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Anti-citrullinated protein antibodies (ACPA) in rheumatoid arthritis : linking genetic predisposition to clinical outcome

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*Anti-citrullinated
protein antibodies (ACPA)
in Rheumatoid Arthritis*

*linking genetic predisposition
to clinical outcome*

Diane van der Woude

**Anti-citrullinated protein
antibodies (ACPA) in
rheumatoid arthritis - linking
genetic predisposition
to clinical outcome**

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**Anti-citrullinated protein antibodies (ACPA)
in rheumatoid arthritis -
linking genetic predisposition to clinical outcome**

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Chapter 1

Introduction

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RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a disease characterized by arthritis of mainly the small joints of the hands and feet, which is thought to be the result of an autoimmune response. It is the most common inflammatory arthritis with a prevalence of 0.5-1.0% in European and North-American populations ¹. There is substantial geographic variation in the occurrence of RA with very high prevalences reported in native American-Indian populations ², and very low prevalences in populations from South-East Asia ³. The disease is approximately three times more frequent in women than in men, and the prevalence increases with age. Besides the potentially destructive arthritis, patients can be affected by various extra-articular features such as secondary Sjögren's syndrome, interstitial lung disease, pericarditis and pleuritis. Fortunately, the advent of tumor necrosis factor (TNF) inhibitors and other biological agents have led to a therapeutic revolution for patients with rheumatoid arthritis ⁴. Instead of having to resign to an inevitably progressive and debilitating disease course, modern-day treatment aims at achieving the lowest possible disease activity and ultimately remission. Nonetheless, rheumatoid arthritis continues to be a major cause of (partial) disability and of loss of productivity, and is associated with substantial economic costs ⁵.

Classification criteria for the disease were first phrased in 1956 ⁶ after Sir Alfred Garrod had introduced the term rheumatoid arthritis in 1876 in an attempt to counteract the unsatisfactory use of designations such as "chronic rheumatism" and "rheumatic gout" ⁷. The purpose of the classification criteria was to facilitate both clinical diagnosis and scientific research. For many years since, the 1987 American College of Rheumatology (ACR) classification criteria have been used to this end, despite the fact that the incorporation of items such as erosive radiographic changes led to limited diagnostic value of these criteria for patients with early arthritis ⁸. In order to facilitate the study of persons with earlier stages of disease, the ACR and the European League Against Rheumatism (EULAR) have recently developed the 2010 classification criteria for RA as shown in Table 1 ⁹. It is worthwhile to note that these criteria are based on patient characteristics which were associated with the decision by the physician to start treatment with methotrexate. These criteria are a reflection of the shift towards increasingly earlier diagnosis and treatment of rheumatoid arthritis.

AUTOANTIBODIES IN GENERAL AND ANTI-CITRULLINATED PROTEIN ANTIBODIES (ACPA) IN PARTICULAR IN RHEUMATOID ARTHRITIS

Rheumatoid factor (RF) is the classic autoantibody associated with RA. It has long been known to be a marker of future RA development ¹⁰. However, the predictive ability of

Table 1. The 2010 ACR/EULAR classification criteria for rheumatoid arthritis

Target population: patients

1) who have at least one joint with definite clinical synovitis (swelling)

2) whose synovitis is not better explained by another disease.

Criteria	Score
A. Joint involvement:	
- 1 large joint	0
- 2-10 large joints	1
- 1-3 small joints (with or without involvement of large joints)	2
- 4-10 small joints (with or without involvement of large joints)	3
- > 10 joints (at least 1 small joint)	5
B. Serology (at least one test result is needed for classification):	
- Negative RF <i>and</i> negative ACPA	0
- Low-positive RF <i>or</i> low-positive ACPA	2
- High-positive RF <i>or</i> high-positive ACPA	3
C. Acute-phase reactants (at least one test result is needed for classification):	
- Normal CRP <i>and</i> normal ESR	0
- Abnormal CRP <i>or</i> abnormal ESR	1
D. Duration of symptoms:	
- < 6 weeks	0
- ≥ 6 weeks	1

A score of ≥ 6/10 is needed for classification of a patient as having definite RA.

Joint involvement refers to any swollen or tender joint on examination. Distal interphalangeal joints, first carpometacarpal joints and first metatarsophalangeal joints are excluded from assessment. RF: rheumatoid factor. ACPA: anti-citrullinated protein antibody (tested as anti-cyclic citrullinated peptide (anti-CCP)), CRP: C-reactive protein, ESR: erythrocyte sedimentation rate.

immunoglobulin M (IgM)-RF is limited by its relatively modest specificity (85%), which entails that many patients who are IgM-RF-positive will not develop RA ¹¹. Recently, a better diagnostic and predictive marker has emerged in the form of anti-citrullinated protein antibodies (ACPA).

ACPA bind to proteins or peptides containing citrulline, which is a post-translational modification of the naturally occurring amino acid arginine. By an enzymatic process called deimination or citrullination which is mediated by a series of enzymes known as peptidyl arginine deiminases (PADs), the positively charged peptidylarginine is converted into the neutral peptidylcitrulline (Figure 1) ¹². The activity of PAD enzymes is dependent on high concentrations of calcium, and deimination can occur both intracellularly concurrent with apoptosis, and extracellularly provided the calcium concentration is high enough ^{13, 14}. Besides the occurrence of endogenous citrullination, recent reports have drawn attention to the possible role of the oral bacterium *Porphyromonas gingivalis* (*P. gingivalis*) which also posses PAD ¹⁵. This pathogen is the major etiologic agent associated with periodontitis; a condition which is significantly increased in patients with longstanding active RA ¹⁶. These associations raise the possibility that citrullination of bacterial and host proteins by *P. gingivalis* may be a mechanism by which citrullinated antigens in RA are generated.

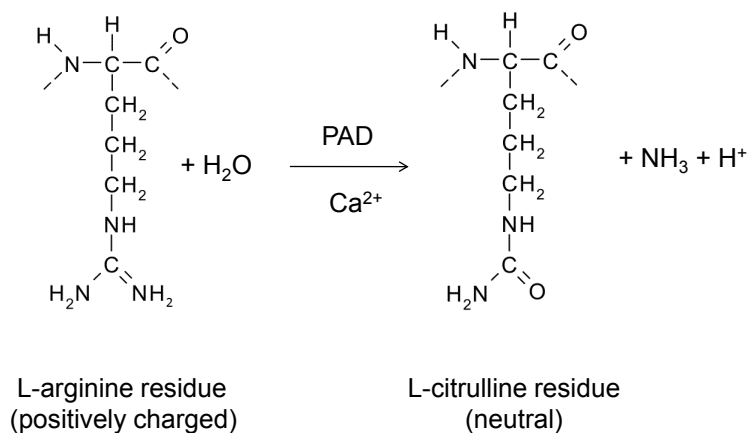


Figure 1. The enzymatic process of citrullination.

The exact function of citrullination is not fully known although it has been described to make proteins more prone to degradation by proteolytic enzymes ¹⁴. The discovery that proteins undergoing processing in antigen-presenting cells (APCs) can be citrullinated before presentation of their peptides to T cells is immunologically interesting, although the exact consequences for the immune response remain unclear ¹⁷. Citrullinated proteins have been detected in a variety of inflamed tissues, such as in inflamed joints in several different forms of arthritis, in lungs and other organs ¹⁸. Furthermore, citrullination has been shown to take place in lining and sublining cells in the joints of patients with RA ¹³.

ACPA were first described as anti-perinuclear factor and anti-keratin antibodies, both of which bind to filaggrin ¹⁹⁻²¹. It was not until several years later that recognition of this antigen was found to be exclusively dependent on the presence of citrulline residues ²². The same was the case for citrullinated vimentin, which was discovered to be the target of the RA-specific anti-Sa antibodies that had been described many years before ^{23, 24}. Based on these findings, several commercial assays that test for the presence of antibodies to cyclic citrullinated proteins (CCP) have been developed and successfully introduced into clinical practice ²⁵. The commercial ACPA tests have been designed to detect most, if not all, ACPA epitope reactivities found in vivo. The test which is currently most widely used in clinical practice is the second generation anti-CCP test, although other targets such as mutated citrullinated vimentin are also employed ²⁶.

DIAGNOSTIC AND PROGNOSTIC VALUE OF ACPA

A comprehensive overview of the diagnostic utility of the anti-CCP and RF tests in RA has been provided by a meta-analysis ^{11, 27}. The most important difference between the two tests was the increased specificity of the anti-CCP assays versus IgM-RF (95% vs. 85%). The sensitivity of anti-CCP2 was similar to that of IgM-RF (67% in patients with established rheumatoid arthritis). In patients with early rheumatoid arthritis (<2 years of disease) the summary sensitivity was 57% ²⁸. The diagnostic characteristics of IgG-RF and IgA-RF, which had previously been reported to be more accurate than IgM-RF, proved to be similar to IgM-RF; thus, these measurements do not provide additional information.

With regard to other rheumatologic diseases, ACPA have been detected in 5-15% of patients with psoriatic arthritis ^{29, 30} and in patients with juvenile idiopathic arthritis, in whom IgG-anti-CCP was most often present in IgM RF-positive polyarthritis which most closely resembles adult RA ³¹. Anti-CCP antibodies have also been reported in patients with non-rheumatologic diseases such as pulmonary TB ³². However, follow-up studies have revealed that sera from these patients also frequently react with unmodified arginine-containing peptide, suggesting that these antibodies are not citrulline-specific ³³. Of healthy controls, 1% tests positive for anti-CCP2-antibodies as was shown in an overview of 144 independent studies ³⁴. It is unclear how many of these people may develop RA in the future.

In view of these data, it is necessary to re-examine how serological markers can be used most effectively in the diagnostic strategy for RA. Determination of anti-CCP2 antibodies, which have a high specificity and a reasonable sensitivity, is a good first option in patients presenting with inflammatory arthritis. However, anti-CCP-assays are still considerably more expensive than measurements of RF in most countries. Whether it is worthwhile to also determine IgM-RF depends on the interpretation of the results. Requiring a positive result on both tests leads to a loss of sensitivity, because fewer patients have both antibodies than either RF or anti-CCP alone. On the other hand, considering a positive result on one test as sufficient for a diagnosis of RA would lead to a considerable reduction in specificity (especially when only using the RF test) in exchange for a small gain in sensitivity. This balance between sensitivity and specificity needs to be kept in mind when deciding whether to measure IgM-RF in addition to anti-CCP2.

The 2010 ACR/EULAR classification criteria for RA have included an extra level of complexity by discerning between low-positive and high-positive IgM RF and ACPA results, where low-positive refers to positive values ≤ 3 times the upper limit of normal (ULN), while high-positive refers to values >3 times the ULN (Table 1). Although it has previously been reported that the presence of high levels of IgM RF would lead to increased sensitivity for RA ³⁵, a recent study showed that a high level of RF has very limited additive prognostic value compared to ACPA-positivity ³⁶. The 2010 criteria for RA therefore could

be improved by omitting the RF level criterion. There are no clear results with regard to the value of anti-CCP levels in this respect ^{37, 38}.

