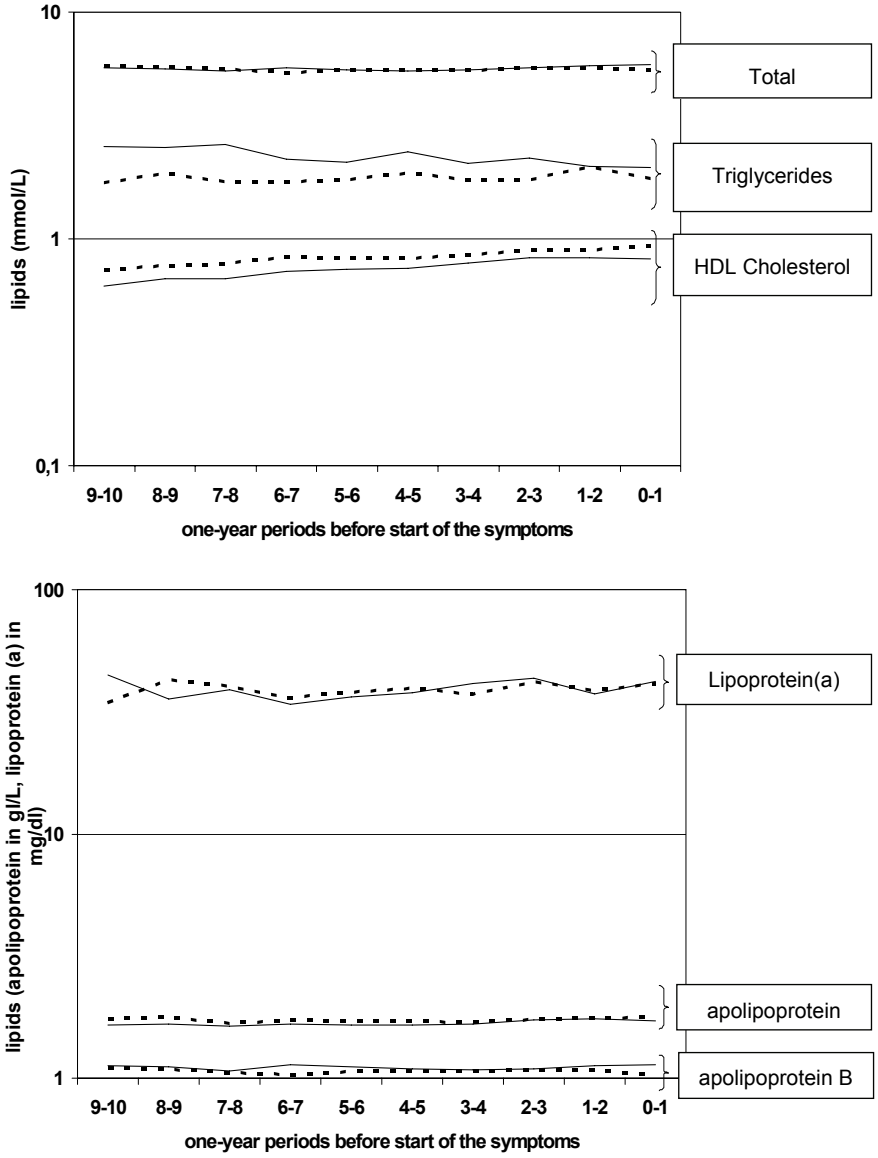

Table 5.1: Multilevel regression analyses of TC, HDLc, apo AI, apo B and Lp(a) levels in patients who later developed rheumatoid arthritisand controls

Lipid	Crude data absolute difference (95% CI)	CRP-adjusted absolute difference (95% CI)	IgM-PRF-adjusted absolute difference (95% CI)	Anti-CCP-adjusted absolute difference (95% CI)
TC (mmol/l)	0.21 (20.00 to 0.42)*	0.22 (0.00 to 0.43)*	0.21 (20.00 to 0.43)*	0.19 (20.02 to 0.41)
HDLc (mmol/l)	20.08 (20.12 to 0.03)‡	20.07 (20.11 to 20.04)‡	20.08 (20.12 to 20.04)‡	20.07 (20.11 to 20.03)‡
Triglycerides (mmol/l)†	0.53 (0.48 to 0.58)‡	0.53 (0.48 to 0.58)‡	0.53 (0.48 to 0.59)‡	0.53 (0.48 to 0.58)‡
Apo AI (g/l)	20.02 (20.08 to 0.04)	20.02 (20.08 to 0.04)	20.01 (20.07 to 0.05)	20.02 (20.08 to 0.04)
Apo B (g/l)	0.06 (0.01 to 0.11)*	0.06 (0.01 to 0.11)*	0.06 (0.01 to 0.11)*	0.06 (0.01 to 0.11)*
Lp(a) (mg/dl)†	3.16 (0.27 to 0.41)	0.33 (0.30 to 0.41)	0.34 (0.28 to 0.41)	0.33 (0.27 to 0.40)

Anti-CCP, anti-cyclic citrullinated peptides; apo AI, apolipoprotein AI; apo B, apolipoprotein B; CRP, C reactive protein; HDLc, high-density lipoprotein cholesterol; IgMRF, immunoglobulin M rheumatoid factor; Lp(a), lipoprotein(a); TC, total cholesterol.
Values are absolute differences between patients who later developed rheumatoid arthritis and controls with 95% CI. No effect modification was found between time and division in patients or controls, so the differences remained constant over time.

* p≤0.05; †For triglycerides and Lp(a) the differences of the geometric means were reported because these variables were non-normally distributed and the natural logarithm was used in the analyses; ‡p<0.001.

To investigate the longitudinal relationship of inflammation and serological markers with the various lipid levels, patients and controls were combined into one group. It was shown that CRP had a significant influence on total cholesterol, HDLc and apo AI levels. An increase of 10 mg/l in CRP level was associated with decreases of 0.1 mmol/l in total cholesterol (p=0.01), 0.04 mmol/l in HDLc (p<0.001) and 0.05 mg/l in apo AI levels (p<0.001). We found no significant associations for the other lipids. Looking at the serological markers, we only found a small significant influence of IgM-RF on apo AI (p=0.02)—that is, an increase of 10 AU/ml in IgM-RF was associated with a decrease of 0.005 g/l in apo AI. The final analysis using this time course model yielded no evidence for a time lag between the lipids and CRP, sPL-A2 or the serological markers. Although the associations reported here were found in the pooled population, it did not matter whether the person was a patient who later developed rheumatoid arthritis or a control, because the same significant relationship between inflammation and serological markers with the lipid levels was found in the separate groups.

Discussion

Our study shows that the lipid profile of blood donors who later developed rheumatoid arthritis is more atherogenic than that of matched controls. This lipid profile is characterised by higher total cholesterol, triglyceride and apo B levels and lower HDLc levels. Even after adjusting for IgM-RF, anti-CCP and CRP levels, the lipid profile remained more atherogenic in patients. This phenomenon starts more than 10 years before the clinical onset of rheumatoid arthritis.

The differences in the various lipid values are small but may have clinical relevance, in the light of results from other studies [28–30]. For instance, in a placebo-controlled study with fibrates, the differences in lipid values between groups receiving active treatment and placebo were similar to those found in our study and the patients treated with fibrates had >20% risk reduction for cardiovascular disease [31].

Contrary to the expectations, CRP had only a marginal influence on the differences in lipid levels between patients and controls. Another acute-phase reactant, sPL-A2, showed a comparable, marginal influence on the differences in lipids between patients and controls. The influence of CRP on lipid levels was equal for both groups—that is, increase in of CRP level was associated with a decrease in total cholesterol, HDLc, apo AI and Lp(a) levels, and with an increase in triglycerides and apo B, resulting in a higher atherogenic index (ie, ratio of total cholesterol to HDLc).

Growing evidence indicates that inflammation has an important role in the pathogenesis of cardiovascular disease, particularly in atherosclerosis.10 In addition to a postulated direct effect of inflammation on endothelial cells, there is mounting evidence that inflammation can also increase the cardiovascular risk by deterioration of the lipid profile. This is supported by the demonstration of a decrease in HDLc and apo AI levels and an increase in triglycerides and apo B levels during an acute-phase response [32]. Other investigators found an association between increase in lipids as oxidised low-density lipoprotein cholesterol and proinflammatory cytokines as CRP, interleukin 6 and tumour necrosis factor a [33]. Our findings confirm these effects of inflammation on the various lipid concentrations. As the changes in CRP only explained a small part of the observed differences in lipid levels that remained after adjusting for CRP, an alternative explanation is required.

Two possible explanations are plausible. Firstly, in view of the association between the development of rheumatoid arthritis and a less favourable lipid profile, a (marginally) deteriorated lipid profile may render a person more

susceptible to inflammation or inflammatory diseases. In other words, one or more of the examined lipids could have a regulatory effect on inflammation. Several reports show anti-inflammatory effects of HDLc and particularly apo AI. It is suggested that apo AI is able to inhibit interactions between T lymphocytes and monocytes, thereby modulating the inflammatory response [34]. Moreover, another study showed the ability of apo AI to inhibit interleukin 1 and tumour necrosis factor a, which further supports the theory of an active modulating role of lipids in inflammation [35].

Secondly, a less favourable lipid profile is related to the development of rheumatoid arthritis by a common or linked background. This could be a socioeconomic (including dietary) or genetic background. A few studies have indicated a genetic predisposition for dyslipidaemia in patients with rheumatoid arthritis.36 Further unravelling of the human genome will probably elucidate this predisposition. Further investigations into genetic markers that could single out the population at risk should be undertaken.

In summary, our study supports the observation that patients with rheumatoid arthritis have a more atherogenic lipid profile even in the preclinical phase of rheumatoid arthritis, which ultimately could explain the increased cardiovascular risk in patients with rheumatoid arthritis. Furthermore, we show that inflammation is associated with a (further) deterioration of the lipid profile. However, contrary to expectations, inflammation can explain only a small part of the observed differences in lipids between people who later develop rheumatoid arthritis and controls. Whether lipids modulate the susceptibility to the development of inflammatory diseases such as rheumatoid arthritis remains to be elucidated.

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Chapter 6

Vitamin D deficiency does not increase the risk of rheumatoid arthritis

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To the Editor:

In an interesting report from the Iowa Women's Health Study by Merlino et al [1], the authors conclude that increased dietary intake of vitamin D is associated with a lower risk of rheumatoid arthritis (RA). Vitamin D levels were estimated using a self-administered dietary questionnaire. However, it is well-known that sun exposure is a much more important source of vitamin D than dietary intake. Moreover, the total amount of vitamin D can be accurately measured by the serum level of 25-hydroxyvitamin D (25[OH]D), which is a more reliable indicator of vitamin D deficiency [2]. Therefore, we tested the hypothesis that serum 25(OH)D levels in healthy blood donors who later developed RA would be lower than those in matched healthy controls.

We studied 79 patients with RA who had donated blood a median of 13 times (range 1–51) at the Sanquin Blood Bank North West Region in Amsterdam before the onset of symptoms, as described previously [3]. From each patient 3 serum samples were selected, 1 each from the time points 1 year, 2 years, and ≥5 years before the start of symptoms. One control donor sample was selected per patient sample. The control samples were matched for age, sex, and time of donation to ensure identical storage conditions and to prevent seasonal influences. We measured 25(OH)D using a binding enzyme-linked immunosorbent assay (ImmunDiagnostik, Benheim, Germany). Serum samples from all 3 time points were not available for all patients. In total, 192 patient samples and 190 samples from control donors were studied.

In the first analysis, the cutoff value for vitamin D deficiency was arbitrarily set at 20 nmoles/liter. The association between RA and vitamin D deficiency at all 3 time points was calculated by chi-square test, using SPSS version 11.0. In the second analysis, the mean difference in vitamin D levels in the RA patients and controls was estimated by random coefficient multilevel analysis with MLwiN (Multilevel Models Project, Institute of Education, University of London, London, UK) [4]. This technique was used because each patient had a different number of measurements at different points in time. Because vitamin D levels were not normally distributed, geometric means were calculated.

The association between vitamin D deficiency and later development of RA is presented in table 6.1. There was no association between these 2 parameters at any time point (P = 0.59, P = 0.44, and P = 0.37 at 1 year, 2 years, and ≥5 years before the start of symptoms, respectively).

Table 6.1: Association between vitamin D deficiency and later development of RA*

	Patients with preclinical RA		Controls		
	Vitamin D deficiency	No vitamin D deficiency	Vitamin D deficiency	No vitamin D deficiency	*P*
1 year before symptoms	15	42	17	38	0.59
2 years before symptoms	14	45	18	42	0.44
≥5 years before symptoms	21	55	16	59	0.37
Total	50	142	51	139	0.86

* Values are the number of subjects. RA = rheumatoid arthritis.

Levels of 25(OH)D in 50 of 192 pre–clinical RA samples (26.0%) were below the threshold of 20 nmoles/liter, compared with 51 of 190 control samples (26.8%) (*P* = 0.86). We also used lower cutoff values (12.5, 10, 7.5, and 5 nmoles/liter) and still found no association between vitamin D deficiency and later RA. The geometric mean vitamin D concentration (estimated by random coefficient analysis) was slightly lower in the patients than in the controls (29.8 versus 32.1 nmoles/liter), but the difference was not statistically significant (*P* = 0.44).