During the disease course of RA, anti-CCP levels generally decline from the baseline value ³⁹. In a study investigating intensive treatment in early RA, anti-CCP levels were even reported to decrease by 50% ⁴⁰. In contrast to IgM-RF however, there is hardly any seroconversion, meaning that despite declining levels, patients who once tested anti-CCP-positive hardly ever become anti-CCP-negative ^{39, 40}. Conflicting results have been reported with regard to the association between anti-CCP levels and disease activity ⁴¹, although the majority of publications have described that a higher level of anti-CCP antibodies at baseline is associated with a higher level of disease activity, a less favorable response to treatment with methotrexate and increased joint destruction ⁴²⁻⁴⁴. Two small longitudinal studies have investigated whether anti-CCP levels fluctuate in parallel with disease activity and were not able to draw definitive conclusions ^{45, 46}. Larger scale investigations of whether anti-CCP level fluctuations may occur prior to or concomitantly with an increase in disease activity are lacking.

In addition to their function as a diagnostic marker for RA, ACPA have also been extensively investigated with regards to their long-term predictive abilities. The presence of ACPA is currently the best predictive marker for the progression of undifferentiated arthritis (UA) to rheumatoid arthritis (RA). In multivariate logistic regression analysis that also accounted for other predictive variables, anti-CCP2 positivity was associated with an odds ratio of 8.1 (95% confidence interval 4.2–15.8; $p < 0.001$) for the development of RA in UA patients ⁴⁷. ACPA-positive RA patients also have a more severe disease course than ACPA-negative RA patients, with more joint destruction and extra-articular manifestations of disease ^{43, 48, 49}.

Several studies have investigated the time-point at which patients develop ACPA. By making use of pre-disease samples from blood-bank donors who later developed RA, these reports were able to demonstrate that ACPA can be detected many years before disease manifestation ^{50, 51} and that ACPA titers increase up to the point of disease onset.

The fact that ACPA appear during the preclinical phase of RA, together with the finding that ACPA can exacerbate arthritis in mice, suggest that anti-citrullinated protein immunity may play a role in the pathogenesis of the disease ⁵². This has prompted investigations into which risk factors are associated with the emergence of the anti-citrullinated protein immune response.

GENETIC RISK FACTORS FOR RHEUMATOID ARTHRITIS

The risk of developing RA is known to be influenced by several genetic risk factors, of which the Human Leucocyte Antigens (HLA) are the most important. The association between RA and the HLA region was originally described more than 30 years ago⁵³, and subsequent studies revealed that several of the HLA-DR alleles are associated with RA, which led to the formulation of the Shared Epitope (SE)-hypothesis in 1987⁵⁴. This hypothesis provided a theoretical background for the observed associations between the HLA region and RA based on the fact that all HLA-DR alleles which predispose to RA have the same or a similar amino acid sequence (shared epitope) at positions 70-74 of the HLA-DRB1 molecule. This sequence is located in the peptide-binding groove of the HLA alleles and may therefore be directly involved in the presentation of peptides to arthritogenic T cells.

After the first descriptions of ACPA, it soon became clear that the SE alleles were solely associated with, and thus only predisposed to, ACPA-positive RA⁵⁵. Several reports showing that certain HLA SE alleles can bind and present citrullinated peptides^{56, 57}, have provided a possible biological explanation for the association between ACPA and the SE alleles. A chemical analysis of an induced murine immune response to citrullinated collagen type II demonstrated that the conformational change in the antigen induced by citrulline was critical for recognition by antibodies⁵⁸, again suggesting that citrulline plays an essential role in the initiation of the immune response. Intriguingly, further research has now shown that the SE alleles mainly predispose to the development of ACPA, rather than to the development of RA⁵⁹. This suggests a pathophysiological sequence of events detailed below in which genetic predisposition results in the development of anti-peptidylcitrulline immunity prior to the onset of RA.

After these seminal findings with regards to the association between the HLA SE alleles and RA, many of the genetic risk factors for RA have now been found to be specific for either ACPA-positive or ACPA-negative RA. The majority of genetic risk factors, such as PTPN22, the TRAF1-C5 locus, the OLIG3-AIP3-locus and STAT4, are preferentially associated with ACPA-positive RA^{60, 61}. Conversely, there are other genetic risk factors that have been described to be exclusively associated with ACPA-negative RA, such as HLA-DR3 and interferon regulatory factor (IRF) haplotypes⁶². As there are no markers available that are specific for this disease subset, it is impossible to determine at present whether these genetic risk factors predispose to ACPA-negative RA or to specific immunological alterations in these patients.

ENVIRONMENTAL RISK FACTORS

In addition to genetic aspects, environmental risk factors are known to contribute to the etiology of RA. Many epidemiological studies have shown an association between cigarette smoking and the development of RA ^{63, 64}. Smoking was found to interact with the HLA SE alleles in the predisposition to RF-positive RA ⁶⁵. However, recent data have shown a striking interaction between smoking and the SE alleles in conferring risk for ACPA-positive, rather than for RF-positive RA ⁶⁶. They also demonstrated an association between smoking and the development of citrullinated antigens in bronchoalveolar lavage fluid cells, thereby providing a possible pathogenetic link between smoking and the development of ACPA-positive RA.

PATHOPHYSIOLOGICAL MODEL OF RHEUMATOID ARTHRITIS

The recent findings that have elucidated the differences in risk factors for ACPA-positive versus ACPA-negative RA have important consequences for the understanding of the pathophysiology of RA, which can be viewed as a multi-stage process (Figure 2).

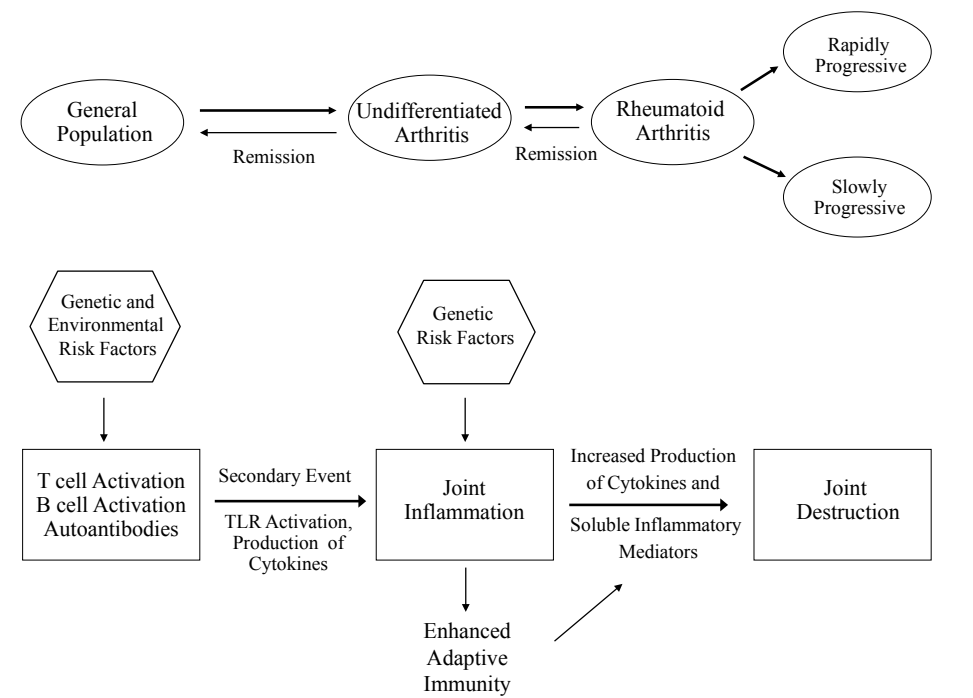


Figure 2: A multi-stage model of the clinical and pathophysiological development of RA.

In the first stage, a genetic predisposition along with environmental factors results in an adaptive immune response with antigen-dependent T and B cell activation. In the case of ACPA-positive RA, the SE alleles together with smoking may lead to the development of an anti-citrullinated protein immune response. A nonspecific environmental trigger could function as a secondary event leading to joint inflammation, which manifests itself as undifferentiated arthritis (UA). Other genetic risk factors involved in, for example, cytokine regulation, play a role in determining the extent and duration of joint inflammation. The inflammatory process itself can subsequently further stimulate the adaptive immune response through the generation of new epitopes by, amongst other processes, citrullination, which has been shown to occur more readily in inflamed joints. In individuals who have previously developed an adaptive immune response (possibly with production of ACPA), the immune cells that now gain access to the joints can enhance the inflammation and lead to the increased production of cytokines and soluble inflammatory mediators. Large numbers of activated CD4-positive T cells accumulate in the joints of patients with RA. The abundant presence of these activated T cells in the joints has several different effects. Through production of cytokines and through cell-surface interactions, the activated T cells stimulate monocytes, macrophages, and synovial fibroblasts to produce other cytokines such as TNF α and to secrete matrix metalloproteinases⁶⁷. Furthermore, the activated T cells stimulate osteoclastogenesis⁶⁸. Finally, the autoreactive T cells are capable of providing help to autoreactive B cells, which can lead to the production of autoantibodies. B cells can also function as antigen-presenting cells and thus can promote T cell activation and perpetuation of the autoimmune response⁶⁹. Activated B cells and autoantibody-producing plasma cells are present in RA synovium, although it is not completely clear if the original B cell activation occurs in the joint or elsewhere. ACPA bound to citrullinated antigens have also been detected in the joints of RA patients^{70, 71}.

Autoantibodies may play a pathogenic role by fixing complement in the joint leading to the release of chemotactic factors such as C5a and the subsequent recruitment of inflammatory cells. Another mechanism by which immune complexes can contribute to joint damage is through the activation of monocytes and macrophages by binding to Fc-receptors on the surface of these cells. This could cause perpetuation of the synovial inflammation and progression of UA to RA and erosive disease. This pathophysiological model, based on the activation of adaptive immunity, currently seems most applicable to ACPA-positive RA.

OUTLINE OF THIS THESIS

There were three main aims of this thesis:

1. to quantify the heritability of ACPA-positive and ACPA-negative RA, and to elucidate the association of distinct genetic and environmental risk factors with both subsets of disease.
2. to characterize the immunological properties of ACPA and to investigate the association between ACPA characteristics and disease progression.
3. to identify predictive factors for the most favorable outcome of RA: sustained remission after discontinuation of disease-modifying anti-rheumatic drugs (DMARDs).

The thesis is divided into three parts corresponding to the three main aims.

Part 1 is dedicated to the investigation of the heritability of RA, and to the exact association of specific genetic risk factors (the HLA-DRB1 alleles) and an environmental risk factor (alcohol) with ACPA-positive and ACPA-negative RA.

As described above, it has been known for many years that the most prominent genetic risk factor for RA is situated at the HLA locus. The HLA-DRB1 alleles that confer the strongest predisposition for RA are also known as the shared epitope (SE) alleles and have been found to selectively predispose to ACPA-positive RA. Other HLA-DRB1 alleles confer protection against disease although, due to conflicting results, it was unclear exactly which alleles are protective⁷²⁻⁷⁴. It was also unknown whether these protective effects might be limited to ACPA-positive or ACPA-negative RA. We therefore performed a meta-analysis of four European populations to investigate precisely which HLA-DRB1 alleles are associated with protection in ACPA-positive and ACPA-negative RA (**chapter 2**).

In addition to the HLA alleles, multiple other genetic risk factors have been described which predispose to RA. It is remarkable that the majority of these genetic risk factors have been found to preferentially confer risk to autoantibody-positive disease. This suggests that the extent to which genetic factors contribute to disease development may differ between ACPA-positive and ACPA-negative RA. In **chapter 3**, we therefore determined the heritability of ACPA-positive and ACPA-negative RA by applying quantitative genetic methods to data from a large twin cohort. Furthermore, we assessed the contribution of the predisposing HLA-DRB1 shared epitope (SE) alleles to the genetic variance of both groups.

Surprisingly, these studies described in chapter 3 revealed that the heritability of ACPA-positive and ACPA-negative disease does not differ, and is 66% for both subsets of disease. The effect of the HLA SE alleles explained a relatively modest 18% of the genetic variance of ACPA-positive RA, raising the question which other genetic risk factors could account for the remaining 82% of “missing heritability”. In light of the findings of chapter 2, which revealed a strong protective effect of HLA-DRB1*13 alleles for ACPA-positive disease, we investigated to what extent these protective HLA effects contribute to the

genetic variance of RA in **chapter 4**. Since a multitude of new genetic risk factors for RA in the form of single nucleotide polymorphisms (SNPs) has been described in the past few years, the effect of 12 well-replicated SNPs was also taken into account.

Besides genetic risk factors, there are also environmental risk factors which are known to predispose to RA, such as smoking. A recent case-control study proposed that another environmental risk factor: alcohol intake, may be associated with a lower risk of developing RA ⁷⁵. This is in line with findings from experimental studies showing a downregulatory effect of alcohol on both innate and adaptive immune responses ⁷⁶⁻⁷⁸. In **chapter 5** we evaluated whether the protective effect of alcohol is specific for RA, or may extend to other forms of inflammatory arthritis as well. Furthermore, we assessed if the protective effect differed between the ACPA-positive and ACPA-negative subset of disease.

Part 2 focuses on the immunological characteristics of ACPA, the influence of genetic and environmental risk factors on the shaping of the ACPA response, and the clinical value of the various commercially available ACPA tests.

Different parts of human antibodies have different functions. While the antigen specificity is determined by the Fab (Fragment antigen binding) region, the Fc (Fragment crystallizable) region is responsible for immune effector functions such as complement activation, antibody-dependent cellular cytotoxicity and binding to cellular Fc-receptors. These Fc-mediated effects are influenced by Fc-linked carbohydrate structures known as glycans. By glycosylation, a posttranslational modification process, different kinds of glycan structures can be attached to the Fc-parts of the antibodies, resulting in several different glycoforms in human serum ⁷⁹. Interestingly, early studies have demonstrated a predominance of IgG-G0 (meaning without galactose) glycoforms in sera of RA patients which correlated with disease activity and reverted to normal levels in patients who achieved (spontaneous) remission ^{80, 81}. To investigate whether ACPA, which are hypothesized to play a crucial role in the pathogenesis of RA, carry a different glycosylation pattern than other human antibodies, we developed a technique that allowed analysis of Fc-linked glycans in an antigen specific manner ⁸². **Chapter 6** describes the results of the analysis of the ACPA glycan profile from serum and synovial fluid.

Human antibodies consist of four polypeptide chains: two heavy chains and two light chains. There are five different types of heavy chains that determine the isotype of the antibody: IgM, IgD, IgG₁₋₄, IgA and IgE. The isotypes differ substantially with regard to their ability to mediate effector mechanisms, such as complement activation and binding to Fc-receptors. The ACPA response has been shown to consist of various isotypes, and the fact that ACPA of the IgM isotype could be detected at various stages of the disease indicated that the anti-citrullinated protein immune response is constantly being (re)activated ⁸³⁻⁸⁶. It was unclear however, if during this process of continuous reactivation throughout disease progression, there was also further maturation of the ACPA response in the sense of isotype switching. In addition, the finding that the ACPA isotype use differed between

patients⁸⁶, raised the question whether changes in isotype use are associated with disease progression. In **chapter 7**, we therefore examined the relationship between ACPA isotypes and disease development and progression. The use of two large, independent cohorts with long-term follow-up data enabled us to investigate the association between the ACPA isotype profile and progression of radiographic damage.

Another antibody characteristic which is crucial for the pathogenicity of autoantibodies, is the fine specificity of antigen recognition⁸⁷. The important pathophysiological consequences of an increase or shift in antigen recognition during the course of an immune response (a phenomenon known as epitope spreading), have been described in for example systemic lupus erythematosus (SLE)⁸⁸. Knowing that ACPA can be detected before the clinical diagnosis of RA, we hypothesized that similar to SLE, epitope spreading of the ACPA response may play a role in the evolution of the disease. We therefore investigated the reactivity pattern of ACPA before disease onset and during disease progression, as described in **chapter 8**. Making use of several different cohorts, which cover the entire spectrum of the disease course ranging from ACPA-positive healthy individuals to long-standing RA, this study provides an overview of the ACPA fine specificity development over time. The clinical effects of individual differences in ACPA fine specificity repertoire were examined in more detail in **chapter 9**. Based on the known association between the HLA SE alleles and some ACPA fine specificities as described below, this association was now investigated with regard to several more citrullinated epitopes. Moreover, an in-depth analysis of the effect of SE gene dose was performed. In order to investigate the clinical outcome of differences in the ACPA fine specificity repertoire, we investigated whether certain ACPA fine specificities were associated with the extent of radiographic joint damage.

As mentioned in the first part of this introduction, the HLA SE alleles and smoking are known to predispose to the development of ACPA-positive RA. The combined effect of HLA SE alleles and smoking has been shown to exceed the sum of their single effects: a phenomenon known as biological interaction⁸⁹⁻⁹¹. Furthermore, a previous study on ACPA fine specificity reported that the HLA SE alleles may also determine the fine specificity of the ACPA response, since they predisposed to the development of antibodies against citrullinated vimentin but not against citrullinated fibrinogen⁹². Based on these results, we wondered whether genetic and environmental risk factors might also interact with regard to their effect on the fine specificity of ACPA. If interaction were to be found in association with ACPA-reactivity to a particular citrullinated protein, this may indicate that the protein under investigation is of etiologic importance as an autoantigen in RA. Partly in response to a publication postulating a specific interaction between genotype, smoking and autoimmunity to citrullinated α -enolase peptide (CEP-1)⁹³, we analyzed the effects of HLA SE alleles and smoking on the ACPA reactivity profile in **chapter 10**. The results of this analysis revealed that the interaction between genotype, smoking and autoimmunity to

citrullinated antigens was not confined to citrullinated α -enolase, but extended to several other citrullinated antigens as well.

Chapter 11 provides additional essential information with regard to these results. We hypothesized that the gene-environment interaction between the HLA SE alleles and smoking affected the development of ACPA-positive RA in general, rather than the development of specific ACPA fine specificities. When the analysis was performed within ACPA-positive patients, the interaction was no longer present, which argues in favor of our hypothesis, and against an effect of the gene-environment interaction on shaping the reactivity of the ACPA response.

While the ACPA fine specificity studies mentioned above were all performed with in-house assays specifically developed for this purpose, we also examined the clinical utility of the ACPA tests which are commercially available in **chapter 12**. The aim of this investigation was to compare the predictive ability of anti-CCP2-, anti-CCP3-, anti-modified citrullinated vimentin (anti-MCV-) and RF-tests with regard to three outcome measures: progression from UA to RA, the rate of joint destruction and achieving sustained DMARD-free remission in RA.

Part 3 of this thesis describes the results of two studies concerning remission in rheumatoid arthritis.

RA patients vary considerably in terms of their disease course and outcome. The spectrum extends from debilitating, destructive joint disease on one side, to remission, the most favorable outcome, on the other side⁹⁴. These days, remission is increasingly becoming an attainable goal of RA treatment, especially since the introduction of biological anti-rheumatic therapy. The potent suppression of disease activity which can be achieved with these novel treatment agents, together with the finding that early aggressive therapy leads to better long-term outcomes, has resulted in new treatment goals striving to achieve remission in as many patients as possible^{95, 96}.

Remission rates after treatment with new therapeutic agents are now often reported, but there are few data on remission after treatment with conventional therapy, such as non-steroidal anti-inflammatory drugs (NSAIDs) and non-biological disease modifying anti-rheumatic drug (DMARD) therapy. These figures are however required for a fair comparison of the remission rates reported in clinical trials of novel agents. Moreover, the investigation of remission as a definitive disease outcome resembling cure is very important from a pathophysiological point of view. Data on patient characteristics associated with remission could lead to new hypotheses about the biological pathways involved in disease persistence and resolution. In **chapter 13**, we therefore used two large independent cohorts of patients treated with conventional therapy to examine the prevalence and predictive factors for disease modifying anti-rheumatic drug (DMARD)-free sustained remission.

The results of this study were extended in a subsequent investigation in which we compared the prevalence and predictive factors for remission in two different patient cohorts. Conventional therapy as mentioned above, was used in one of the cohorts, while the other cohort was treated according to a tight control strategy. Tight control entails a treatment strategy aiming at a predefined goal of minimal disease activity. This strategy has been shown to lead to improved functional status and high remission percentages, irrespective of the exact initial choice of therapy ⁹⁷. In **chapter 14**, we investigated whether this tight control strategy aiming at a low disease activity score ($DAS < 1.6$) resulted in more drug-free remission, or led to drug-free remission in patients who were more difficult to treat.

Chapter 15 provides a summary of the results and a discussion of the implications of the studies described in this thesis.