RA is caused by an interplay of genetic and environmental factors. Vitamin D deficiency has been suggested as an environmental risk factor, based on vitamin D levels in patients with RA as well as studies of animal models of arthritis. More than 60% of RA patients have vitamin D levels below the normal range [5–7]. Als et al found lower serum vitamin D

concentrations in RA patients than in healthy controls [8]. Furthermore, pretreatment with analogs of vitamin D decreased the incidence of collagen-induced arthritis in mice [9] and rats [10, 11]. This observation in animal models suggests an immunomodulatory effect of 1,25-vitamin D, the active metabolite. In accordance with these results, Merlino et al found that vitamin D intake was inversely associated with the risk of RA onset. However, the present results do not show any difference in vitamin D levels between patients with preclinical RA and well-matched controls. This discrepancy may be a result of the different methods of assessing vitamin D levels. In the present study, vitamin D was measured in serum, whereas Merlino et al used questionnaires to measure dietary vitamin D intake, without taking sun exposure into account. We believe that direct measurement of vitamin D in serum is a more accurate estimate of vitamin D levels than is a dietary questionnaire.

In conclusion, we found no difference between 25(OH)D serum levels in patients who later developed RA and healthy donors. This suggests that vitamin D does not have an important role in the pathogenesis of RA.

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Chapter 7

Bone metabolism is altered in preclinical rheumatoid arthritis

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Submitted

Abstract

Objective: Increased levels of autoantibodies and inflammation markers occur years before onset of symptoms of rheumatoid arthritis (RA). The present study investigates whether presence of autoimmunity and inflammation in preclinical RA is accompanied by alterations in bone metabolism.

Methods: Seventy-nine patients had donated blood before onset of RA. From each patient 3 samples were selected: 1, 2 and 5 years or longer before onset of symptoms, respectively, together with one control sample. The following markers were measured: 1) markers for bone formation: osteocalcin (OC) and N-terminal propeptide of type I collagen (P1NP), 2) a marker of bone resorption: β-C-telopeptide (β-CTX), and 3) regulators of osteoclast activity: receptor activator of NFκB ligand (RANKL) and osteoprotegerin (OPG). The mean values at the specified time points of the different markers in the patient and control groups were compared with random coefficient analysis. Variables differing statistically significantly from controls were then tested for their possible association with future radiographic progression using linear regression analysis.

Results: Correcting for age, gender, time of blood donation, autoantibodies and inflammation, preclinical RA patients had increased mean levels of P1NP and OPG compared with the control group. Preclinical levels of P1NP and OPG were negatively associated with radiographic progression after the onset of the symptoms of RA, but these associations were not statistically significant.

Conclusion: The asymptomatic phase of RA is characterized not only by autoimmunity and increased inflammation, but also by a parallel alteration of bone metabolism.

Introduction

The chronic inflammation of the joints in rheumatoid arthritis (RA) may lead to the destruction of cartilage and bone. Thirty percent of patients with newly diagnosed RA already have joint damage that is visible on radiographs after a median symptom duration of only three months [1]. Before the era of modern combination therapies, this figure increased to 75% two years after the onset of the disease [2]. Radiographic damage is associated with a loss of functional capacity in a later stage of the disease [3, 4]. Since joint damage is irreversible but potentially avoidable by modern treatment, it is important to diagnose and treat RA early, preferably before joint damage occurs [5, 6].

The development of radiographic damage in RA can be predicted by measurements of markers and regulators of bone metabolism in blood and urine. Measurements of bone formation in RA patients have produced varying results, whereas measurements of bone resorption in these patients mostly show increased values. The activity of bone formation by the osteoblast can be measured among others by osteocalcin (OC) and by the N-terminal telopeptide of type I procollagen (P1NP). RA patients have significantly lower OC levels in comparison with healthy controls [7], however, differences in OC levels between RA patients with and without radiographic progression could not be found [7, 8]. Contrarily, elevated P1NP levels were found in RA patients compared with healthy controls [9]. The association between P1NP levels and radiographic progression has not yet been studied in RA. The activity of bone degradation by the osteoclast can be measured by, among others, the C-terminal crosslink of type I collagen (β-CTX), a collagen C-telopeptide breakdown product. RA patients have significantly higher urine and serum β-CTX levels compared with healthy controls [7, 9]. Serum β -CTX is associated with radiographic damage in early arthritis [7, 8] and higher β-CTX concentrations were found in erosive arthritis patients in comparison with non-erosive arthritis patients [8]. In addition, urine β-CTX levels are significantly correlated with future radiographic progression [10-12].

The activation of the osteoclast is regulated by the Receptor Activator of Nuclear Factor Kappa B (NFκB) ligand (RANKL) and osteoprotegerin (OPG), in combination with Receptor Activator of NFκB (RANK). RANKL is an osteoclast-activating cytokine that is produced by osteoblasts and activated T-cells, whereas OPG, a soluble receptor produced by a variety of tissues including the cardiovascular system, lung, kidney, intestine, and bone, as well as hematopoietic and immune cells, prevents osteoclast activation. RA patients have higher serum levels of both OPG and RANKL in comparison with matched healthy controls [13]. In the COBRA-study, high RANKL and low OPG levels were associated with an increased risk of radiographic damage in early arthritis patients [14].
In a previous study we found increased levels of rheumatoid factor (IgM-RF) and/or antibodies against cyclic citrullinated peptides (anti-CCP) in one half of blood donors, starting at median 5 years before they developed the first symptoms of RA [15]. We also demonstrated increased levels of acute phase reactants in these patients years before the onset of symptoms, regardless of their autoantibody status [16, 17]. The present study was performed to investigate whether this preclinical subtle increase of inflammation has an influence on markers of bone metabolism. Therefore, we tested whether concentrations of markers of bone metabolism and regulators of osteoclast activity in preclinical RA patients differ from healthy controls at three time points in the preclinical phase of RA. In case of any differences between preclinical RA patients and controls, we investigated whether these markers and/or regulators were associated with radiographic damage after the onset of the symptoms of RA.

Patients and methods

Patients

Since 1984, the Sanquin Blood Bank North West Region in Amsterdam, The Netherlands, has stored 1-ml aliquots of serum from donated blood at -30˚C. From 5000 patients registered with RA at the Jan van Breemen Institute, 79 patients were identified who had donated blood, in general 2-4 times per year, before the onset of RA, as described previously [15] . All blood donations from a particular RA patient in the period 1984-1999 were traced. For each RA sample, 1 control sample was selected, matched for gender, age, and time of blood donation to ensure identical storage conditions. Also, the most recent available

radiographs of hand and feet of the 79 RA patients were collected. The study was approved by the local Institutional Review Board.

Procedures

In previous studies we used all patient samples (median 13 samples per patient) and for each patient sample a matched control sample [15-17]. In the present study, from each later RA patient three serum samples were selected, if available: at 1 year, 2 years and 5 years or longer before the start of the symptoms, respectively, together with the corresponding matched control sample. The percentage of controls positive for IgM-RF and anti-CCP and the median CRP concentrations of the controls in this study were comparable with the findings in the previous studies [15-17].

The following markers were measured: 1) two markers for bone formation: OC and P1NP, 2) a marker of bone resorption: β-CTX, and 3) two counteracting regulators of osteoclast activity: RANKL and OPG. All markers were measured using commercial assays according to the instructions of the manufacturers. OC (N-mid), P1NP and β-CTX were measured with an Elecsys 2010 (Roche Diagnostics, Mannheim, Germany). Total RANKL and OPG were measured with appropriate ELISA kits from Immundiagnostik, Bensheim, Germany. IgM-RF, anti-CCP, secretory phospholipase A_2 (sPLA2) and C-reactive protein (CRP) were measured in previous studies [15-17].

All available radiographs were scored according to the Sharp/van der Heijde method [17] by an experienced rheumatologist (DvS), who was blinded for all preclinical test results.

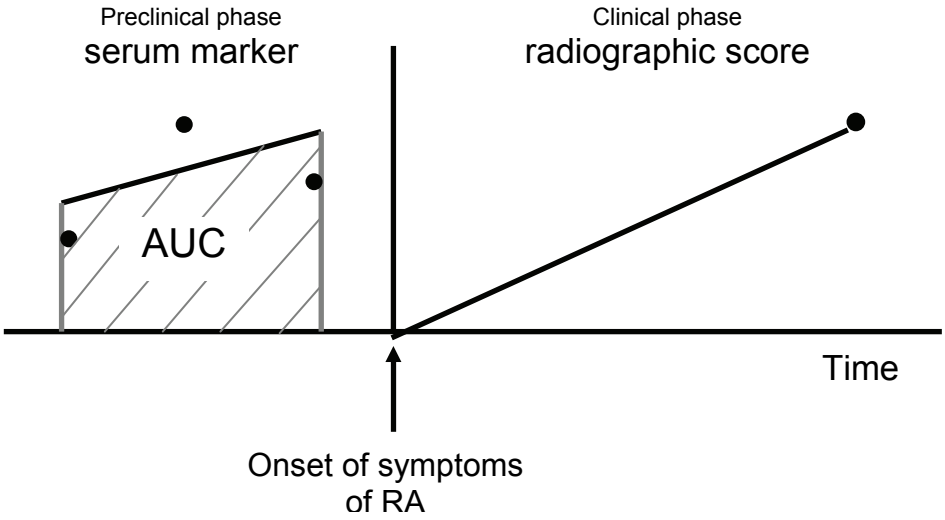
Analysis

In the first analysis, only preclinical RA patients with samples at all three time points were selected with matched controls. At all time points the mean concentrations of OC, P1NP, β-CTX and OPG of the group of preclinical RA patients were compared with those of the group of healthy controls by using independent t-tests. Median RANKL levels were compared between the groups of patients and controls with Mann-Whitney U tests.

The first analysis was performed with only the patients who had samples available at all three time points and was not corrected for potential confounding factors, i.e. gender, age, the presence of autoantibodies and levels of markers of inflammation. Therefore, comparable analyses were performed with all patient samples in the period of 0-6 years before the onset of symptoms and in matched control samples. In this second analysis, differences in OC, P1NP, β-CTX, OPG and RANKL over time in preclinical RA patients were compared with controls with

random coefficient analysis. This method was used to correct for the different number of samples per patient and the variation in the time of the blood donation [18]. Samples obtained six years or longer before the onset of symptoms were not used because of their small number. All regression models were corrected for age, gender, time of donation, autoantibodies (IgM-RF and anti-CCP) and markers of inflammation (CRP and sPLA2). Because the distribution of RANKL concentrations was skewed to the right, the natural log of RANKL concentrations was used in the analysis.

Figure 7.1: Graph of the analysis of the association per individual between preclinical serum markers and radiographic scores



AUC: area under the curve

If a bone marker or a regulator of osteoclast activity differed between preclinical RA patients and healthy controls in the random coefficient analysis, this parameter was associated with future radiographic progression within the group of preclinical RA patients. For each patient, individual regression lines were calculated for the specific markers and/or regulators of bone metabolism. These individual regression lines were used to calculate the mean individual area under the curve (AUC) per year (see figure 7.1). Not for all patients it was possible to calculate an individual regression line. When a particular parameter was not

measured in an individual, the mean AUC per year of the whole group was used. When only one value of a parameter was available, the mean slope of the whole group was used to measure the individual mean AUC per year. The mean AUC per year of the bone markers and regulators were associated with the mean radiographic progression per year (i.e. the most recent Sharp/ van der Heijde score divided by the number of years with symptoms of the patient at the time of this radiological measurement) with linear regression analysis. The association was also corrected for age at the start of the symptoms of RA, gender and the presence of autoantibodies in the preclinical phase.
Linear regression analyses were carried out with SPSS 14.0. Random coefficient analyses were performed with MLwiN (Multilevel Models Project, Institute of Education, University of London, London, UK).

Results

Patient characteristics
Seventy-nine patients (62% female; mean age at onset of the symptoms 51 years) who had been blood donor before the onset of RA were identified. A median of 13 serum samples per patient (range 1–51) was available; the median time between the first donation and the onset of the symptoms was 7.5 years (range 0.1–14.5 years). In total, 1078 patient sera and 1071 matched control sera were identified. From each patient 3 samples were selected: at 1 year, 2 years and 5 years or longer before the start of the symptoms, respectively, together with one matched control donor sample. In this study, 192 patient sera and 191 matched control sera were tested. For 32 patients it was not possible to select a sample at all three time points; 18 patients had two samples and 14 patients had one sample available. For one patient sample there was no matched control sample.
Radiographs of hands and feet were available for 71 out of 79 patients (90%). The mean time between the start of the symptoms and the radiographs was 7.2 years. Median Sharp/van der Heijde score at that time point was 16 (IQ-range 5-49).