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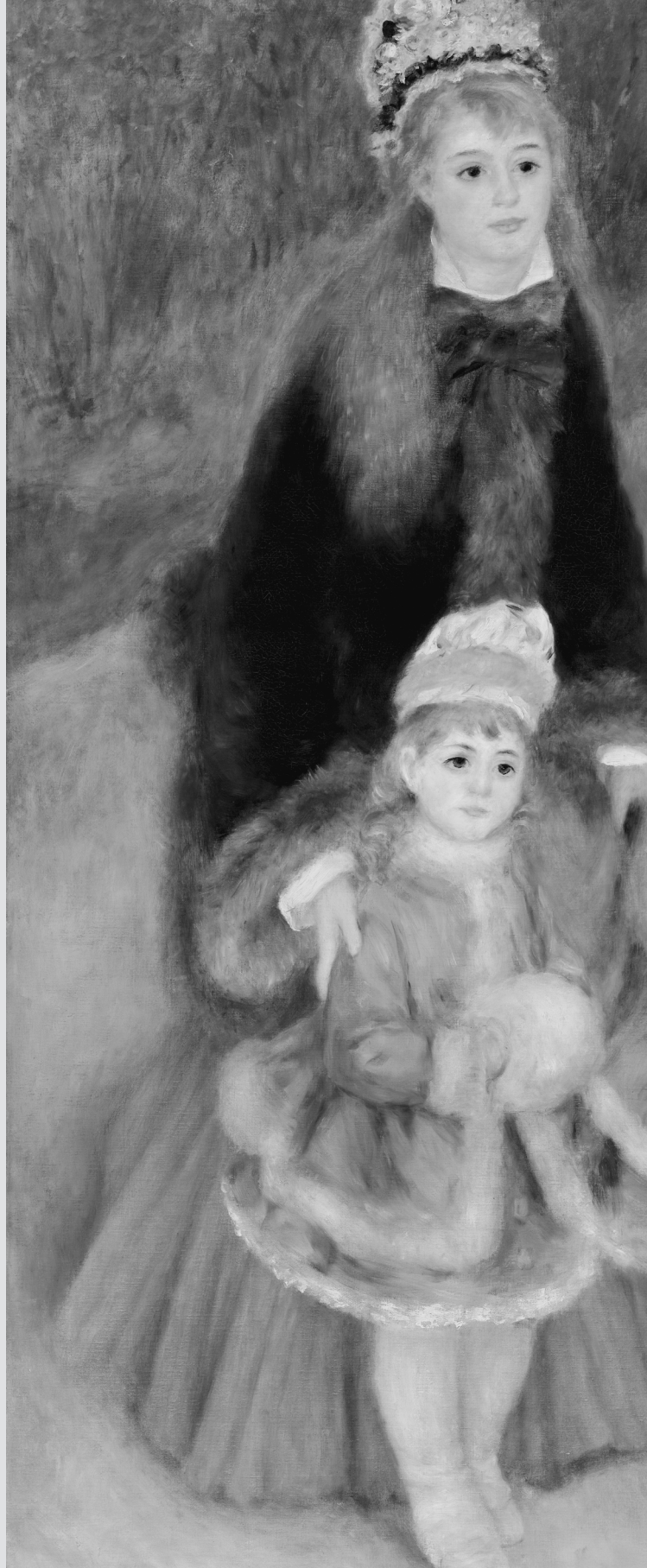
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Part I

**Risk factors for and heritability
of rheumatoid arthritis**



Chapter 2

Protection against anti-citrullinated protein antibody-positive rheumatoid arthritis is predominantly associated with HLA-DRB1*1301

A meta-analysis of HLA-DRB1 associations with ACPA-positive and ACPA-negative rheumatoid arthritis in four European populations

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ABSTRACT

Objective The protective effect of HLA-DRB1 alleles on the development of rheumatoid arthritis (RA) is poorly understood. We performed a meta-analysis of four European populations to investigate which HLA-DRB1 alleles are associated with protection in anti-citrullinated protein antibody (ACPA)-positive and ACPA-negative RA.

Methods More than 2800 patients and 3000 controls with information on HLA-DRB1 typing and ACPA-status were collected from four European countries: Norway, Sweden, the Netherlands and Spain. The odds ratio's (ORs) and 95% confidence intervals (CI) associated with the different HLA-DRB1 alleles were analyzed in a combined meta-analysis, focused on protective alleles and classifications. The analysis of ACPA-positive RA was stratified for the shared epitope (SE) alleles to correct for skewing due to this association.

Results In ACPA-positive RA, the only alleles which conveyed protection after stratification for SE, were HLA-DRB1*13 alleles (OR (95%CI): 0.54 (0.38-0.77)). The protective effect of the allele classifications based on the DERA and D70 sequence was no longer present after exclusion of DRB1*13 (OR (95%CI) D70: 0.97 (0.75-1.25)), indicating that DRB1*13, rather than the DERA or D70 sequence as such is associated with protection. Within the DRB1*13 alleles only DRB1*1301 was associated with protection (OR (95%CI): 0.24 (0.09-0.59)). Protection appeared to follow a North-South gradient with the strongest association in Northern countries. In ACPA-negative RA, there were no robust associations with HLA-DRB1 alleles.

Conclusion Our data do not support any of the classifications of protective alleles and indicate that protection against ACPA-positive RA is predominantly associated with HLA-DRB1*1301.

INTRODUCTION

The Human Leucocyte Antigens (HLA) are the most prominent genetic risk factor for Rheumatoid Arthritis (RA). The association between RA and the HLA region was originally discovered based on the observation that lymphocytes from RA patients were not reactive against cells from other RA patients in mixed lymphocyte cultures ^{1,2}. This meant that RA patients had certain HLA alleles in common, which were less prevalent in control populations. Serological testing subsequently revealed that the HLA-D-Related (DR)w4 alloantigen, but not HLA-A, B or C antigens were associated with RA ³. Later studies demonstrated that several of the HLA-DR alleles were associated with RA, which led to the formulation of the Shared Epitope (SE)-hypothesis in 1987 ⁴. This hypothesis provided a theoretical background for the observed associations between the HLA region and RA based on the fact that all HLA-DR alleles which predispose to RA have the same or a similar amino acid sequence (shared epitope) at positions 70-74 of the HLA-DRB1 molecule. This sequence is located in the peptide-binding groove of the HLA alleles and may therefore be directly involved in the presentation of peptides to arthritogenic T cells. However, due to the complexity of the HLA region, the association between the HLA region and RA is multifaceted and it is now known that not all HLA SE alleles contribute to RA to the same extent ^{5,6}. Nonetheless, the formulation of the HLA SE hypothesis has provided a rationale for combining several HLA molecules in analyses and has thereby enabled further well-powered investigations of the contribution of the HLA region to the risk of RA ⁷.

The discovery of anti-citrullinated protein antibodies (ACPA) has led to a paradigm shift in the investigation of genetic risk factors for RA. The HLA SE alleles were found to not predispose to RA as such, but rather to ACPA-positive disease, which is present in approximately two-thirds of patients with RA according to the ACR criteria ⁸. These observations are very important for the pathophysiological concept of disease development, since they indicate that the HLA SE alleles may be involved in the induction of the ACPA-response ⁹. Several SNPs known to be associated with RA, such as PTPN22, were also discovered to be specifically associated with ACPA-positive RA ^{10,11}. In contrast, DRB1*03 has been reported to be associated with ACPA-negative disease ^{12,13}, although not all studies have confirmed this association ¹⁴. These differences in underlying risk factors indicate that ACPA-positive and ACPA-negative RA may constitute distinct disease entities with a different underlying pathogenetic background.

In addition to the HLA-DRB1 alleles which contribute to RA susceptibility, other HLA-DRB1 alleles confer protection against disease ¹⁵⁻¹⁷. These protective HLA-DRB1 alleles have been categorized according to several different classifications, analogous to the shared epitope classification of susceptibility alleles. The three best-known classifications postulate that HLA-DRB1 alleles with a protective effect harbor the "shared sequence"

DERAA at position 70-74¹⁸, or an aspartic acid (D) at position 70¹⁶, or an isoleucine (I) at position 67 of the HLA-DRB1 molecule¹⁵. The DERRA and D70 alleles have been shown to be protective both in the presence and the absence of SE alleles^{19, 20}, which demonstrates that their protective effect is not solely due to the absence of SE alleles^{21, 22}. A new classification of HLA-DRB1 alleles has recently been put forward which incorporates both predisposing and protective effects^{23, 24}. Although this has provided some interesting nuances with regard to the predisposing effect of the different shared epitope alleles, it is unclear if this classification accurately describes the protective HLA effects⁷.

The multitude of classifications of protective HLA alleles illustrates that a protective effect of HLA alleles in RA is now well-accepted. However, it is still unclear which HLA alleles exactly are protective. Geographical differences in the prevalence of HLA alleles have led to conflicting results, which have been further complicated by the use of different classifications. Furthermore, it is as yet unclear whether protective effects are present in ACPA-positive as well as in ACPA-negative disease.

For these reasons, we wished to determine the contribution of individual HLA-DRB1 alleles to RA, both with respect to susceptibility and protection in a meta-analysis across European populations. Using data from four different populations, being from Norway, Sweden, The Netherlands and Spain, we investigated the HLA-DRB1 associations with ACPA-positive and ACPA-negative RA in more than 2800 patients and 3000 controls. A significant protective effect of HLA-DRB1*13 was found which remained present after stratification for the SE effect. Moreover, the protective effect of HLA-DRB1*13 was only observed for ACPA-positive and not for ACPA-negative disease. An in-depth analysis of the protective classifications revealed that the protective effect of DERRA and D70 alleles was limited to the HLA-DRB1*13 alleles and was in fact only observed for DRB1*1301. Together, our data do not support any of the classifications described above and indicate that protection is mainly associated with DRB1*1301.

MATERIALS AND METHODS

Populations

Data on cases and controls were contributed by cohorts from four different European countries: Norway, Sweden, the Netherlands and Spain. The protocol of each cohort was approved by the relevant local ethics committee and all participants provided informed consent. More than 97% of cases and controls in all four cohorts was of Caucasian descent.

The Norwegian dataset consisted of RA patients who participated in the Oslo RA registry (ORAR) or the European Research on Incapacitating Disease and Social Support (EURIDISS) cohort. For the ORAR, which was initiated in 1992, inclusion criteria were a diagnosis of RA according to the 1987 ACR classification criteria and a residential address

in Oslo. The EURIDISS, which commenced in 1991, enrolled consecutive RA patients with a maximum disease duration of 4 years at baseline. Both RA cohorts have been described in detail elsewhere ^{25, 26}. Controls were randomly selected from the Norwegian Bone Marrow Registry and cases and controls were matched for gender at a group level.

The Swedish Epidemiological Investigation of RA (EIRA) cohort recruited cases and controls aged 18-70 from May 1996 to December 2003 from a geographically defined area in the south and central regions of Sweden. Patients were seen by rheumatologists at private as well as general health care units and were eligible for inclusion if they fulfilled the 1987 ACR criteria for RA. Controls were randomly selected from a national population register and were matched to the cases for age, sex and residential area. More details on the EIRA have been described in previous publications ²⁷.

Data on Dutch cases were provided by two inception cohorts of early arthritis patients, the Leiden Early Arthritis Clinic (EAC) and the BehandelStrategieën (BeSt) trial. The Leiden EAC was initiated in 1993 and included patients with recent-onset arthritis (less than 2 years of complaints) treated at the Leiden University Medical Center (LUMC). For the BeSt study, patients with arthritis with a maximum disease duration of 2 years and active disease were recruited at 20 centers in the western part of the Netherlands from 2000 to 2002. Only patients with a diagnosis of RA were included in the present study. These cohorts are described in further detail elsewhere ^{28, 29}. Dutch controls were randomly selected by the section Immunogenetics and Transplantation Immunology (ITI) of the Department of Immunohematology and Blood Transfusion, LUMC.

The Spanish dataset consisted of RA patients fulfilling the ACR criteria for RA, who were recruited from 4 Spanish hospitals in Granada, Seville, Lugo and Madrid. Blood donors and bone marrow donors from the same cities were included as healthy controls. Controls were matched to patients by age and sex. More characteristics of the Spanish dataset have been presented in previous reports ³⁰.