Bone markers and regulators of osteoclast activity
Forty-six out of 79 (58%) preclinical RA patients had samples at all three time points. The mean bone marker levels of preclinical RA patients and matched controls at one, two and five years before the start of the symptoms of RA are

shown in table 7.1. OPG was increased in preclinical RA patients compared with controls at all three time points (P<0.001). In preclinical RA patients at one year and two years before the onset of symptoms of RA, P1NP and RANKL levels were increased, but these difference were not statistically significant (for P1NP p=0.12 and p=0.07, and for RANKL p=0.14 and p=0.09, respectively). β-CTX was increased in preclinical RA patients one year before the start of symptoms, but this difference was also not statistically significant (p=0.15).
In the next analysis, all patient samples and matched controls in the time period of 0-6 before the start of the symptoms of RA were used to correct the differences between preclinical RA patients and controls for potential confounding variables in a multivariate regression analysis. The regression models for the bone markers and the regulators of osteoclast activity are shown in table 7.2. After correction for age, gender, time of blood donation, autoantibodies and markers of inflammation, the group of preclinical RA patients had statistitically significantly increased mean levels of P1NP and OPG compared with the control group (an increase of 5.0 ng/ml and 4.1 pmol/l for P1NP and OPG, respectively). Differences in median RANKL and mean β-CTX levels between the patient and control groups in the first univariate analysis (table 7.1) were no longer found after correction for age, gender, autoantibodies and inflammation.

Table 7.1: Mean bone marker levels of preclinical RA patients and matched controls at one, two and five years before the start of the symptoms of RA

	Patients		Controls		p-value
1 year before onset symptoms					
P1NP (ng/ml)	46.5	(19.1)	39.6	(22.1)	0.12
OC (ng/ml)	19.9	(7.9)	18.3	(8.3)	0.36
β-CTX (ng/ml)	0.13	(0.08)	0.11	(0.05)	0.15
OPG (pmol/l)	10.0	(5.5)	5.2	(1.5)	0.00
RANKL (pmol/l)*	653.5	(401.0-1582.0)	491.5	(330.3-1179.0)	0.14
2 years before onset symptoms					
P1NP (ng/ml)	49.4	(22.0)	40.6	(23.0)	0.07
OC (ng/ml)	20.6	(8.7)	18.8	(7.9)	0.30
β-CTX (ng/ml)	0.10	(0.06)	0.11	(0.07)	0.75
OPG (pmol/l)	8.6	(2.9)	4.5	(1.3)	0.00
RANKL (pmol/l)*	645.5	(387.3-1617.0)	437.0	(318.8-1064.8)	0.09
5 years before onset symptoms					
P1NP (ng/ml)	46.3	(19.9)	43.3	(23.2)	0.51
OC (ng/ml)	18.8	(6.4)	18.5	(8.0)	0.86
β-CTX (ng/ml)	0.11	(0.07)	0.10	(0.05)	0.20
OPG (pmol/l)	7.6	(2.5)	4.6	(1.3)	0.00
RANKL (pmol/l)*	619	(393.3-1544.8)	570.5	(347.3-1098.5)	0.35

P1NP = N-terminal telepeptide of type I procollagen, OC = Osteocalcin, β-CTX = C-terminal crosslink of type I collagen, OPG = Osteoprotegerin, RANKL = Receptor activator of NFκB ligand.
* Median and inter-quartile range

Table 7.2: Association between bone markers and the presence of preclinical RA, corrected for time of donation, age, gender, presence of autoantibodies and inflammation

	B (standard error)									
	P1NP		OC		CTX		OPG		Ln RANKL	
Preclinical RA versus controls	**5.02**	**(2.00)**	1.06	(0.74)	0.0081	(0.0067)	**4.10**	**(0.33)**	-0.02	(0.10)
Time of donation (years)	0.40	(0.60)	0.12	(0.22)	-0.0001	(0.0020)	-0.18	(0.09)	0.00	(0.03)
Age (years)	-0.07	(0.13)	0.09	(0.05)	0.0001	(0.0004)	**0.05**	**(0.02)**	0.00	(0.01)
Gender (female vs male)	2.64	(2.98)	1.44	(1.07)	-0.0037	(0.0094)	0.32	(0.35)	-0.06	(0.13)
sPLA2 (ng / ml)	0.32	(0.24)	0.15	(0.09)	-0.0004	(0.0008)	0.00	(0.04)	**0.03**	**(0.01)**
CRP (mg / l)	**-0.65**	**(0.25)**	**-0.31**	**(0.09)**	-0.0009	(0.0008)	0.06	(0.04)	-0.01	(0.01)
IgM-RF (IU / ml)	-0.05	(0.03)	-0.01	(0.01)	-0.0001	(0.0001)	-0.01	(0.00)	**0.01**	**(0.00)**
Anti-CCP (AU / ml)	0.00	(0.00)	0.00	(0.00)	0.0000	(0.0000)	0.00	(0.00)	0.00	(0.00)
Intercept	40.52	(8.95)	11.74	(3.21)	0.1112	(0.1112)	2.20	(1.05)	6.24	(0.40)

Statistically significant B's are in bold; The models are calculated with random coefficient analysis.
P1NP = N-terminal telepeptide of type I procollagen, OC = Osteocalcin, β-CTX = C-terminal crosslink of type I collagen, OPG = Osteoprotegerin
RANKL = Receptor activator of NFκB ligand, sPLA2 = secretory phospholipase A_2, CRP = C-reactive protein, IgM-RF = rheumatoid factor, Anti-CCP = antibodies against cyclic citrullinated peptides.

Association between preclinical serum markers and radiographic progression

If a bone marker or a regulator of osteoclast activity differed between preclinical RA patients and healthy controls in the multivariate analysis, this parameter was associated with future radiographic progression within the group of preclinical RA patients. Based on the previous analysis, P1NP and OPG are potential predictors of radiographic progression in preclinical RA patients, since the mean levels of these two markers were increased in preclinical RA patients. For these two markers, the mean individual AUC per year of the preclinical serum markers was associated with the mean progression per year of the Sharp/van der Heijde score. After correction for age, gender and the presence of preclinical autoantibodies, preclinical levels of P1NP and OPG were negatively associated with radiographic progression after the onset of the symptoms of RA (B=-0,60 (p=0.12) and B=-0,09 (p=0.16) for P1NP and OPG, respectively), but these differences were not statistically significant (Table 7.3).

Table 7.3: Association of P1NP and OPG and future radiographic progression in preclinical RA, corrected for age, gender and preclinical presence of autoantibodies

	B (standard error)			
	P1NP		OPG	
AUC rediographic damage (Sharp / van der Heijde score)	-0,60	(0,38)	-0,09	(0,07)
Gender (female versus male)	**9,67**	**(4,64)**	0,39	(0,81)
Age at start of the symptoms of RA (years)	0,01	(0,22)	0,06	(0,04)
Preclinical IgM-RF positivity	1,79	(5,72)	-0,44	(0,99)
Preclinical anti-CCP positivity	0,76	(4,85)	-0,44	(0,84)
Intercept	38,25	(13,25)	6,84	(2,30)

Statistically significant B's are in bold; The models are calculated with linear regression analyses.
P1NP = N-terminal telepeptide of type I procollagen, OPG = Osteoprotegerin, AUC = area under the curve, IgM-RF = rheumatoid factor, Anti-CCP = antibodies against cyclic citrullinated peptides.

Not all patients had two or more measurements for each parameter, which is necessary to calculate an individual regression line. Therefore, we used the mean slope of the whole group for these patients to calculate the individual mean AUC per year. This method was compared with two other methods: 1) only using the AUC of the patients who had two or three measurements of the specific parameter, and 2) using a slope of zero (beta = 0). The calculated B's did not differ between the three statistical methods (data not shown) and therefore we used the described method to increase the power of the analysis since more patients could be included.

Discussion

In this group of preclinical RA patients a number of abnormalities were found in serum markers of bone metabolism and osteoclast regulation. OPG was increased compared with controls at one, two and five years before the start of the symptoms of RA. Statistically non-significant findings were increased levels of P1NP and RANKL (at one year and two years before the onset of symptoms) and β-CTX (at one year before the start of symptoms). With multivariate analysis, correcting for age, gender, time of blood donation, autoantibodies and inflammation, preclinical RA patients proved to have increased levels of P1NP

and OPG compared with controls. Preclinical levels of P1NP and OPG were also negatively associated with radiographic progression after the onset of the symptoms of RA, but these differences were not statistically significant.
The levels of OC and β-CTX (indicating the activity of bone formation and degradation, respectively) in preclinical RA patients were similar to those found in healthy controls. This is in contrast to studies in RA patients with established and active disease, in which lower serum OC levels and higher urine and serum β-CTX levels were found compared with healthy controls [7, 9, 10, 19]. P1NP levels were increased in preclinical RA, which is in line with results in RA patients [9]. This suggests that significant differences in concentrations of markers of bone formation can be detected above a certain level of “disease activity” in the presymptomatic phase of RA. Also, elevated levels of OPG were found, which indicates an altered, probably increased, osteoclast activity in the preclinical phase.
We found a trend for a negative association between preclinical levels of P1NP and OPG and radiographic joint damage after the onset of the symptoms of RA. This suggests that a low level of bone formation as well as a low level of inhibition of osteoclast activation in the preclinical phase of RA is associated with more radiographic damage after the start of the symptoms of RA. In theory, it might be expected that preclinical inflammation would lead to increased preclinical bone degradation as a forerunner of radiographic damage in the clinical phase, indicating that bone resorption is upregulated by inflammatory processes. Instead of increased bone degradation, however, we found increased OPG. One possible explanation of the observed phenomena could be that the elevation of OPG is an - albeit failing - compensation mechanism for a slight and not measurable increase in bone degradation. In the clinical phase of early RA, one study found that high RANKL and low OPG levels are associated with an increased risk of radiographic damage, which is not in line with the results of the present study [14]. The possibility of bone damage occurring in the preclinical phase of RA is supported by the recent demonstration of erosive infiltration of joint margins in the asymptomatic phase of collagen induced arthritis, an animal model of RA [20].
The present study has some limitations. Differences in the mean levels of markers and/or regulators of osteoclast activity between patients and controls could be influenced by the menopausal state or the use of bone active agents, although we expect that the number of patients using bone active agents was probably low, since osteoporosis is often undertreated, particularly during the time of the study (1984-1999). It was not possible to correct for these potential confounding factors, because the controls were anonymous donors and only gender, age and the time

of blood donation are known. Additional information on the preclinical RA patients was collected by chart review and also in this group, menopausal state or the use of bone active agents could not be measured reliably. Since the controls were matched for age and gender, it is not expected that this has influenced the difference between patients and controls, although it could have influenced the absolute test values in both groups. Also, it was not possible to use fasting samples for this study since the blood bank recommends donors to eat before they donate blood. It is unknown whether this has influenced the results. Furthermore, data from only 71 patients could be used to study the association between preclinical levels of serum bone markers and regulators of osteoclast activity and radiographic progression after the start of the symptoms of RA. This could be the reason for the non-significant associations between the preclinical bone markers and radiographic progression in this study. Finally, it must be noted that the findings of this study can be used only in a population of patients and are not suitable for decision-making in individual patient care.

In conclusion, the present study of the asymptomatic phase of RA adds evidence of an alteration of bone metabolism parallel to the increased inflammation and autoimmunity described earlier in this situation. In at least a part of the patients, the process of joint destruction is already underway when they experience the first symptoms of the disease.

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Chapter 8

HLA-DR4 and antibodies against cyclic citrullinated peptides are associated in preclinical rheumatoid arthritis

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Submitted

Abstract

Objective: The association between genetic markers (HLA-DR4 and SE) and the presence of auto antibodies (IgM-RF and anti-CCP) was determined in healthy persons before the start of symptoms.

Methods: In 56 pre-clinical RA patients HLA-DRB1 SE-alleles were determined by HLA-DQ-typing and associated to the onset of anti-CCP antibodies and rheumatoid factor in the years before RA symptoms started. Blood samples donated before symptoms commenced were obtained from the Sanquin Blood Bank.

Results: Of the 56 pre-clinical RA patients, 26 were positive for anti-CCP (46%), 13 for IgM-RF (23%), 32 for HLA-DR4 (57%) and 47 for SE (84%). Anti-CCP was significantly associated with the presence of HLA-DR4 (p=0.03, OR: 3.5; 95% CI 1.1-11.0). However, the association between anti-CCP and SE did not reach significance (p=0.11, OR: 3.7; 95% CI 0.7-19.4) probably due to the small number of patients. IgM-RF was not statistically significantly associated with HLA-DR4 (p=0.31, OR: 2.0; 95% CI 0.5-7.3) and the association with SE did just not reach significance (p=0.07, OR could not be calculated due to the absence of patients with the combination IgM-RF+ and SE-).

Conclusion: In preclinical RA, anti-CCP and carriership of HLA-DR4 are positively associated whereas a trend is observed for IgM-RF and SE alleles.

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease of unknown origin, characterised by chronic joint inflammation leading to destruction of bone and cartilage, reduction of functional capacity and increased mortality (1, 2). Since structural joint damage is irreversible, early recognition and treatment of the disease is very important (3, 4) and tests to predict future RA in healthy persons at risk are needed. In a recent study it was found that one half of RA patients had autoantibodies, IgM Rheumatoid Factor (IgM-RF) and antibodies against cyclic citrullinated peptide (anti-CCP), a median of 4.5 years before the onset of symptoms (5). Particularly with the anti-CCP test it is possible to predict the development of RA in healthy individuals with an increased risk of developing RA, such as first degree family members of RA patients or persons with arthralgia without joint swelling or other signs of inflammation (5).
The cause of RA is multifactorial, most likely a combination of genetic and environmental factors. The main genetic factor is the presence of specific HLA-class II alleles (HLA-DR4 and DR1) encoding the shared epitope (SE) which are present more often among RA patients (SE carrier ship frequency 60-70%) compared with the healthy population (SE carrier ship frequency 42%) (6, 7). Therefore, genetic factors could be useful for the detection of RA in a healthy population. This study was designed to associate genetic markers (HLA-DR4 and SE) with the presence of auto antibodies (IgM-RF and anti-CCP) in healthy individuals before the start of symptoms of RA.

Patients and methods

From 5000 patients registered with RA at the Jan van Breemen Institute, a large outpatient rheumatology clinic, 79 patients were identified who had donated blood before the onset of RA, as described previously (5). IgM-RF and the first generation anti-CCP have been determined previously (5). Patients were considered as being positive for IgM-RF and/or anti-CCP if they were positive at least once for these tests before the start of symptoms. DNA was collected from 56 out of these 79 RA patients. Unfortunately, in the other 23 RA patients genetic material could not be collected because of death (n=3), moving home

(n=19) or non-response (n=1). The study was approved by the local Medical Ethical Committee.
The carrier ship of SE in the Dutch population (41%) was estimated in a previous study (7) and compared with the preclinical RA patients. The presence of HLA-DRB1*04 (HLA-DR4) and SE (HLA-DRB1*04, HLA-DRB1*01 and DRB1*10) were inferred from HLA-DQA1 and DQB1 typing performed by PCR-Single Strand Conformation Polymorphism method (8, 9).
Associations between the SE-alleles and the auto antibody tests (IgM-RF and anti-CCP) were calculated by Chi square and odds ratios (OR) were determined in SPSS 14.0.

Results

A median of 10 (range, 1 – 51) serum samples for auto-antibodies measurement, per patient was available the first of which was taken at a median of 8.1 years (range, 0.1 – 14.5 years) before the onset of the symptoms. The studied group had a lower mean age and contained more men in comparison to the group of persons who dropped out, but the differences did not reach statistical significance (p=0.13 and p=0.16, respectively). Of the 56 pre-clinical RA patients, 26 were positive for anti-CCP (46%), 13 for IgM-RF (23%), 32 for HLA-DR4 (57%) and 47 for SE (84%). The numbers of patients with the test combinations are shown in table 8.1. Anti-CCP was significantly associated with the presence of HLA-DR4 (p=0.03, OR: 3.5; 95% CI 1.1-11.0). The association between anti-CCP and SE, however, did not reach significance (p=0.11, OR: 3.7; 95% CI 0.7-19.4). IgM-RF was not statistically significantly associated with HLA-DR4 (p=0.31, OR: 2.0; 95% CI 0.5-7.3) and the association with SE did just not reach significance (p=0.07, OR could not be calculated due to the absence of patients with the combination IgM-RF+ and SE-).

Table 8.1: Association between autoantibodies and HLA-DR4 and shared epitope (SE) in preclinical RA patients

	anti-CCP+	anti-CCP-	p-value	IgM-RF+	IgM-RF-	p-value
HLA-DR4+	19	13	0.03	9	23	0.31
HLA-DR4-	7	17		4	20	
SE+	24	23	0.11	13	34	0.07
SE-	2	7		0	9	

Discussion

In pre-clinical RA a positive association between anti-CCP and carrier ship of one or two HLA-DRB1*04 alleles was observed. These results are in accordance with the observation by Berglin et al. (10) who described 59 subjects with pre-clinical RA (of whom 45 were women) in whom a very high relative risk (OR 66.8, 95% CI 8.3-539.4) for future development of clinical RA was found based on the presence of anti-CCP antibodies and carrier ship of DRB1*0404 and DRB1*0401 SE alleles.

A study of carrier ship of SE alleles in anti-CCP positive arthralgia patients compared with anti-CCP positive early arthritis and established RA patients showed a significantly higher frequency of SE alleles in the arthritis patients compared with the arthralgia patients (9). These results confirm the findings of the present study which shows that a combination of SE alleles and HLA-DR4 and anti-CCP or RF predict the onset of arthritis at an early stage. In established (early) RA more studies stress the importance of the serological antibodies in combination with SE alleles. In early RA, the carrier ship of one or two SE-alleles showed a strong correlation with the production of anti-CCP antibodies (OR 3.3, 95% CI 1.8-6.0 and OR 13.3 95% CI 4.6-40.4 respectively) (11). This group also described a strong association between the number of SE-alleles and the production of anti-CCP antibodies. A small study from Germany described a stronger association of the production of anti-CCP with HLA-DR4 than with HLA-DR1 positive RA patients (12). The association of a major DR4 positive SE allele, HLA-DRB1*0401, was described as very strong because 90% of the RA patients carrying this allele were anti-CCP-positive. Not all RA patients carrying the HLA-DRB1*0401 allele, however, were anti-CCP-positive.

It can be concluded in established RA that serological tests are related to the genetic markers of RA at the MHC, but more tests can be added in the future like PTPN22 , TRAF-1 C5, STAT 4 that seem to be related to the anti-CCP positivity in RA (13). The fact that the strong relationship between the presence of SE and the serological markers anti-CCP and IgM-RF was expected but could not be confirmed in this study was probably due to the fact that the number of available DNA samples that could be obtained several years after the blood donation unfortunately was limited. Several efforts were made to isolate DNA out of the sera because some patients could not be retrieved or had passed out. Unfortunately insufficient DNA could be extracted to perform HLA-typing. However, this study in preclinical RA shows a positive association between HLA-DR4 and anti-CCP which is in accordance with the studies in arthralgia patients

and in established RA. Moreover, the prevalence of carrier ship of SE-alleles (84%) was also significantly higher in this pre-clinical RA group compared with the healthy population (42%) (7). These facts support the hypothesis that in order to detect RA at a very early stage of the disease it can be useful to add tests for genetic markers like SE to the serological tests like the anti-CCP antibodies and IgM-RF, especially if medical interventions at such an early phase of the disease are planned to inhibited the onset of RA.

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Chapter 9

Antibodies to citrullinated human fibrinogen (ACF) have diagnostic and prognostic value in early arthritis

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Abstract

Background: The anti-cyclic citrullinated peptide (CCP) test has a high sensitivity and specificity for rheumatoid arthritis, although CCP is not the physiological target of the autoantibodies. Citrullinated fibrin is abundant in inflamed synovium.

Objective: To assess the diagnostic and prognostic value of antibodies against citrullinated fibrinogen (ACF), a soluble precursor of fibrin, in comparison with IgM-rheumatoid factor (IgM-RF) and the second generation anti-CCP test.

Methods: In 379 patients with early arthritis (258 rheumatoid and 121 undifferentiated), the sensitivity, specificity, and positive predictive value of ACF, anti-CCP, and IgM-RF for diagnosing rheumatoid arthritis were calculated. Multivariate logistic regression analysis was used to assess the diagnostic and prognostic value (radiographic progression after two years) of the tests.

Results: The sensitivities of the ACF, anti-CCP, and IgM-RF tests were 55.8%, 57.8%, and 44.6%, with specificities of 92.6%, 94.2%, and 96.7%, respectively. Approximately 30% of the IgM-RF negative patients were positive for ACF or anti-CCP or both. The ACF and anti-CCP test had a high agreement in early arthritis ($k = 0.84$). Of all baseline characteristics, the ACF test and the anti-CCP test were the best predictors for diagnosing rheumatoid arthritis at one year (odds ratio (OR) = 10.3 and 10.6, respectively) and for radiographic progression after two years (OR = 12.1 and 14.8).

Conclusions: ACF is as sensitive as anti-CCP and more sensitive than IgM-RF in diagnosing rheumatoid arthritis in early arthritis. The ACF test is also a good predictor of radiographic progression, with a performance similar to the anti-CCP test. The ACF test and the anti-CCP test are especially valuable in IgMRF negative arthritis.

Introduction

Rheumatoid arthritis is a systemic autoimmune disease of unknown origin. To prevent joint destruction, early diagnosis and treatment is required. The diagnosis can be made by the 1987 classification criteria of the American College of Rheumatology (ACR) [1], but these criteria have a low sensitivity in early arthritis [2].

There are a few rheumatoid arthritis specific antibodies. These include the so called antiperinuclear factor (APF), antifilaggrin antibodies (AFA), and antikeratin antibodies (AKA). The epitopes recognised by APF, AFA, and AKA were found to be generated by a post-translational modification— namely deimination of the natural amino acid arginine to the amino acid citrulline by activity of peptidylarginine deiminase [3]. Based on that knowledge, Schellekens and co-workers developed an enzyme linked immunosorbent assay (ELISA) using a cyclic citrullinated peptide (CCP) derived from the sequence of human filaggrin as substrate [4]. The assay was later improved (the second generation anti-CCP test) and sensitivities of 70–80% at specificities of 98–99% have been reported in established rheumatoid arthritis and controls [5, 6]. Sensitivity in early arthritis cohorts for the diagnosis rheumatoid arthritis varies between 40% and 70% [4, 7–11]. Although the anti-CCP ELISA has a reasonable sensitivity, the cyclic citrullinated peptide is not the physiological target of the autoantibodies.

Citrulline containing antigens are expressed in rheumatoid arthritis synovium [12, 13]. Moreover, B cells that actively secrete anti-CCP are specifically present in bone marrow and synovial fluid of anti-CCP seropositive patients with rheumatoid arthritis [14, 15]. When searching for the nature of citrullinated proteins in rheumatoid synovial tissue, Masson-Bessière et al identified citrullinated α and β chains of fibrin as the target for APF, AFA, or AKA positive sera [16]. Subsequently, antibodies to in vitro citrullinated fibrinogen, a soluble precursor of fibrin, have been described as a serological criterion for the early diagnosis of rheumatoid arthritis when compared with rheumatoid factor (RF) and the first generation anti-CCP assay [17].

To study the diagnostic and prognostic value of anticitrullinated fibrinogen (ACF) in early arthritis in comparison with IgM-RF and the second generation anti-CCP test, two studies were undertaken: a cross sectional analysis of patients with established rheumatoid arthritis and nonrheumatoid controls, and a diagnostic and prognostic study on patients from an early arthritis clinic (EAC).

Methods

Patients

To calculate the cut off values of the ACF test, anti-CCP test, and the IgM-RF test at 99% specificity, the following groups of patients were tested: 239 established cases of rheumatoid arthritis (53 active and 186 in clinical remission), 91 rheumatology clinic patients without arthritis, and 91 healthy controls. The rheumatoid patients fulfilled the ACR criteria for rheumatoid arthritis [1]. These calculated cut off values were then used to study the diagnostic and prognostic value of the ACF test in comparison with the IgM-RF and anti-CCP tests in a study population.

This study population consisted of 379 consecutive patients aged >18 years, with peripheral arthritis of two or more joints and a symptom duration of two years or more; they were referred to the EAC of the Jan van Breemen Institute, a large rheumatology clinic in Amsterdam, between 1995 and 1998. Patients who had previously been treated with a disease modifying anti-rheumatic drug (DMARD) and those with spondylarthropathy, reactive arthritis, crystal induced arthropathy, systemic lupus erythematosus, Sjögren's syndrome, or osteoarthritis were excluded. The diagnosis of rheumatoid arthritis after one year follow up was made in 258 patients by an experienced rheumatologist (BD), who was blinded to the results of the ACF and anti-CCP tests. The remaining 121 non-rheumatoid patients were classified as having undifferentiated arthritis (73 with polyarthritis and 48 with oligoarthritis).

Disease indices

The following data were collected during the first visit: demographic characteristics, disease duration, disease activity by disease activity score (DAS28) [18], pain by visual analogue scale (VAS), and functional status by the health assessment questionnaire (HAQ) [19]. Laboratory assessments at baseline included erythrocyte sedimentation rate (ESR), C-reactive protein, IgM-RF, ACF, and anti-CCP. Radiographs of hands and feet were obtained at baseline and after two years. The number of erosions and the joint space narrowing were scored according to the Sharp/van der Heijde method [20] by an experienced rheumatologist (DvS), who was blinded to all baseline variables.