Genotyping

The genotyping procedures of the HLA-DRB1 alleles have been described in previous publications ^{14, 20, 30, 31}. High-resolution, 4-digit typing was available for the entire Norwegian and Spanish cohorts. For the Dutch cohort, low-resolution typing was complemented by 4-digit typing of the DRB1*04 alleles, and by specific probes to detect the presence of the SE or DERAA sequence in individuals carrying DRB1*01, DRB1*10, DRB1*11 or DRB1*13 alleles. DRB1*1301 and *1302 were differentiated in part by 4-digit typing, and in part on the basis of their known, specific associations with HLA-DRB3 and -DQB1 alleles which were determined in the entire dataset.

Similarly, for the Swedish dataset high-resolution typing of all DRB1*04 alleles was performed. All alleles containing a DERAA sequence in the Swedish cohort were identified by help of an interpretation table for the HLA-DRB1 low resolution kit. This allowed

the ascertainment of the following allelic groups in this cohort: DRB1*0103, DRB1*0402, DRB1*11-DEAAA (*1102 or *1103) and DRB1*13-DEAAA (*1301 or *1302). Unfortunately, this did not allow the differentiation of DRB1*1301 from DRB1*1302.

Shared epitope alleles and protective classifications were defined according to Table 1.

Serological measurements

ACPA were determined by the measurement of antibodies against cyclic citrullinated peptides by a second-generation ELISA (anti-CCP2) (for the ORAR: DIASTAT; Axis-Shield Diagnostics, Dundee, UK. For the EURIDISS: INOVA, San Diego, CA, USA. For the Swedish, Dutch and Spanish cohorts: Immunoscan RA Mark 2; Eurodiagnostica, Malmö/Arnhem, Sweden/The Netherlands). These different anti-CCP2-assays have been shown to provide very similar results ³². Samples with a value above the cut-off as specified by the manufacturer were considered positive.

Statistical analysis

For each of the cohorts, we used logistic regression analysis to calculate the odds ratio's (ORs) and 95% confidence intervals of developing ACPA-positive and ACPA-negative RA in association with the different HLA-DRB1 alleles. To take into account the matching of cases and controls in the study design, the analyses in the Swedish cohort were corrected for residential area, since age and gender have been shown to have no effect on the distribution of HLA alleles ³³. A dominant genetic model which estimates the effect of the presence of a certain allele, irrespective of the presence of 1 or 2 copies, was used for all analyses. This model provided a better fit of the data than an additive allele model which assumes the effect in homozygotes to be considerably larger than the effect in heterozygote individuals.

Due to the strong predominance of SE alleles among ACPA-positive patients, all other alleles are inherently less prevalent in patients than in controls. This results in seemingly protective effects of these alleles, which are in fact merely caused by skewing due to the large difference in the prevalence of SE alleles. In order to obtain an accurate estimate of the effect of the non-SE alleles, the analyses for these alleles in ACPA-positive patients were therefore stratified for the presence of SE in the following manner. For each non-SE allele (e.g. DRB1*03) the six possible combinations of this allele with SE alleles were investigated:

- | | | |
|--------------------|--------------|---------------|
| A) DRB1*03/DRB1*03 | B) DRB1*03/x | C) x/x |
| D) SE/SE | E) SE/x | F) SE/DRB1*03 |

For the SE-negative stratum, the presence of DRB1*03 was compared to the absence of DRB1*03, hence the effect in group A and B was investigated using group C as the reference category. This corresponds to the dominant genetic model as described above. For the SE-positive stratum, the risk associated with the genotype F (SE/DRB1*03) was

analyzed using group E (SE/x) as the reference category, to adjust for the risk associated with the presence of 1 SE allele.

Subsequently, we performed a meta-analysis using the effect sizes (β) and standard errors of the different cohorts. To account for the fact that there was significant statistical heterogeneity (Q-statistic, p -value<0.10) in a small number of the analyses, a random effect approach ³⁴ was applied for all comparisons. This method allows between-study heterogeneity and incorporates it in the calculations. The data were analyzed per cohort with the Statistical Package for the Social Sciences (SPSS) 16.0. For the meta-analysis, we used the freely available software environment for statistical computing R.

RESULTS

The study cohort consisted of 2806 RA patients and 3772 controls from four different European populations. The distribution of cases and controls across the cohorts was: Norway: 788 cases and 898 controls, Sweden: 827 cases and 934 controls, the Netherlands: 844 cases and 1213 controls, and Spain: 347 cases and 727 controls. All patients fulfilled the 1987 ACR criteria for RA. The proportion of patients which was ACPA-positive was very similar in all cohorts and ranged from 58% to 62%.

The classifications of predisposing and protective HLA-DRB1 alleles, which have been described to be associated with RA, are listed in Table 1. The Shared Epitope (SE) classification incorporates several HLA-DRB1 alleles which confer a high risk for ACPA-positive disease, with reported odds ratio's (ORs) ranging from 4.6 to 11.3 ^{30, 35}.

The DERAA, D70 and I67 alleles have been claimed to be associated with protection from RA, with ORs of 0.50, 0.23 and 0.14 (for DERAA presence ²⁰, D70 homozygosity ¹⁶ and I67 homozygosity respectively ¹⁵). As shown in Table 1 there is considerable overlap between the protective classifications. The frequencies of the presence of the separate HLA-DRB1 alleles in the four control populations are also presented in Table 1, as well as the frequencies of the allele-classifications.

Associations between HLA-DRB1 alleles and ACPA-negative RA

Table 2 shows the results of the meta-analysis for ACPA-negative RA. Although the data show a predisposing effect of DRB1*03 and DRB1*04, as well as a possible protective effect of DRB1*07 and DRB1*15 on ACPA-negative RA, these associations were only weakly significant. With regard to the effects of DRB1*03 and DRB1*07 there were marked geographical differences. The previously reported association between DRB1*03 and susceptibility was present in the two Scandinavian cohorts and in the Dutch cohort, but was absent in Spain. However, a recent extensive study in Sweden ¹⁴ did not reveal a predisposing effect of DRB1*03 on ACPA-negative RA, indicating that more studies will be

Table 1. Frequencies and classifications of HLA-DRB1 alleles according to predisposition and protection in RA

Allele	Shared epitope	DERAA				D70				I67				Frequency in controls in %*			
		No	Sw	Nl	Sp	No	Sw	Nl	Sp	No	Sw	Nl	Sp	No	Sw	Nl	Sp
*0101	X													20			13
*0102	X													0.9	19	21	8.8
*0103						X			X	X				2.1	1.2	0.5	2.1
*03	-					-			-	-				25	24	22	25
*0401	X													22	24	16	4.3
*0402						X			X	X				0.3	0.8	0.2	2.8
*0403	-					-			-	-				2.0	1.5	2.5	5.8
*0404	X													13	7.6	6.5	5.8
*0405	X													0.4	0.9	0.7	3.9
*0407	-					-			-	-				0.6	1.3	1.3	1.2
*0408	X													1.9	1.3	0.6	0.4
*07									X	X				17	16	20	27
*08									X					8.5	9.0	7.0	5.4
*09	-					-			-	-				2.7	3.2	2.0	2.1
*1001	X													2.0	2.1	3.0	4.1
*1101									X					6.3	7.4	14	20
*1102						X			X					0.3			2.9
*1103						X			X					0.4	1.2	1.0	1.2
*1104									X					0.9			3.6
*12									X	X				5.2	4.3	5.0	1.9
*1301						X			X	X				15		14	13
*1302						X			X	X				9.2	24	12	6.6
*1303									X	X				0.4	1.0	1.4	4.4
*1454	-					-			-	-				3.1	4.1	7.0	4.1
*15										X				29	29	27	19
*16									X					0.6	1.2	2.0	2.5
Freq. in controls in %		No	Sw	Nl	Sp	No	Sw	Nl	Sp	No	Sw	Nl	Sp	No	Sw	Nl	Sp
		53	49	44	37	26	30	26	27	56	60	66	75	67	67	66	63

Rare alleles with a median prevalence in controls < 0.5% were not listed.

No = Norway, Sw = Sweden, Nl = Netherlands, Sp = Spain.

* In the Swedish and Dutch cohorts, four-digit typing was not available for all alleles as described in the Methods section. The frequencies were therefore listed in the following manner: sum of DRB1*0101 and DRB1*0102, sum of all DRB1*10 alleles (in the row of *1001), sum of DRB1*1101 and DRB1*1104 (in the row of *1101), sum of DRB1*1102 and DRB1*1103, (for the Swedish cohort: sum of DRB1*1301 and DRB1*1302) and sum of all DRB1*14 alleles (in the row of *1454).

It has recently been shown that the majority of individuals previously genotyped as DRB1*1401 in fact carry the genotype DRB1*1454 ⁴⁴. In anticipation of probable genotyping revisions we have therefore listed DRB1*1454 as the most common DRB1*14 allele.

Table 2. HLA-DRB1 associations with ACPA-negative RA, meta-analysis results

HLA-DRB1 allele	ACPA-negative patients OR (95% CI)	p-value
*01	1.06 (0.90-1.25)	0.46
*03	1.39 (1.01-1.93)	0.05
*04	1.17 (1.00-1.37)	0.05
*07	0.67 (0.48-0.95)	0.03
*08	0.97 (0.59-1.60)	0.90
*09	0.90 (0.54-1.50)	0.68
*10	1.04 (0.67-1.60)	0.86
*11	1.01 (0.79-1.29)	0.95
*12	0.84 (0.59-1.20)	0.34
*13	0.87 (0.73-1.03)	0.10
*14	1.09 (0.80-1.48)	0.58
*15	0.78 (0.65-0.94)	0.01
*16	1.04 (0.42-2.58)	0.94

required in order to draw definitive conclusions about this association. With regard to the protection conferred by DRB1*07, there appeared to be a North-to-South gradient with the strongest protective effect in Norway and no observable effect in Spain.

To perform a more detailed analysis of possible protective alleles, we also investigated the effects of the three classifications proposed to be associated with protection: the DERA- , D70- and I67- alleles .The DERA- alleles did not convey a protective effect for ACPA-negative RA, while both the D70- and I67- alleles showed a modest protective effect (OR (95%CI) 0.75 (0.59-0.96) and 0.70 (0.53-0.94) respectively).

Associations between HLA-DRB1 alleles and ACPA-positive RA

Table 3 displays the results of the meta-analysis for ACPA-positive RA. Since our aim was to specifically investigate protective effects, the table only lists the four-digit subtype analysis of alleles which have been reported to be associated with protection. Due to the preponderance of SE alleles among ACPA-positive patients, all other alleles are inherently less prevalent in patients than in controls. This leads to seemingly protective effects of these alleles, which are in fact merely the result of skewing caused by the large difference in the prevalence of SE alleles. In order to obtain an accurate estimate of the effect of the non-SE alleles, the analyses for these alleles were therefore stratified for the presence of SE.