Antibody measurements

Antibodies to CCP were measured using the second generation immunoscan rheumatoid arthritis ELISA kit (Eurodiagnostica, Arnhem, Netherlands, cut off

value 25 AU/ml). The assay was carried out according to the manufacturer's protocol. IgM-RF was measured on an ES300 immunochemistry analyser (Roche Diagnostics, Almere, Netherlands) as described before [10].
IgG antibodies to citrullinated fibrinogen were detected by ELISA using citrullinated fibrinogen as immunosorbent [17]. Plasminogen-free fibrinogen (Calbiochem, Breda, Netherlands) was depleted of IgG using protein G sepharose. IgG-free fibrinogen was citrullinated in vitro using rabbit skeletal muscle peptidylarginine deiminase (PAD) (Sigma, Zwijndrecht, Netherlands): 7 U/mg fibrinogen in 0.1 M Tris-HCl (pH 7.4), 10 mM $CaCl_2$, and 5 mM DTT for two hours at 37˚C [21]. Citrullination was controlled by a mobility shift of α and β chain of fibrinogen detected by SDS gel electrophoresis followed by western blotting with a positive serum [16]. Microtitre plates (MaxiSorp, Nunc, Roskilde, Denmark)
coated with citrullinated fibrinogen (10 µg/ml phosphate buffered saline (PBS)) were incubated for one hour at room temperature with diluted sera in duplicate (1:50 in PBS, 0.2% gelatine, 0.05% (vol/vol) Tween 20). After incubation with horseradish peroxidase conjugated mouse monoclonal antihuman IgG (MH16MIXME, Sanquin, Amsterdam, Netherlands) for one hour at room temperature, 3,3', 5,5' tetramethylbenzidine (10 mg/ml in DMSO) 1:100 diluted in 0.11 M acetate buffer pH 5.5 supplemented with 10 µl/10 ml of a 3% H2O2 solution was added. The reaction was stopped with 2M H2SO4 and absorbance at 450 nm was measured. All washing steps were carried out with PBS, 0.1% Tween 20. The antibody titre is expressed in AU/ml using a pool of IgM-RF positive sera as calibrator in eight dilutions. Coefficients of intra-assay and interassay variation were below 20% both for the same batch of citrullinated fibrinogen and for different batches.
Nissinen et al reported that a majority of patients with recent onset rheumatoid arthritis and 44% of SLE patients were positive in an anti-PAD ELISA [22]. Because the PAD enzyme used to citrullinate fibrinogen is not removed from the antigen preparation, antibodies to PAD might influence the results. This is, however, unlikely, as we did not find positive reactions with SLE sera.

Data analysis

First, in the group of established rheumatoid arthritis patients and controls, the area under the receiver operating characteristic (ROC) curve of ACF, anti-CCP, and IgM-RF was calculated and the sensitivities of the tests were compared at three specificities (95%, 98.5%, and 99%). The cut off value of the three tests was calculated at 99% specificity. These values were used in the other statistical analyses.

Second, sensitivity, specificity, and positive predictive value (PPV) of ACF, anti-CCP, and IgM-RF were calculated in the group of 379 early arthritis patients. Sensitivity expresses the percentage of rheumatoid patients positive for the test, while specificity is calculated from the percentage of test negative patients with undifferentiated arthritis. The baseline characteristics of the rheumatoid patients and the patients with undifferentiated arthritis were compared using Student's t test, the Mann–Whitney U test, and the χ^2 test, as appropriate.
Finally, multivariate logistic regression analysis was used to assess the diagnostic and prognostic value of the ACF test in patients with early arthritis. The diagnostic value of the test was assessed by predicting the diagnosis of rheumatoid arthritis or undifferentiated arthritis at a one year follow up, while the prognostic value was assessed by predicting radiographic progression at two years of follow up. Radiographic progression was defined as an increase in the Sharp/van der Heijde score by at least 5 after two years follow up [23], those with smaller increases being classified as not progressive. Variables associated with the diagnosis of rheumatoid arthritis in the univariate analysis ($p<0.10$) were entered into the models as independent variables. The analyses were carried out with a backward logistic regression analysis in SPSS 11.5.

Results

The basic characteristics of the ACF test, the anti-CCP test, and the IgM-RF test in the patients with established rheumatoid arthritis and the controls are given in table 9.1. At a specificity of 99%, the sensitivities of the ACF test and the anti-CCP test were 67.8% and 71.1%, respectively. At this specificity, the IgM-RF test was only 41.8% sensitive. As a control for ACF, IgG depleted fibrinogen was coated at 10 µg/ml and the ELISA was carried out as described above. Subtracting the extinction obtained from the fibrinogen coat from that of citrullinated fibrinogen before calculation did not influence the specificity and sensitivity (data not shown). Cut off values of the tests at 99% specificity were 140 U/ml for ACF, 25 U/ml for anti-CCP, and 45 IU/ml for IgM-RF. To compare all tests at the same specificity, these high cut off values were chosen for further analysis in an early arthritis population.

Table 9.1: Sensitivity of IgM-RF, anti-CCP, and ACF in patients with established rheumatoid arthritis and controls

Test	Specificity			Area under ROC curve	Confidence interval
	95%	98.5%	99%		
IgM-RF	49.8%	45.2%	41.8%†	0.726	0.678 to 0.774
Anti-CCP	72.0%	71.1%	71.1%‡	0.903	0.873 to 0.932*
ACF	72.4%	67.8%	67.8%§	0.893	0.861 to 0.925*

* p<0.05 v IgM-RF.
Cut off values of the tests: †IgM-RF, 45 IU/ml; ‡anti-CCP, 25 U/ml; §ACF, 140 U/ml.
ACF, antibodies to citrullinated human fibrinogen; CCP, cyclic citrullinated peptide; RF, rheumatoid factor; ROC, receiver operating characteristic.

Table 9.2 shows the baseline characteristics of that population. The group of patients with rheumatoid arthritis was significantly older (p,0.01), had higher mean ESR and C-reactive protein levels (p<0.001), a higher mean DAS (p<0.001), a higher median radiographic damage score (p<0.01), and a worse mean HAQ (p<0.001) than the group of patients with undifferentiated arthritis.

Table 9.2: Baseline characteristics of the early arthritis population, separated into rheumatoid arthritis (RA) and undifferentiated arthritis (UA)

Baseline characteristics	Total group (n = 379)	RA (n = 258)	UA (n = 121)	p Value
Age (years)†	56.1 (15.6)	57.6 (14.8)	52.8 (16.6)	*
Female (n (%))‡	260 (68.6)	181 (70.2)	79 (65.3)	NS
Disease duration (years)§	0.4 (0.3 to 0.7)	0.4 (0.3 to 0.7)	0.4 (0.3 to 0.6)	NS
ESR (mm/h)†	31.8 (22.8)	36.1 (23.2)	22.8 (19.0)	**
C reactive protein (mg/dl)§	15 (4 to 35)	18.5 (6.3 to 44)	6 (2 to 18)	**
DAS28 score†	4.8 (1.3)	5.2 (1.2)	4.1 (1.2)	**
Sharp/van der Heijde score§	1 1 (0 to 6)	2 (0 to 6)	0 (0 to 4)	*
HAQ score†	1.0 (0.8)	1.2 (0.8)	0.8 (0.6)	**

Value are mean (SD) or median (interquartile range).
* p<0.01; **p<0.001.
† Student's t test; ‡χ^2 test; §Mann–Whitney U test.

Table 9.3: Sensitivity, specificity, and positive predictive value (PPV) of ACF, anti-CCP, and IgM-RF for the clinical diagnosis of rheumatoid arthritis in early arthritis

	Sensitivity (%)	Specificity (%)	PPV (%)
Early arthritis (n = 379)			
IgM-RF >45	44.6	96.7	96.6
ACF >140	55.8	92.6	94.1
Anti-CCP >25	57.8	94.2	95.5
IgM-RF negative early arthritis (n = 260)			
ACF >140	28.7	94.9	87.2
Anti-CCP >25	30.8	96.6	91.7

ACF, antibodies to citrullinated human fibrinogen; CCP, cyclic citrullinated peptide; RF, rheumatoid factor; ROC, receiver operating characteristic.

The sensitivity, specificity, and PPV for the diagnosis of rheumatoid arthritis of the tests described are shown in table 9.3. Sensitivities varied between 44.6% and 57.8%, and specificities between 92.6% and 96.7%. About 30% of the IgM-RF negative early arthritis patients were positive for ACF or anti-CCP or both. The ACF and anti-CCP tests had a very high agreement in early arthritis. ACF and anti-CCP were single positive in 29 of 379 patients (k=0.84, data not shown); 16 patients were single positive for anti-CCP (81.3% rheumatoid arthritis) and 13 patients were single positive for ACF (61.5% rheumatoid arthritis).

Complete two year follow up data were available from 296 of the 379 early arthritis patients (78.1%). These patients used a median of one DMARD (range one to five) during the period of follow up; 62% of the patients used methotrexate. The reasons for loss to follow up were: non-compliance (n=31); discharge from the clinic because of remission (n=17); moving home (n=10); death (n=9); and miscellaneous reasons (n=16). The group of patients lost to follow up had similar baseline characteristics as the group which completed the follow up, except for the median baseline Sharp/van der Heijde score which was higher in noncompleters than in completers (6 v 1, $p<0.001$). Also, the non-completers were less often positive for IgM-RF (18.1% v 35.1%), anti-CCP (25.6% v 45.1%), or ACF (26.8% v 43.7%) ($p<0.01$ for all tests) than the completers.

Table 9.4: Results of logistic regression analysis of baseline variables to predict rheumatoid arthritis at one year in early arthritis

	B	SE	Odds ratio (Exp(B))	95% CI	Accuracy
Constant	-3.355	0.622			
Anti-CCP ≥ 25	1.536	0.587	4.6	1.5 to 14.7	
IgM-RF ≥ 45	1.521	0.662	4.6	1.3 to 16.7	78.0%
ACF ≥ 140	1.443	0.614	4.2	1.3 to 14.1	
DAS28	0.800	0.145	2.2	1.7 to 3.0	
Pain (VAS)	-0.014	0.007	0.99	0.97 to 0.99	
Variables not in equation	**p value**				
Age	0.204				
ESR	0.484				
C reactive protein	0.089				
HAQ	0.371				
Sharp/van der Heijde	0.611				

ACF, antibodies to citrullinated human fibrinogen; CCP, cyclic citrullinated peptide; CI, confidence interval; DAS28, 28 joint disease activity score; HAQ, health assessment questionnaire; RF, rheumatoid factor; ROC, receiver operating characteristic; VAS, visual analogue scale.

In the univariate analysis, all baseline variables were significantly associated with the diagnosis of rheumatoid arthritis at one year ($p<0.05$, data not shown). Variables predictive of the diagnosis of rheumatoid arthritis in the logistic regression analysis were anti-CCP, IgM-RF, ACF, DAS28, and VAS pain (table 9.4). Because of the very high agreement between the ACF test and the anti-CCP test, two alternative predictive models were calculated with the same independent variables, but one without anti-CCP and one without ACF (data not shown). In the model without anti-CCP, the ACF test was the best predictor of diagnosis rheumatoid arthritis (odds ratio (OR)=10.3; 95% confidence interval (CI), 3.9 to 26.7) and in the model without ACF, the anti-CCP predicted diagnosis rheumatoid arthritis best (OR=10.6; 95% CI, 4.1 to 27.8).

Baseline variables with a significant association with radiographic progression at two years of follow up were ACF, anti-CCP, IgM-RF, ESR, C reactive protein, DAS28, HAQ, and the Sharp/van der Heijde score ($p<0.001$, data not shown). Variables predictive of radiographic progression in the logistic regression analysis were anti-CCP, ESR, and the Sharp/van der Heijde score at baseline, with anti-CCP as the best predictor (OR=14.8) (table 9.5).

Table 9.5: Results of logistic regression analysis of baseline variables to predict radiographic progression at two years in early arthritis

	B	SE	Odds ratio (Exp(B))	95% CI	Accuracy
Constant	23.468	0.438			
Anti-CCP ≥ 25	2.694	0.364	14.8	7.2 to 30.2	
Sharp/van der Heijde	0.102	0.029	1.1	1.0 to 1.2	80.2%
ESR	0.024	0.007	1.02	1.01 to 1.04	
Variables not in equation	***p value***				
IgM-RF ≥ 45	0.376				
ACF ≥ 140	0.099				
DAS28	0.412				
C reactive protein	0.616				
HAQ	0.481				

ACF, antibodies to citrullinated human fibrinogen; CCP, cyclic citrullinated peptide; CI, confidence interval; DAS28, 28 joint disease activity score; HAQ, health assessment questionnaire; RF, rheumatoid factor; ROC, receiver operating characteristic; VAS, visual analogue scale.

The ACF test was removed by this model, owing to the very high agreement between the ACF test and the anti-CCP test. Thus a second model for predicting radiographic progression was calculated without anti-CCP as an independent variable (table 9.6). In this model, ACF, ESR, and the Sharp/van der Heijde score at baseline were most predictive of radiographic progression, with the ACF test as the best predictor (OR=12.1).

Table 9.6: Results of logistic regression analysis of baseline variables (without anti-CCP) to predict radiographic progression at two years in early arthritis

	B	SE	Odds ratio (Exp(B))	95% CI	Accuracy
Constant	23.239	0.403			
ACF ≥ 140	2.494	0.343	12.1	6.2 to 23.7	
Sharp/van der Heijde	0.090	0.029	1.1	1.0 to 1.2	81.7%
ESR	0.024	0.007	1.02	1.01 to 1.04	
Variables not in equation	**p value**				
IgM-RF ≥ 45	0.553				
DAS28	0.291				
C reactive protein	0.955				
HAQ	0.447				

ACF, antibodies to citrullinated human fibrinogen; CCP, cyclic citrullinated peptide; CI, confidence interval; DAS28, 28 joint disease activity score; HAQ, health assessment questionnaire; RF, rheumatoid factor; ROC, receiver operating characteristic; VAS, visual analogue scale.