The well-known association between SE alleles and susceptibility to ACPA-positive RA is confirmed by our data, as well as the hierarchy in the strength of this association, being DRB1*04>*10>*01. We also observed predisposing effects of DRB1*09, *15 and *16 , although the effect of *09 and *16 alleles was limited to SE-negative individuals. Despite the fact that some of these associations were relatively weak, they nonetheless suggest that

Table 3. HLA-DRB1 associations with ACPA-positive RA, meta-analysis results

HLA-DRB1 allele	OR (95% CI)	p-value	Stratification, OR (95% CI)	p-value
*01	1.35 (1.10-1.66)	0.004		
*0101 and *0102	1.44 (1.18-1.74)	<0.001		
0103	0.31 (0.13-0.75)	0.009	#	
*03	0.64 (0.55-0.75)	<0.001	SE neg: 1.05 (0.80-1.38)	0.71
			SE pos: 1.18 (0.95-1.46)	0.14
*04	3.76 (2.93-4.84)	<0.001		
*0402	1.48 (0.81-2.71)	0.21	#	
other *04	3.74 (2.86-4.91)	<0.001		
*07	0.56 (0.47-0.67)	<0.001	SE neg: 1.04 (0.79-1.38)	0.76
			SE pos: 0.89 (0.69-1.14)	0.35
*08	0.50 (0.36-0.70)	<0.001	SE neg: 0.73 (0.46-1.14)	0.17
			SE pos: 1.00 (0.50-1.98)	0.99
*09	1.43 (0.94-2.16)	0.10	SE neg: 3.25 (2.06-5.12)	<0.001
			SE pos: 1.76 (1.00-3.11)	0.05
*10	2.37 (1.56-3.60)	<0.001		
*11	0.56 (0.46-0.68)	<0.001	SE neg: 0.93 (0.64-1.36)	0.72
			SE pos: 0.90 (0.67-1.19)	0.45
*1102 and *1103	1.01 (0.61-1.67)	0.97	SE neg: 1.46 (0.63-3.37)*	0.38
			SE pos: 1.53 (0.75-3.12)	0.24
other *11	0.49 (0.39-0.62)	<0.001	SE neg: 0.80 (0.49-1.29)	0.36
			SE pos: 0.70 (0.51-0.97)	0.03
*12	0.60 (0.42-0.84)	0.003	SE neg: 0.76 (0.37-1.54)	0.44
			SE pos: 1.10 (0.69-1.77)	0.68
*13	0.33 (0.25-0.45)	<0.001	SE neg: 0.54 (0.38-0.77)	<0.001
			SE pos: 0.57 (0.41-0.81)	0.002
*1301 and *1302	0.31 (0.22-0.45)	<0.001	SE neg: 0.45 (0.27-0.74)	<0.001
			SE pos: 0.54 (0.34-0.83)	0.006
other *13	1.01 (0.48-2.10)	0.99	SE neg: 2.35 (0.63-8.72)	0.20
			SE pos: 1.56 (0.65-3.71)**	0.32
*14	0.48 (0.34-0.68)	<0.001	SE neg: 0.52 (0.28-0.98)##	0.04
			SE pos: 1.02 (0.46-2.24)##	0.96
*15	0.69 (0.57-0.82)	<0.001	SE neg: 1.51 (1.17-1.95)	0.001
			SE pos: 1.25 (1.02-1.55)	0.04
*16	1.21 (0.73-1.99)	0.46	SE neg: 2.91 (1.12-7.56)	0.03
			SE pos: 1.51 (0.69-3.27)	0.30

Printed in bold are effects with a p-value below 0.01. For the non-SE alleles, only those associations with a p-value ≤ 0.005 after stratification for SE, are printed bold.

* No cases in Leiden cohort.

Stratification for SE led to absence of cases or controls in the different strata in two of the four cohorts, therefore not performed.

** No cases in Norwegian cohort.

HLA-DRB1*1402 alleles have a SE motif and were exceedingly rare in all cohorts (prevalence below 0.3% in controls). Therefore the HLA-DRB1*14 effect was also stratified for SE, after exclusion of individuals with DRB1*1402 alleles.

the effect of HLA-DRB1 alleles on susceptibility to ACPA-positive RA may extend beyond the SE alleles.

Intriguingly, the DRB1*13 alleles appeared to be the only alleles associated with protection (Table 3). In both the SE-negative and SE-positive stratum, the protective effect of DRB1*13 not only remained present, but was also associated with a considerable effect size (OR 0.54 and 0.57 respectively) (Figure 1).

In the subtype analysis of some of the protective alleles, e.g. in the case of DRB1*0103 and DRB1*0402, stratification resulted in a lack of cases or controls in several cohorts,

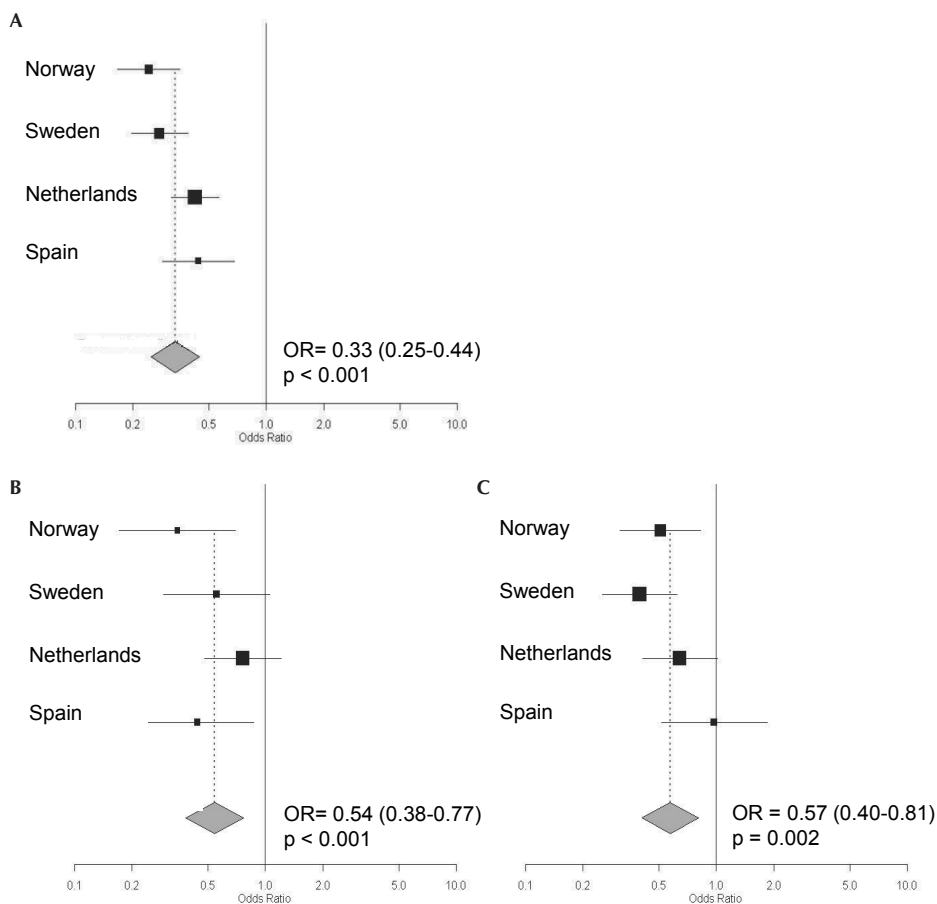


Figure 1: The effect of DRB1*13 on ACPA-positive RA.

Forest plots depicting the ORs and 95% CIs of the four separate cohorts and the combined estimate of the random effects meta-analysis.

Figure 1A: Unstratified analysis.

Figure 1B: SE-negative stratum.

Figure 1C: SE-positive stratum.

rendering a meta-analysis ineffective. We can therefore not formally exclude that these alleles may be associated with a protective effect, although in the case of *0402 this is unlikely based on the result of the non-stratified analysis.

The protective effect of DRB1*13 and protective classifications on ACPA-positive RA All protective classifications described for RA include DRB1*13 or some of the DRB1*13 suballeles (Table 1). When analyzed according to the different protective classifications, the protective effect of the I67 alleles did not remain significant after stratification for SE. Both the D70 and the DERA alleles were associated with a protective effect, which

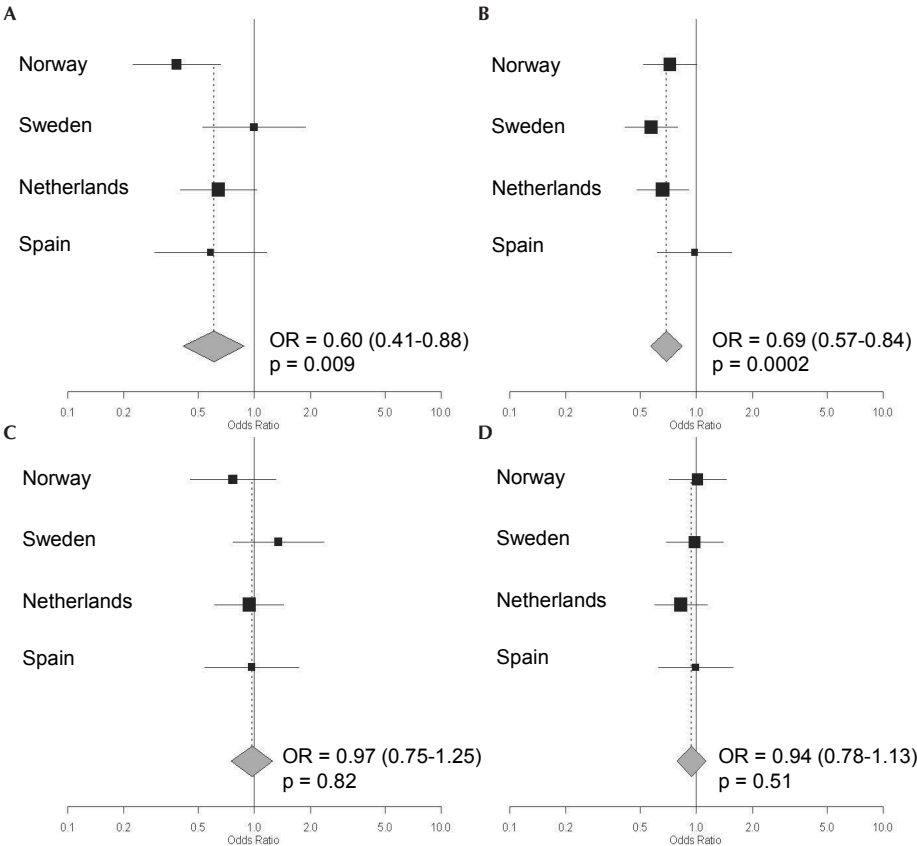


Figure 2: The effect of D70 alleles with and without DRB1*13 alleles in ACPA-positive RA. Forest plots depicting the ORs and 95% CIs of the four separate cohorts and the combined estimate of the random effects meta-analysis.

Figure 2A: Effect of all D70 alleles, SE-negative stratum.

Figure 2B: Effect of all D70 alleles, SE-positive stratum.