Discussion

The diagnostic and prognostic value of antibodies directed against citrullinated fibrinogen was compared with that of the second generation anti-CCP test in an early arthritis cohort. For diagnosing rheumatoid arthritis, the ACF test was as sensitive as the second generation anti-CCP test and more sensitive than the IgM-RF test. About 30% of the IgM-RF negative patients with early arthritis were positive for the ACF test and therefore this test will be useful, especially in IgM-RF negative early arthritis patients.

Despite the higher sensitivity of the ACF test and the second generation anti-CCP test compared with the IgM-RF test for diagnosing rheumatoid arthritis, the specificity of the IgM-RF test was slightly higher. This reflects a small percentage of patients diagnosed with undifferentiated arthritis and having autoantibodies to citrullinated proteins. Such patients could eventually develop rheumatoid arthritis, as has been suggested by the high positive predictive value of anti-CCP in a prospective study of patients with early arthritis [24]. In an earlier study in the same cohort, Jansen et al found a sensitivity of 42.6% and a specificity of 97.5% for the first generation anti-CCP test [10]. In this early arthritis population, both the ACF test and the second generation anti-CCP test were more sensitive for the diagnosis rheumatoid arthritis. In an early rheumatoid population, Nogueira

et al found a sensitivity for antibodies to citrullinated fibrinogen of 64.6% at 98.5% specificity [25], which is in line with the results of the present study. In multivariate analysis, we found that the anti-CCP test was the best predictor of the diagnosis rheumatoid arthritis, followed by the IgM-RF test and the ACF test (odds ratios around 4.5). Because of the high agreement of ACF and anti-CCP, it will not be useful to combine the two tests to predict the diagnosis of rheumatoid arthritis. Therefore, two other models were calculated with the same independent variables, but with only one of the two tests. In these models, baseline ACF and anti-CCP were similarly good predictors of the diagnosis rheumatoid arthritis one year later, with odds ratios of approximately 10.5.

The prognostic value of the ACF test was evaluated with multivariate logistic regression analyses using two year follow up data. ACF was a good predictor of radiographic progression at the two year follow up, nearly as good as the anti-CCP test (OR=12.1 v 14.8). Compared with previous reports on the prognostic value of citrulline specific autoantibodies, including the first and second generation anti-CCP test, an odds ratio of 12–14 is remarkably high [8, 11, 26–28].

The baseline characteristics of the non-completers were similar to those of the completers, except for the Sharp/van der Heijde score and the three antibody tests. The noncompleters had a higher median Sharp/van der Heijde score than the completers at baseline, although they were positive less often for IgM-RF, anti-CCP, and ACF. There may have been a coincidental selection of patients with a high Sharp/van der Heijde score at baseline, and a subsequent mild course of the disease, resulting in remission and loss to follow up. As the Sharp/van der Heijde score predicts radiographic progression, the selective loss to follow up may have led to an overestimation of the odds ratios of the anti-CCP test and the ACF test in predicting radiographic progression in our early arthritis population.

The results of this study underline the high disease specificity for antibodies to citrullinated proteins and peptides. However, the present data provide no explanation of how the antibody response develops in rheumatoid arthritis. There was no difference in sensitivity between the anti-CCP test and the ACF test in early arthritis and in established rheumatoid arthritis. In the vast majority of patients both ACF and anti-CCP were found. ACF and anti-CCP were both single positive in 8% of EAC patients. The agreement between the tests is surprising as citrullinated fibrin (fibrinogen is the soluble precursor of fibrin) has been described as a physiological substrate for antibodies recognising citrulline containing epitopes [16]. As has been shown for several other autoimmune disorders (reviewed by Doyle and Mamula [29]), antibodies might preferentially

recognise a modified physiological target—that is, citrullinated fibrin— early in the disease. Later the antibody response could spread towards less restricted epitopes [30]. Responses to uncitrullinated fibrinogen could be detected in our study population and were higher in patients than controls. However, they never reached the degree of positivity that was found with citrullinated fibrinogen. It is known that rheumatoid arthritis specific antibodies can be detected several years before the onset of clinical symptoms [31–33]. Although the participants from the EAC in Amsterdam had a short disease duration at the time of testing for ACF and anti-CCP, differences between the two responses might be difficult to detect. The ACF response in patient samples taken before clinical signs of the disease could shed light on how the antibody response develops. Alternatively, other citrullinated proteins—for example, vimentin—might trigger the initial immune response in rheumatoid arthritis [5]. Citrulline containing peptides, derived from the sequence of vimentin, have been shown to be efficiently presented by the rheumatoid arthritis associated HLA-DRB1*0401 MHC class II molecule to T cells in a transgenic mouse model [34]. The data point towards an important role of citrulline as an anchor amino acid. Whether the overall sequence might be of less importance has to be elucidated in further studies.

In conclusion, the ACF test is useful for establishing the diagnosis of rheumatoid arthritis and is a good predictor of radiographic progression in early arthritis, comparable to the second generation anti-CCP test. Both tests are especially valuable in IgM-RF negative early arthritis.

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Chapter 10

Summary and general discussion

Summary of the main findings

The purpose of this thesis was to study the preclinical phase of RA to find serological parameters that might help to predict the onset of RA. The studies were performed by using serial blood samples of blood donors who developed RA later. This blood donor pre-RA cohort with matched controls was the result of a collaboration between the Sanquin Blood Bank North West Region, which had stored serum from all blood donations since 1984, and the Jan van Breemen Institute (JBI), both located in Amsterdam, The Netherlands. At the JBI, a large regional rheumatology clinic, 79 RA patients were identified who had previously been blood donor. Of these, a median of 13 serum samples was available. Serological markers were used to predict the development of RA in the preclinical phase of the disease and to study pathogenetic events. Also, the value of autoantibodies to predict RA and future radiographic damage in early arthritis was studied with data from the Early Arthritis Clinic at the JBI.

Preclinical autoantibodies (Chapter 2)
In previous studies, autoantibodies have been demonstrated in serum samples from healthy subjects up to 10 years before they developed RA. However, the time course for the development of antibodies before onset of clinical RA is unknown, nor is it known which antibody, or combinations of antibodies, might be most sensitive or specific for predicting future development of the disease. Serum samples from pre-clinical RA patients were tested for IgM-RF and first-generation anti-CCP. Thirty-nine patients (49%) were positive for IgM-RF and/or anti-CCP on at least one occasion before the development of RA symptoms, a median of 4.5 years (range 0.1–13.8) before symptom onset. Of the 2,138 control samples, 1.1% was positive for IgM-RF, and 0.6% was positive for anti-CCP. The finding of an elevated serum level of IgM-RF or anti-CCP in a healthy individual implies an increased risk for the development of RA. IgM-RF and anti-CCP testing with appropriately high specificity may assist in the early detection of RA in high-risk populations.

Preclinical inflammation (Chapters 3 and 4)

Preclinical inflammation was investigated in two studies. In the first study, the presence of serologic signs of inflammation in patients with preclinical RA was investigated with serial measurements of highly sensitive CRP. For the periods 0–1 year, 1–2 years, and 4–5 years before the onset of symptoms, the median CRP concentration was increased in preclinical RA patients compared with controls. Furthermore, the CRP concentration increased significantly over time in patients with preclinical RA. The increase was most common within the 2 years before the onset of symptoms. The preclinical increase in CRP levels was observed both in donors with and without serologic abnormalities. The difference in CRP concentrations between preclinical RA patients and controls may be a significant factor in the development of later symptomatic inflammation. However, there were only small differences in CRP levels between preclinical RA patients and controls. Therefore, the findings of this study can be used only in a population of patients and are not suitable for decision-making in individual patient care.

In the second study, the temporal relationship between onset of inflammation (measured by sPLA2 and CRP) and the presence of autoantibodies (IgM-RF and anti-CCP) was investigated in preclinical RA. IgM-RF and anti-CCP concentrations were significantly associated ($p<0.001$) with concentrations of sPLA2, CRP, and the combination of sPLA2 and CRP at the same time point. However, we found no stronger association between the two autoantibody tests and the three inflammation measures 1, 2, and 3 years before or after a time point than for measurements at the same time, in the whole group or in subgroups of IgM-RF and anti-CCP positive patients. In conclusion, both the acute phase response and autoantibody formation often develop years before the first symptoms of RA. These phenomena are probably closely connected in time.

Preclinical lipid profile (Chapter 5)

RA is characterised both by inflammation and an increased cardiovascular risk. Active early RA is associated with dyslipidaemia, which may partially explain the enhanced cardiovascular risk. However, it is unknown when this dyslipidaemia starts. Therefore, levels of total cholesterol, HDLc, triglycerides, apo AI, apo B and Lp(a) were determined in 1078 serum samples of 79 blood donors who later developed RA and compared with 1071 control samples, matched for age, sex and storage time. Samples of patients who later developed RA showed, on average, 4% higher total cholesterol, 9% lower HDLc, 17% higher triglyceride and 6% higher apo B levels than matched controls ($p< 0.05$). The magnitude of

the differences in lipid levels between groups explained by CRP was limited. It was concluded that patients who later develop RA have a considerably more atherogenic lipid profile than matched blood donors for at least 10 years before onset of symptoms.

Preclinical vitamin D levels (Chapter 6)

It was recently reported that higher dietary intake of vitamin D as measured by questionnaire was associated with a lower risk of RA. However, it is well-known that sun exposure is a much more important source of vitamin D than dietary intake. Since the total body amount of vitamin D can be accurately estimated by serum levels of 25-hydroxyvitamin D (25(OH)D), we tested the hypothesis that 25(OH)D serum levels of blood donors who developed RA later would be lower than matched controls. From each patient serum samples were selected from the time points 1 year, 2 years and 5 years or longer before the start of the symptoms, respectively, together with one control donor sample per patient sample. At all time points there was no association between vitamin D deficiency and later RA. The geometric mean 25(OH)D concentration was slightly lower in the patients compared with the controls (29.8 vs. 32.1 nmol/l), but the difference was not statistically significant. It was concluded that there is no difference between 25(OH)D serum levels in patients who later develop RA and healthy donors, which suggests that vitamin D does not play an important role in the pathogenesis of RA.

Preclinical bone markers and regulators of osteoclast activity (chapter 7)

This chapter describes a study in which it was tested whether the presence of autoimmunity and inflammation in preclinical RA is accompanied by alterations in bone metabolism. In preclinical RA patients and controls, the following markers were measured: 1) markers for bone formation: osteocalcin (OC) and N-terminal propeptide of type I collagen (P1NP), 2) a marker of bone resorption: β-C-telopeptide (β-CTX), and 3) regulators of osteoclast activity: receptor activator of NFκB ligand (RANKL) and osteoprotegerin (OPG). Correcting for age, gender, time of blood donation, autoantibodies and inflammation, the group of preclinical RA patients had increased mean levels of P1NP and OPG compared with the control group. Preclinical levels of P1NP and OPG were negatively associated with radiographic progression after the onset of the symptoms of RA, but these associations were not statistically significant. It appears that the presymptomatic phase of RA is characterized not only by autoimmunity and increased inflammation, but also by a parallel alteration of bone metabolism.

HLA-DR4 and Shared Epitope in preclinical RA (Chapter 8)
The association between genetic markers (HLA-DR4, now mostly measured as the shared epitope, SE) and the presence of autoantibodies (IgM-RF and anti-CCP) was determined in preclinical RA. Of the 56 preclinical RA patients of whom DNA was available, 26 were positive for anti-CCP (46%), 13 for IgM-RF (23%), 32 for HLA-DR4 (57%) and 47 for SE (84%). Anti-CCP was significantly associated with the presence of HLA-DR4 (p=0.03, OR: 3.5; 95% CI 1.1-11.0). However, the association between anti-CCP and SE did not reach significance (p=0.11, OR: 3.7; 95% CI 0.7-19.4), probably due to the small number of patients. IgM-RF was not statistically significantly associated with HLA-DR4 (p=0.31, OR: 2.0; 95% CI 0.5-7.3) and the association with SE did just not reach significance (p=0.07, OR could not be calculated due to the absence of patients with the combination IgM-RF+ and SE-). In conclusion, anti-CCP and carriership of HLA-DR4 and the SE allele are positively associated in preclinical RA. Since there was no DNA available of controls, it was not possible to use HLA-DR4 and SE for a risk calculation for the development of RA in the preclinical phase of the disease.