Figure 2C: Effect of D70 alleles after exclusion of DRB1*13, SE-negative stratum.

Figure 2D: Effect of D70 alleles after exclusion of DRB1*13, SE-positive stratum.

remained present after stratification for SE (Figure 2A and 2B), yet it was remarkable that, apart from DRB1*13, none of the alleles with a D at position 70 or a DERAA-motif appeared to confer protection by themselves (Table 3). We therefore investigated if the protection associated with the group of D70 or DERAA alleles as a whole, could be explained solely by the protective effect of the DRB1*13 alleles. To this end, we excluded the DRB1*13 alleles from the analysis and re-analyzed the effect of all alleles with a D at position 70 on ACPA-positive RA. As can be seen in Figure 2C and 2D, these alleles were not protective, despite the fact that they had a D at position 70. The same was the case for the DERAA alleles (data not shown). These data indicate that the presence of a D at position 70 or of

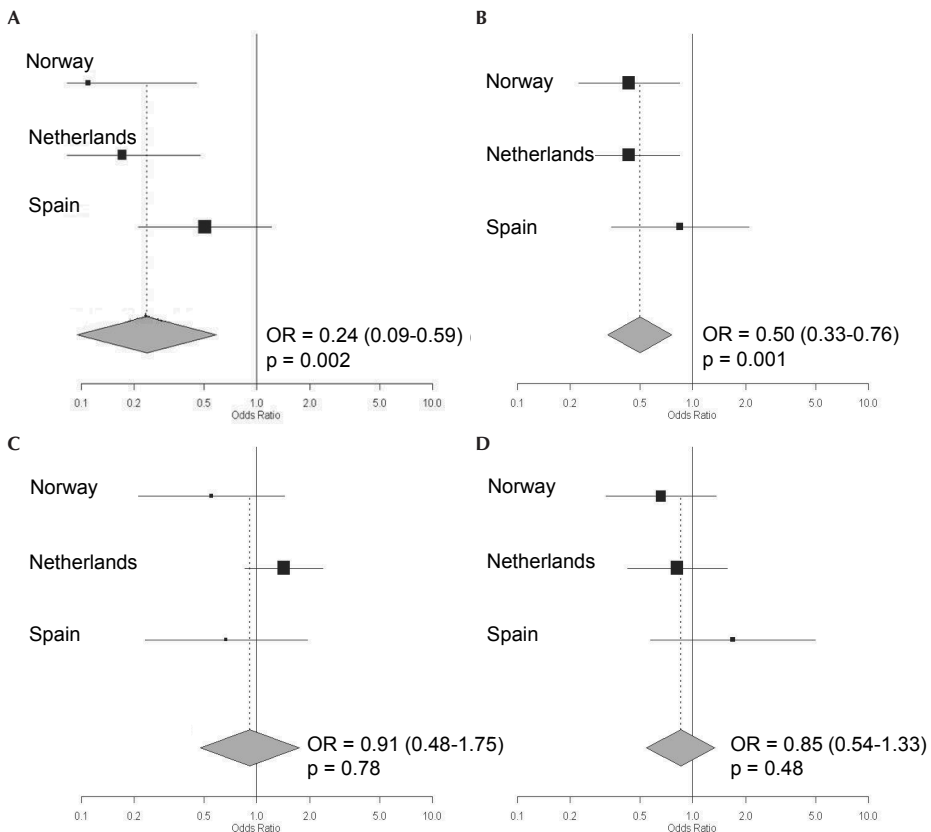


Figure 3: The effect of DRB1*1301 and *1302 in ACPA-positive patients.

Forest plots depicting the ORs and 95% CIs of the three separate cohorts with high-resolution typing of the DRB1*13 alleles, and the combined estimate of the random effects meta-analysis.

Figure 3A: Effect of DRB1*1301, SE-negative stratum.

Figure 3B: Effect of DRB1*1301, SE-positive stratum.

Figure 3C: Effect of DRB1*1302, SE-negative stratum.

Figure 3D: Effect of DRB1*1302, SE-positive stratum.

the DERA sequence as such does not result in protection from ACPA-positive RA, but rather, that DRB1*13 appears to be associated with protection.

In light of the strong protective effect conveyed by DRB1*13, we investigated if the presence of a DRB1*13 allele could annul the predisposition associated with a SE allele. The risk of SE/DRB1*13 heterozygote individuals compared to the risk of individuals carrying neither SE nor DRB1*13 alleles, was however still increased (OR (95%CI): 2.14 (1.64-2.80)). Although this effect will vary according to the difference in risk associated with the different SE alleles and in different cohorts, the presence of one DRB1*13 allele does not compensate the risk associated with the presence of one SE allele in meta-analysis.

Next, we analyzed whether the DRB1*13 association was confined to DRB1*13 alleles which contain a DERA sequence: DRB1*1301 and*1302 (DRB1*1304 was not present in the study populations). As can be seen in Table 3, the protective effect was limited to the DRB1*1301 and*1302 alleles, although the analysis for the other DRB1*13 alleles was possibly hampered by relatively small numbers of cases and controls. Complete four-digit typing of DRB1*13 was available for three of the four populations included in this meta-analysis (Norway, Netherlands and Spain). Subtype analysis revealed that the protective effect of DRB1*1302 was no longer present after stratification for the SE (Figure 3). DRB1*1301 therefore was the only allele which was consistently associated with protection from ACPA-positive RA.

DISCUSSION

HLA alleles contribute to the susceptibility of RA in various ways. As a consequence of the highly polymorphic nature of the HLA-region in the population, it has been difficult to dissect the contribution of the various HLA alleles to RA-susceptibility. Previously, several different classifications have been developed in order to summarize the predisposing and protective effects of the HLA alleles with regards to RA. Our data confirm the predisposing effect of the SE alleles and also corroborate the differential effect sizes with which different HLA SE alleles predispose to ACPA-positive disease ^{5,6}. Furthermore, our results indicate a contribution of DRB1*09 and DRB1*15 to ACPA-positive disease. The relatively modest effect of DRB1*15 requires replication in further studies, before any firm conclusions can be drawn. The predisposing effect of DRB1*09 to ACPA-positive RA on the other hand, has been described in other populations as well ³⁶. Therefore, it may be appropriate to include DRB1*09 in the list of susceptibility genes for ACPA-positive RA.

More importantly, however, our results confirm the association of HLA-DRB1 alleles with protection and considerably refine the definition of protective alleles. Our data indicate that the protective effect is only apparent for the DRB1*13 allelic group. Analysis of the different allele-classifications which have been developed to capture the protective effects

of HLA-DRB1 alleles in RA ^{15, 16, 18} revealed that the protective effect of the DERA and D70 classification could largely be attributed to the DRB1*13 alleles. This underscores the relative importance of the protective effect mediated by DRB1*13 in comparison to other alleles, and also raises the question whether the classifications of protective effects may need to be reconsidered.

Further analysis of the DRB1*13 alleles showed that protection was only apparent for the DRB1*1301 allele. Although our study included over 2700 patients and 3000 controls from four large data-sets from four different European populations, we cannot exclude that smaller protective effects may also be present for other alleles than DRB1*1301 which could not be detected in the present investigation. Previous studies in other ethnicities have also reported protective effects of other HLA alleles such as DRB1*1302 and DRB1*14 for RA, although not all of these results were stratified for ACPA-status or corrected for the effect of the HLA SE alleles ^{37, 38}. It would be interesting to know to what extent the protective effects differ among different populations.

The present study clearly confirms that the SE alleles are only associated with ACPA-positive RA. The same is true for the association between DRB1*13 or *1301 and protection. The present study thus demonstrates once more that the associations between HLA-DRB1 alleles and ACPA-positive versus ACPA-negative RA are very different both quantitatively and qualitatively. Regarding protective effects of HLA-DRB1 alleles in ACPA-negative RA, a recent study in a limited number of patients has reported that the DERA alleles may be protective in this subset as well ³⁹. We could not confirm this finding in the present study, although the weak protective effects we observed for DRB1*07 and DRB1*15 in ACPA-negative RA do not exclude the presence of HLA-mediated protection in ACPA-negative disease.

In the current investigation, the effect of the presence of the different HLA-DRB1 alleles was investigated separately. The risk for ACPA-positive RA in individuals heterozygous for the SE was assessed as part of the stratified analysis, but we cannot make conclusions about the risk associated with heterozygosity for the various other HLA-DRB1 alleles. It is conceivable that combinations of certain alleles may confer susceptibility or protection, as was described in a recent report, in which the combination of DRB1*03 and DRB1*13 alleles was found to be associated with an increased risk of ACPA-negative disease ¹⁴. A meta-analysis of heterozygosity effects may therefore yield very interesting results in the future.

For the statistical analysis, a dominant allele model was applied in the present study. An alternative would have been to use an additive allele model, which assumes substantially larger effects in homozygous versus heterozygous individuals. Although the dominant allele model provided the best fit to the data in the current analysis, other reports have favored an additive model ⁷. Discrepancies between publications may therefore be partly attributable to differences in statistical methods. A meta-analysis such as presented here is

helpful in this respect because it overcomes these statistical differences and provides an overview of the results of four cohorts analyzed in the same way.

As can be seen from Figures 1A and 3B, there was a tendency towards a North-South gradient in the strength of the associations of several HLA-DRB1 alleles. Associations were often the strongest in Norway and Sweden, slightly less strong in the Netherlands and the weakest or sometimes even absent in Spain. This was the case for both predisposing and protective alleles in both ACPA-positive and ACPA-negative disease. For HLA-associated susceptibility, these same geographical differences can be observed in previous publications^{40, 41}, but they have not been described for the protective effects of HLA in RA. If the existence of this gradient proves to be real, it may be a factor which needs to be taken into account when comparing data from different populations. It may also serve to reconcile some of the seemingly conflicting data which have been reported in different populations. Furthermore and perhaps most importantly, it may provide clues to candidate environmental factors that may be involved in the pathogenesis of the disease.

The main reason to perform studies such as the current meta-analysis, is to obtain mechanistic insight into the contribution of the HLA-region to RA. This has provided important new insights in the past, such as the realization that the HLA SE alleles do not contribute to RA as such, but rather to ACPA-positive disease⁸. More recently it was also shown that the presence of the HLA SE alleles influences both the magnitude and specificity of the ACPA-response^{9, 42}. Together, these observations indicate that the HLA SE alleles are primarily involved in shaping of the ACPA-response presumably by facilitating T-cell help to ACPA-producing B-cells. Intriguingly, our data show that the protective effects associated with the presence of DRB1*13 are also most prominent in the ACPA-positive group of RA patients. These observations would be in line with the notion that the predisposing effect of the HLA SE alleles and the protective effect of DRB1*13 act within the same biological pathway. Indeed, the presence of DRB1*13 considerably lowered the predisposing effects of the HLA SE alleles in individuals heterozygote for both, although the predisposing effect of the HLA SE alleles was not annulled. In case these effects target the same biological pathway, the presence of DRB1*13 may perhaps also influence the specificity and magnitude of the ACPA-response.