Autoantibodies in early arthritis (Chapter 9)
The anti-CCP test has a high sensitivity and specificity for RA, although CCP is not the physiological target of the autoantibodies. Citrullinated fibrin is abundant in inflamed synovium. The objective of this study was to assess the diagnostic and prognostic value of anti-citrullinated fibrinogen (ACF), a soluble precursor of fibrin, in comparison with IgM-RF and the second generation anti-CCP test. The sensitivities of the ACF, anti-CCP, and IgM-RF tests were 55.8%, 57.8%, and 44.6%, with specificities of 92.6%, 94.2%, and 96.7%, respectively. Approximately 30% of the IgM-RF negative patients were positive for ACF or anti-CCP or both. The ACF and anti-CCP test had a high agreement in early arthritis (kappa = 0.84). Of all baseline characteristics, the ACF test and the anti-CCP test were the best predictors for diagnosing RA at one year (OR = 10.3 and 10.6, respectively) and for radiographic progression after two years (OR = 12.1 and 14.8). This study shows that ACF is as sensitive as anti-CCP and more sensitive than IgM-RF in diagnosing rheumatoid arthritis in early arthritis. The ACF test is also a good predictor of radiographic progression, with a performance similar to the anti-CCP test. The ACF test and the anti-CCP test are especially valuable in IgM-RF negative arthritis.

Discussion

Based on the findings of the different studies in this thesis, it can be concluded that several processes start years before the onset of the symptoms of RA in many patients, which eventually lead to the development of clinical disease. Interaction of genetic and environmental factors results in the production of antibodies (rheumatoid factors and various types of anti citrullinated protein antibodies (ACPA)), inflammatory mediators such as CRP and sPLA2, alterations of bone metabolism and dyslipidemia before disease onset. The research design made it possible to determine the time course of several serum markers in preclinical RA patients. What do these findings mean in relation to the aetiology of RA? And what are the possible consequences of the results described in this thesis for the prediction of RA in healthy populations and populations at risk?

Aetiology of rheumatoid arthritis

The genetic base of RA is estimated to be 60% [1]. Therefore, environmental factors must also play an important role. Clinically apparent disease is preceded by a steady increase in inflammatory activity during a few years, accompanied by elevations of various cytokines [2, 3]. Alterations in bone metabolism occur in the same period and are probably a direct result of the inflammation. The inflammation itself in (preclinical) RA is an aspecific phenomenon, similar to other inflammatory disease states. The most specific characteristic of RA, the presence of ACPA, occurs on average before the elevation of RF and in this study up to 14 years before the first symptoms. This makes ACPA a likely candidate as a key player in the pathogenesis of at least the more severe forms of the disease, more so than RF.

Recent evidence points to an interaction between the genetic makeup, notably the shared epitope, and ACPA [4]. Less strong associations with RA have been found for the PTPN22 [5], STAT4 [6] and TRAF1/C5 [7] polymorphisms. The physiologic basis of the latter genes in disease susceptibility is not yet fully clear. Of interest, the finding that dyslipidemia is present at least 10 years before the symptoms, points to either a shared genetic background of RA and dyslipidemia, or to longstanding unhealthy lifestyle factors in the later patients. Either way, the wellknown increased risk of cardiovascular disease in RA patients may well originate before the disease becomes manifest.

Indeed, the long duration of the preclinical period suggests that environmental factors must have a prolonged or repeated influence on a situation with an

immunological imbalance causing a chronic and increasing inflammation. In this respect, especially the combination of smoking and SE presence have been implicated [8]. Tobacco smoke would produce inflammation and citrullination in the lung in SE positive individuals. The next intensification step would be the break of tolerance to citrullinated antigens with ensuing systemic autoimmunity and possibly activation of inflammation at other sites of citrullination, such as the joints [9]. Unhealthy dietary habits could come into the play by favouring a background state of low grade inflammation, as the blood of preclinical RA patients was shown to contain on average lower concentrations of several antioxidants [10]. Viral infections may also contribute, again probably in an unspecific manner by inducing a pro-inflammatory state, since no single infection has been reliably related to the development of RA. To conclude this short discussion of environmental and genetic risk factors, an association was found between periodontal disease and RA (reviewed in [11]). This suggests either a specific effect of the involved micro organism *Porphyromonas gingivalis*, which is capable of inducing citrullination, or risk-enhancement by the systemic effect of the local inflammatory state [12].

Limitations of the research design

The preclinical phase of RA is difficult to study, because the affected individuals have no complaints yet and therefore do not seek medical attention. Given the low incidence of RA (0.2-0.4 per 1000 per year [13]), prospective studies in healthy individuals are impossible, because for a sufficient number of cases several years follow-up are necessary in an extremely large study population. Therefore, alternative research designs are necessary to study the preclinical phase, such as the use of a high risk population (examples: multi-case families [14] or Pima Indians [15]), or the use of stored preclinical serum samples of a RA cohort [16, 17].

With the latter alternative research design, the existence of a preclinical phase has been clearly demonstrated. However, the number of patients and the number of serum samples in these studies were limited as a result of which the course of preclinical markers and determination of the start of the appearance of these markers was unknown. Thus a reliable prediction of RA in healthy individuals or individuals at risk becomes difficult. The present research design had the advantage of often multiple available samples. Still, blood donors donate blood only 3-4 times per year, as a result of which studying the role of intercurrent infections is difficult, because the chance of finding evidence of short infectious episodes in the sera is very small.

The used research design also has other disadvantages. First, the time of the start of the complaints was determined by chart review. Since patients can have complaints for a long period at the first visit to the rheumatologist [18], it is difficult to define the exact moment of the start of the complaints. However, it is unlikely that this has biased the results of the studies in this thesis, since RA has a long preclinical phase. Secondly, additional information about lifestyle, such as smoking and nutrition, and menopausal status of the females was lacking. In the patient group it was possible to obtain this information from chart review, but the controls were anonymous. Therefore, statistical analyses could not be corrected for these parameters. Since the sera of patients and controls were matched on age, gender and time of blood donation, it is not expected that this will have influenced differences between patients and controls. Finally, only serum samples were available, as a result of which other relevant parameters, such as genetic profiling, could not be performed in all patients despite additional efforts to extract DNA from peripheral blood cells from the serum.
In the future, the present design should be complemented by the study of prospective cohorts of individuals at risk.

Prevention of RA in healthy individuals
RA management has become focussed on detecting and treating RA as early as possible in order to prevent structural joint damage, a situation which is called secondary prevention. The results from this thesis can be used for the development of primary prevention of RA, i.e. prevention of the development of RA in healthy individuals.
Before primary prevention of RA is possible, it is important that people at risk can be traced by using one or more tests. Of all measured parameters in this thesis, only the presence of antibodies, particularly ACPA, can be used to predict future RA in healthy individuals. None of the other preclinical parameters appeared to be suitable for predicting RA, because differences between patients and controls were too small. The additional value of genetic factors is unclear, since DNA was not available of the controls. In a recent study, It was found that SE in combination with a positive anti-CCP increased the risk of future RA [19], but the number of used controls was to low to make a reliable estimate of the risk. Given the high association between SE and ACPA, it is plausible that a genetic measurement will not have additional value above the anti-CCP test for calculating the risk on RA. To test the additional value of genetic parameters, the association between genetic factors and ACPA in healthy individuals has to be studied.

However, the predictive value of ACPA in a healthy population is limited. The risk of developing RA within five years for healthy persons with a positive anti-CCP test was estimated at 5%. To raise the predictive value of a positive test result, the test must be carried out in a healthy population at risk. For this purpose, healthy first degree family members of RA patients could be useful, since in these individuals the risk of developing RA increases to 70%. The prevalence of RA is about 1% in the Netherlands [20], resulting in approximately 150,000 RA patients with varying numbers of family members. This large group of healthy individuals at risk can serve as a research population for the improvement of predicting future RA in healthy individuals. When predicting future RA in the healthy population is possible, this can result in population-based screening of individuals at risk and subsequently the testing of preventive treatment.

Preventing RA in arthralgia patients

A related approach is to try to prevent the development of RA in patients with arthralgia. Arthralgia patients do not have arthritis by clinical standard, but experience painful and stiff joints, which may be the first symptoms of RA. It is likely that these patients have an increased risk of developing RA, although the exact incidence of RA in this patient group is unknown. Therefore, it is plausible that also in this group RA specific antibodies have a good predictive value for the future development of RA. General practitioners (GP's) will play a vital role in the detection of these patients at risk, since GP's have a gatekeeper role for access to specialized care in the Netherlands and the GP is the first professional to be consulted for health problems. To optimise possible prevention of RA in this stage of the disease, the GP should distinguish arthralgia of hands and feet with morning stiffness ("inflammatory arthralgia") from other causes of joint pain, test these patients for ACPA and IgM-RF and refer patients with a positive test result to a rheumatologist [21].

Conclusion

The results from this thesis can be used for the development of primary prevention of RA, i.e. prevention of RA development in "healthy" individuals without arthritis. Of all measured parameters in this thesis, only the presence of antibodies, particularly ACPA, can be used to predict future RA in healthy individuals. A prevention strategy of RA in individuals without arthritis seems to be possible only in high risk populations such as persons with first degree family members with RA or patients with arthralgia. Research in these persons at risk will result in a better understanding of the aetiology of the disease and improvement of the prediction of RA in individuals without arthritis. When these

future RA patients can be traced and a treatment can be found to postpone or prevent the development of RA, this will improve the quality of life of a large number of future RA patients as well as reduce the high medical and societal costs associated with this disease.

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Samenvatting

Achtergrond en methode (Hoofdstuk 1)

Reumatoïde artritis (RA) is een auto-immuunziekte, die gekarakteriseerd wordt door chronische ontsteking van de gewrichten met als gevolg afbraak van bot en kraakbeen, functieverlies en ook verhoogde comorbiditeit en sterfte. De prevalentie van de ziekte is ongeveer 1% in de Nederlandse bevolking, waarbij vrouwen ongeveer twee keer zo vaak de aandoening hebben in vergelijking met mannen.

De reden waarom RA ontstaat is onbekend, maar genetische factoren lijken samen met omgevingsfactoren een belangrijke rol te spelen in de pathogenese van de ziekte. Uit studies met tweelingen is gebleken dat ongeveer 60% van het ontstaan van RA verklaard kan worden door genetische factoren. Hierbij spelen vooral genen van het major histocompatibility complex, HLA-DR4 en de shared epitope (SE), een grote rol, maar uit recent onderzoek is gebleken dat ook een aantal andere genen geassocieerd zijn met het hebben van RA, zoals het PTPN22 gen, de 6q23 regio, het STAT4 gen en de TRAF en C5 genen. Omgevingsfactoren die een mogelijke rol spelen in de ontwikkeling van RA zijn onder meer roken, infecties en hormonale factoren.

De gewrichtsschade die ontstaat bij RA is onherstelbaar en zorgt, vooral in een later stadium van de ziekte, voor een verslechterde kwaliteit van leven voor de patiënt. Daarom is het van belang dat de ziekte vroeg opgespoord wordt, omdat door vroege en agressieve behandeling gewrichtsschade te voorkomen is. Toch heeft, ondanks de steeds vroegere opsporing van de ziekte, nog een derde van de RA-patiënten bij het eerste bezoek aan de reumatoloog gewrichtsschade. Omdat de afbraak van bot en kraakbeen al erg snel na de start van de klachten ontstaat, is het van groot belang om toekomstige RA-patiënten al op te kunnen sporen voordat de klachten beginnen. Daarom werd in dit proefschrift de preklinische fase van RA-patiënten onderzocht met als doel het voorspellen van RA in deze fase van de ziekte en het vergroten van het inzicht in de ontstaanswijze van RA.

Voor de studies in dit proefschrift is gebruik gemaakt van seriële bloedmonsters van bloeddonoren die later RA hebben ontwikkeld. De onderzoeken zijn voortgekomen uit een samenwerking tussen de Sanquin Bloedbank Noord-West en het Jan van Breemen Instituut (JBI) in Amsterdam. De Sanquin Bloedbank heeft sinds 1984 van elke bloeddonatie serum opgeslagen. Het JBI heeft een grote polikliniek reumatologie en van alle patiënten werden er 79 RA-patiënten geïdentificeerd die voor de start van de klachten bloed hebben gedoneerd bij de bloedbank. Per patiënt waren er mediaan 13 serummonsters beschikbaar voor onderzoek en voor ieder patiëntenmonster werd een controle monster gezocht, gematched voor leeftijd, geslacht en het tijdstip van de bloeddonatie. Daarnaast

werden gegevens van vroege artritis patiënten uit de 'Early Arthritis Clinic' van het JBI gebruikt om de voorspellende waarde van autoantistoffen voor de diagnose RA en radiologische schade te bestuderen.

Preklinische autoantistoffen (Hoofdstuk 2)
Uit eerder onderzoek is naar voren gekomen dat autoantistoffen al jaren voor de start van de klachten van RA te vinden zijn in serummonsters. Door beperkte beschikbaarheid van monsters is het echter nog onbekend hoe het beloop van deze autoantistoffen is voor het ontstaan van de ziekte en welke autoantistoffen het beste gebruikt kunnen worden voor het voorspellen van RA in gezonde personen. De serummonsters van de preklinische RA-patiënten werden in deze studie getest op reumafactor (IgM-RF) en op antistoffen tegen gecitrullineerde eiwitten (anti-CCP). Negenendertig patiënten (49%) werden positief getest op één of beide antistoftesten op een mediaan tijdstip van 4.5 jaar (range 0.1 tot 13.8) voor de start van de klachten. Van de 2,138 controles, was 1.1% positief voor IgM-RF en 0.6% was positief op de anti-CCP test. De aanwezigheid van autoantistoffen (IgM-RF en anti-CCP) al jaren voor de start van de klachten impliceert dat gezonde mensen met autoantistoffen in het bloed een verhoogd risico lopen op het krijgen van RA. Dit zou kunnen leiden tot een snellere opsporing van RA in met name hoge risicogroepen.

Preklinische ontsteking (Hoofdstuk 3 en 4)
In twee studies is de mate van ontsteking bij preklinische RA-patiënten bestudeerd. In de eerste studie werd preklinische ontsteking gemeten met behulp van een sensitieve CRP test. In de periodes 0-1 jaar, 1-2 jaar en 4-5 jaar voor de klachten was de mediane CRP concentratie verhoogd bij preklinische RA-patiënten in vergelijking met controles. De CRP concentratie steeg significant over de tijd bij de patiënten met de hoogste waarden in de twee jaar voor de start van de klachten. De verhoogde CRP concentratie werd gevonden bij preklinische patiënten met en zonder de aanwezigheid van autoantistoffen. Ondanks dat de concentratieverschillen tussen patiënten en controles erg klein waren, kan dit een belangrijke voorloper zijn van ontsteking in het klinisch stadium van de ziekte. CRP concentraties zijn echter niet bruikbaar voor voorspelling van de ziekte op individueel niveau.
In de tweede studie werd de tijdsrelatie tussen de start van preklinische ontsteking (gemeten met CRP en sPLA2) en de aanwezigheid van autoantistoffen (IgM-RF en anti-CCP) bestudeerd. De concentraties van deze antistoffen was significant geassocieerd met concentraties van sPLA2, CRP en de combinatie van sPLA2 en CRP op dezelfde momenten voor de start van de klachten. Er

werden geen sterkere associaties gevonden tussen de autoantistoftesten en de drie ontstekingsparameters 1, 2 en 3 jaar voor of na het moment van het meten van de autoantistoffen. Hieruit werd geconcludeerd dat de acuut fase respons en de aanmaak van autoantistoffen al jaren voor de eerste symptomen van RA ontstaan en dat deze processen zich waarschijnlijk gelijktijdig ontwikkelen.

Het preklinische lipidenprofiel (Hoofdstuk 5)
RA wordt gekarakteriseerd door verhoogde ontsteking en een verhoogd risico op hart- en vaatziekten. Dit verhoogde risico kan deels verklaard worden door de associatie van ziekteactiviteit en dyslipidemie, maar het is onbekend wanneer deze dyslipidemie ontstaat in het ziekteproces. Daarom werd in de preklinische fase van RA het totaal cholesterol, HDLc, triglyceriden, apo AI, apo B en Lp(a) gemeten en vergeleken met gematchte controles. Bloedmonsters van preklinische RA-patiënten hadden gemiddeld een 4% hoger totaal cholesterol gehalte, een 9% lager HDLc, gehalte, 17% hogere triglyceriden concentratie en een 6% hoger apo B niveau in vergelijking met de controlemonsters ($p< 0.05$). De verschillen tussen de patiënten en controles konden maar beperkt verklaard worden door CRP. Geconcludeerd kon worden dat RA-patiënten al minimaal 10 jaar voor de start van de klachten een meer atherogeen lipidenprofiel hebben in vergelijking met de controles.

Het preklinische vitamine D gehalte (Hoofdstuk 6)
In een recent onderzoek werd een hogere inname van vitamine D via de voeding geassocieerd met een lager risico op het krijgen van RA. Het is echter bekend dat blootstelling aan zonlicht een groter aandeel heeft in de vitamine D aanmaak dan voeding. Omdat de hoeveelheid vitamine D in het lichaam accuraat gemeten kan worden door de 25-hydroxyvitamine D (25(OH)D) concentratie in het serum, werd in deze studie 25(OH)D concentraties van preklinische RA patiënten vergeleken met gematchte controles. Van iedere patiënt werd een serummonster geselecteerd op 1 jaar, 2 jaar en 5 jaar of langer voor de start van de klachten met een bijbehorend controlemonster. Op alle momenten in de preklinische fase werd er geen associatie gevonden tussen vitamine D deficiëntie en het later ontwikkelen van RA. Het geometrisch gemiddelde van de 25(OH)D concentratie was bij de patiënten lager in vergelijking met de controles (29.8 vs. 32.1 nmol/l), maar dit verschil was niet statistisch significant. Geconcludeerd werd dat de 25(OH)D concentratie niet verschilt tussen patiënten en controles. Dit suggereert dat vitamine D geen belangrijke rol speelt in de pathogenese van RA.

Preklinische botmarkers en regulatoren van osteoclast activiteit (Hoofdstuk 7)
In deze studie werd onderzocht of de aanwezigheid van autoantistoffen en ontsteking in de preklinische fase van RA leidt tot veranderingen van het botmetabolisme. Bij preklinische RA-patiënten en controles werden de volgende markers gemeten: 1) markers voor botaanmaak: OC en P1NP, 2) een marker voor botresorptie: β-CTX, en 3) regulatoren van de osteoclastactiviteit: RANKL en OPG. Na correctie voor leeftijd, geslacht en het moment van de bloedafname, autoantistoffen en ontsteking hadden de preklinische RA-patiënten gemiddeld hogere waarden van P1NP and OPG vergeleken met de controles. De preklinische P1NP and OPG concentraties waren negatief geassocieerd met radiologische progressie na de start van de klachten, maar deze associaties waren niet statistisch significant. Geconcludeerd kan worden dat in de preklinische van RA niet alleen autoantistoffen en ontsteking aanwezig zijn, maar dat ook het botmetabolisme afwijkend is.

HLA-DR4 en de Shared Epitope in preklinische RA (Hoofdstuk 8)
In deze studie is de associatie onderzocht tussen genetische markers (HLA-DR4 en SE) en de aanwezigheid van autoantistoffen (IgM-RF en anti-CCP) in preklinische RA-patiënten. Van de 56 preklinische patiënten, waar DNA van beschikbaar was, waren 26 patiënten positief voor anti-CCP, (46%), 13 voor IgM-RF (23%), 32 voor HLA-DR4 (57%) and 47 voor SE (84%). Anti-CCP was significant geassocieerd met HLA-DR4 ($p=0.03$, OR: 3.5). De associatie tussen anti-CCP en SE was niet statistisch significant ($p=0.11$, OR: 3.7), wat mogelijk veroorzaakt wordt door het kleine aantal patiënten in deze studie. IgM-RF was niet statistisch significant geassocieerd met HLA-DR4 ($p=0.31$, OR: 2.0) en SE ($p=0.07$, OR onbekend omdat er geen patiënten waren met de combinatie IgM-RF+ and SE-). Geconcludeerd kan worden dat in preklinische RA de aanwezigheid van anti-CCP positief is geassocieerd met HLA-DR4 en SE. Omdat er geen DNA beschikbaar was van controles was het niet mogelijk om de gemeten genetische markers te gebruiken voor predictie van RA in de preklinische fase van de ziekte.

Autoantistoffen bij vroege artritis patiënten (Hoofdstuk 9)
De anti-CCP test heeft een hoge sensitiviteit en specificiteit voor RA, maar CCP is niet het fysiologische doelwit voor de autoantistoffen. Zo is bijvoorbeeld gecitrullineerd fibrine gevonden in ontstoken synovium. Daarom is in deze studie de diagnostische en prognostische waarde van antistoffen tegen gecitrullineerd fibrinogeen (ACF) onderzocht bij vroege artritis patiënten en vergeleken met

IgM-RF en de tweede generatie anti-CCP test. De sensitiviteit van de ACF, anti-CCP en IgM-RF testen waren respectievelijk 55.8%, 57.8%, and 44.6%, met bijbehorende specificiteit van respectievelijk 92.6%, 94.2%, and 96.7%. Ongeveer 30% van de IgM-RF negatieve patiënten bleken positief voor ACF, anti-CCP of beiden. De ACF en anti-CCP test had bij vroege artritis patiënten een hoge mate van overeenstemming (kappa = 0.84). Van alle kenmerken op baseline waren de ACF test en de anti-CCP test de beste voorspeller voor de diagnose RA na 1 jaar follow-up (OR = 10.3 en 10.6) en radiologische progressie na 2 jaar follow-up (OR = 12.1 en 14.8). Uit dit onderzoek kan geconcludeerd worden dat de ACF test even sensitief is als de anti-CCP test, maar sensitiever is in vergelijking met de IgM-RF test voor de diagnose RA bij vroege artritis patiënten. De ACF test is, net als de anti-CCP test, een goede voorspeller van radiologische progressie. Verder lijken de ACF test en de anti-CCP test met name bruikbaar voor het stellen van de diagnose RA bij IgM-RF negatieve artritis patiënten.

Conclusies van dit proefschrift (Hoofdstuk 10)

Op basis van de resultaten van de verschillende studies in dit proefschrift kan geconcludeerd worden dat er al verschillende processen gaande zijn jaren voordat de klachten van RA ontstaan. Een interactie van het genetisch profiel met omgevingsfactoren resulteert in de productie van autoantistoffen (reumafactor en verschillende antistoffen tegen gecitrullineerde eiwitten (ACPA's), ontstekingsparameters (zoals CRP en sPLA2), veranderingen van het botmetabolisme en dyslipidemie.

De behandeling van RA is met name gefocust op het zo vroeg mogelijk opsporen en behandelen van RA om gewrichtsschade zoveel mogelijk te beperken. Met de resultaten van dit proefschrift kan een start gemaakt worden met preventie van RA voordat de ziekte ontstaat, oftewel primaire preventie van RA. Voordat primaire preventie van RA mogelijk is, moeten mensen met een verhoogd risico op de ziekte opgespoord kunnen worden met een of meerdere testen. Van de gemeten parameters in dit proefschrift kunnen alleen antistoftesten, en voornamelijk ACPA, gebruikt worden voor het voorspellen van RA in gezonde individuen. Bij de andere gemeten preklinische parameters waren de verschillen tussen de patiënten en controles te klein om te kunnen gebruiken voor het voorspellen van RA. De aanvullende waarde van genetische testen is onbekend, aangezien van de controles geen DNA beschikbaar was. Door de hoge associatie tussen SE en ACPA is het echter plausibel dat genetische factoren geen aanvullende waarde hebben boven antistoffen voor het voorspellen van RA bij gezonden mensen.

De voorspellende waarde van ACPA in de gezonde bevolking is beperkt. Het risico op het ontwikkelen van RA binnen vijf jaar is voor gezonde mensen met een positieve anti-CCP test geschat op ongeveer 5%. Om de voorspellende waarde van deze test te verhogen, dient de test uitgevoerd worden in hoog risico populaties, zoals gezonde personen met eerstegraads familieleden met RA. In een dergelijke populatie wordt het risico op het ontwikkelen van RA namelijk geschat op ongeveer 70%. Een andere aanpak voor preventie van RA is het voorkomen van het krijgen van de aandoening bij patiënten met artralgie. Artralgie patiënten hebben nog geen gewrichtsontsteking maar al wel pijnlijke en stijve gewrichten, wat de eerste symptomen kunnen zijn van RA. Het is aannemelijk dat dergelijke patiënten een verhoogde kans hebben op het ontwikkelen van RA, waardoor autoantistoffen ook in deze populatie een goede voorspellende waarde zouden kunnen hebben voor het ontwikkelen van RA. Voor de opsporing van deze patiënten is een belangrijke rol weggelegd voor de huisarts, aangezien dit de eerste professional is die deze patiënten op het spreekuur ziet. Om preventie van RA in dit stadium van de ziekte mogelijk te maken, zal de huisarts artralgie van handen en voeten in combinatie met ochtendstijfheid (inflammatoire artralgie) moeten kunnen onderscheiden van andere soorten gewrichtspijn, deze patiënten testen voor ACPA en IgM-RF en vervolgens de positief geteste patiënten verwijzen naar de reumatoloog.
Naast herkenning en opsporing, zal er ook een (kosten-)effectieve interventie moeten worden ontwikkeld, voordat er werkelijk gesproken kan worden van primaire preventie van reumatoïde artritis.

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Curriculum Vitae

Markus Marianus Jacobus Nielen werd geboren op 6 februari 1977 in Roosendaal en Nispen. Hij behaalde zijn VWO diploma in 1995 op het Norbertus College te Roosendaal. Daarna ging hij Gezondheidswetenschappen studeren aan de Universiteit Maastricht in Maastricht, waar hij in 2000 afstudeerde op de afstudeerrichting Bewegingswetenschappen. Van april 2001 tot augustus 2005 was hij werkzaam als onderzoeker op het Jan van Breemen Instituut in Amsterdam, waarvan de resultaten zijn beschreven in dit proefschrift. Hij behaalde tijdens deze periode tevens zijn master epidemiologie aan het VU medisch centrum in Amsterdam. Na zijn promotieonderzoek is hij gaan werken als onderzoeker op het Nederlands instituut voor onderzoek van de gezondheidszorg (NIVEL) in Utrecht, waar hij momenteel nog steeds werkzaam is op het themagebied huisartsgeneeskundige zorg.