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Chapter 3

Quantitative heritability of ACPA-positive and ACPA- negative rheumatoid arthritis

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ABSTRACT

Objective The majority of genetic risk factors for rheumatoid arthritis (RA) are associated with anti-citrullinated protein antibody (ACPA)-positive RA, while far fewer genetic risk factors have been identified for ACPA-negative RA. To quantify the contribution of genetic risk factors in general and of the predisposing HLA-DRB1 shared epitope (SE) alleles in particular to these two subsets of RA, we computed the heritability and assessed the contribution of HLA SE alleles.

Methods 148 RA twin pairs, of which at least one twin had RA, were tested for ACPA and typed for HLA-DRB1 genotypes. Heritability was assessed by including a bivariate normally distributed random effect representing the contribution of unobserved genetic factors to RA susceptibility in a logistic regression model with the correlation between the random effects fixed according to twin zygosity. The contribution of HLA SE alleles to the genetic variance was assessed by a similar model using genotype-specific population prevalences.

Results The heritability of RA was 66% (95% confidence interval (CI) 44-75%). For ACPA-positive RA the heritability was 68% (CI: 55-79%) and for ACPA-negative RA 66% (CI: 21-82%). The HLA SE alleles explained 18% (CI: 16-19%) of the genetic variance of ACPA-positive RA, and only 2.4% (CI: 1.6-10%) of ACPA-negative RA.

Conclusion The heritability of ACPA-positive and ACPA-negative RA is comparable. These data indicate that genetic predisposition plays an important role in the pathogenesis of ACPA-negative RA, for which most individual genetic risk factors remain to be identified.

INTRODUCTION

Several new genetic risk factors for rheumatoid arthritis (RA) have been identified in the past few years. Candidate gene studies and new techniques such as whole genome scans have led to the discovery of various new genetic variants which predispose to RA, in for example the genomic regions encoding PTPN22, STAT4 and TRAF1/C5¹⁻³. Interestingly, the majority of the genetic factors which predispose for RA have been found to preferentially confer risk to autoantibody-positive disease. The importance of distinguishing autoantibody-positive from autoantibody-negative RA is increasingly being recognized due to the recent findings concerning anti-citrullinated protein antibodies (ACPA).

ACPA were first described as anti-perinuclear factor and anti-keratin antibodies, that both bind to citrullinated filaggrin⁴⁻⁷. Since then, ACPA have been found to be the most predictive factor for the future development of RA⁸⁻¹⁰. Due to its superior specificity and predictive characteristics as compared to IgM RF, ACPA are increasingly being used for diagnostic purposes¹¹. Approximately two-thirds of patients with established RA are ACPA-positive, and these patients are characterized by a destructive phenotype with higher rates of joint destruction as compared to ACPA-negative RA patients¹².

ACPA-positive and ACPA-negative disease differ not only with regard to clinical disease phenotype, but are also associated with different genetic and environmental risk factors^{13, 14}. The HLA-DRB1 shared epitope (SE) alleles for example, which confer the highest risk of all known genetic risk factors, predispose specifically to the development of an anti-citrullinated protein immune response, rather than to the development of RA¹⁵. PTPN22 gene variants, as well as the C5-TRAF locus, have been found to be predominantly associated with ACPA-positive RA, although an association with ACPA-negative RA cannot be excluded^{2, 16}. It is possible that studies which include a larger number of ACPA-negative patients will also find an association between these genetic risk factors and ACPA-negative RA, such as has been shown for STAT4¹⁷. At this moment however, the number of genetic risk factors which has been described to be predominantly associated with ACPA-negative RA is more limited, and includes HLA-DR3 haplotypes and interferon regulatory factor 5 (IRF5)^{14, 18}.

Not only genetic, but also environmental risk factors differ between ACPA-positive and ACPA-negative RA. Recent data have shown an interaction between smoking and the SE alleles in conferring risk for ACPA-positive RA^{19, 20}. The differences in clinical phenotype and underlying risk factors have lead to the concept that ACPA-positive and ACPA-negative RA constitute distinct entities with different pathophysiological mechanisms of disease²¹. The extent to which genetic factors contribute to disease development may differ between these two subsets, and thus far, more genetic factors have been found to be associated with ACPA-positive than with ACPA-negative RA.

To investigate the contribution of genetic factors to disease development, twins are a valuable source of information. Twin studies provide two measures for the influence of genetic factors on disease development: the concordance and the heritability. The concordance represents the frequency with which the twin brothers or sisters of diseased individuals are also affected. While the concordance is influenced by the prevalence of the disease (or disease subset), heritability is not. The heritability supplies a quantitative measure for the amount of variation in disease susceptibility which can be explained by genetic factors. Another advantage of heritability estimates compared to concordance rates, is that the heritability is based on the combined data of both mono- and dizygotic twins. This makes heritability modeling a more powerful method, especially when using small populations such as RA twin cohorts. For RA, the heritability has been assessed at approximately 60% ²². There are no data available however for the heritability of autoantibody-positive versus autoantibody-negative RA.

In this study, we determined the heritability of ACPA-positive and ACPA-negative RA by applying quantitative genetic methods to data from a large twin cohort. In addition, we assessed the contribution of the predisposing HLA-DRB1 shared epitope (SE) alleles to the genetic variance of both groups.

METHODS

Study population

A nationwide study of twin pairs of which one or both had RA was performed in the United Kingdom in 1989 ²³. Twin pairs were recruited by two parallel strategies. All UK rheumatologists were contacted and requested to ask all patients with RA whether they were a twin. Secondly, there was a simultaneous multi-media campaign, inviting patients who had been diagnosed with RA and who had a living twin to contact a study centre. Both members of each twin pair were visited at home by trained research nurses who recorded detailed history and demographic characteristics and performed joint examinations. Blood samples were collected of which serum was stored and lymphocytes were separated for HLA typing. High resolution HLA-DRB1 genotyping was performed enabling the assignment of RA-associated HLA SE alleles ²⁴.

RA was diagnosed according to the 1987 revised ACR criteria ²⁵. Twin pairs were only eligible for inclusion if at least one twin satisfied these criteria. Zygosity status was verified by DNA fingerprinting on all same-sexed pairs using non-radioactive Southern blotting with a multilocus probe ²⁶.

ACPA (Anti-CCP2) assays

Total IgG anti-CCP2 was measured in serum samples by enzyme-linked immunosorbent assay (ELISA) (Immunoscan RA Mark 2; Eurodiagnostica, Arnhem, The Netherlands). Samples with a value above 25 units/ml were considered positive according to the manufacturer's instructions.

Statistical analysis

Summary statistics were generated to investigate the prevalence of ACPA-positive and ACPA-negative RA and the occurrence of the HLA SE alleles. Concordance figures were calculated by dividing the number of pairs in which both twins were affected by the total number of twin pairs.

To calculate the heritability of RA, as well as of ACPA-positive RA and ACPA-negative RA, we used a logistic regression model with a normally distributed random effect²⁷⁻²⁹. In this case, the normally distributed random effect represents the contribution of unobserved genetic factors to RA susceptibility. The correlation between the outcomes of twins due to genetic factors is modeled according to the extent to which a twin pair shares this random effect. Monozygotic twin pairs share all their genome and therefore they also share all their random effect. Dizygotic twins share half of their genome, hence they also share half of the random effect in the model. The variance of the random effect (genetic variance) is a measurement for the total contribution of genetic factors to the outcome. The heritability is defined as the genetic variance divided by the total variance of the outcome. The variance of the random effect with 95% confidence intervals was estimated using profile likelihood, i.e. for a grid of genetic variances the likelihood was computed and the value for which the likelihood curve obtained its maximum is the maximum likelihood estimate of the genetic variance. Since the twin pairs were selected for having at least one affected twin, the prevalences of RA, ACPA-positive RA and ACPA-negative RA were fixed to their population prevalences (see Table 3 for the values used) (see Appendix A for further details of the heritability modeling approach).

Subsequently, we computed the contribution of SE to the total genetic variance of RA, as well as of ACPA-positive RA and of ACPA negative RA. This measure can be obtained by using a method similar to the method for computing the heritability. Instead of using the general population prevalence, now the genotypic specific population prevalences are used (see Table 3). For this model the variance of the random effect represents the genetic factors other than the genotype under study (see Appendix B for further details of the calculation of the contribution of SE to the genetic variance).

All computations were performed in the freely available software environment for statistical computing R.

RESULTS

Concordance

The characteristics of the study cohort, which consisted of 64 monozygotic and 84 dizygotic twin pairs as described previously ²², are displayed in Table 1. The patient characteristics of the monozygotic and dizygotic twins are comparable with regard to demographic factors, autoantibody status and radiographic severity of RA.

Table 1. Patient characteristics

Patient characteristic †	Monozygotic twins (64 pairs with 74 RA patients)	Dizygotic twins (84 pairs with 87 RA patients)
Age in years, mean (SD)	41 (14)	37 (13)
Female gender, n (%)	65 (88%)	71 (82%)
Rheumatoid factor IgM positive, n (%)	58 (78%)	74 (85%)
Anti-CCP2 positive, n (%)	54 (73%)	71 (82%)
Erosive disease, n (%)	55 (74%)	69 (79%)

† For dichotomous variables, the number and the percentage of patients are listed, relative to the total number of RA patients for whom information about the characteristic under investigation was available.

The concordance of RA, which represents the frequency with which the twin brothers or sisters of RA patients are also affected, was calculated for the monozygotic and dizygotic twin pairs (Table 2). For monozygotic twins the concordance is 15.6% versus 3.6% for dizygotic twins. Based on the results of the anti-CCP2 measurements, the concordance of the ACPA-positive and ACPA-negative subsets was also determined. In this cohort, twins who were concordant for RA all had the same ACPA status, meaning that there was no twin pair consisting of two affected individuals in which one individual had ACPA-positive disease and one individual had ACPA-negative disease. The concordance of ACPA-positive RA is comparable to the concordance of RA, while the concordance of ACPA-negative RA is lower than that of RA and ACPA-positive disease.

Table 2. Concordance of total RA, ACPA-positive and ACPA-negative disease.

	Monozygotic twins 64 pairs			Dizygotic twins 84 pairs		
	concordant	discordant	concordance (%)	concordant	discordant	concordance (%)
RA	10	54	15.6	3	81	3.6
ACPA+ RA	8	38	17.4	3	65	4.4
ACPA- RA	2	16	11.1	0	16	0

Table 3. Population prevalences used for heritability modeling

Characteristic	Prevalence	Reference
Prevalence of RA	1%	32
Prevalence of ACPA-positive RA	0.67%	11
Prevalence of ACPA-negative RA	0.33%	11
Prevalence of ACPA in healthy controls	1%	33
Prevalence of SE in ACPA-positive RA patients	84%	30
Prevalence of SE in ACPA-negative RA patients	59%*	*
Prevalence of SE in healthy controls	44%	31

The prevalence for each genotype was fixed to the specific population prevalence as reported in the United Kingdom^{30,31} or, if not available from the UK, in studies from other Caucasian populations^{11,32,33}. * personal communication from the Wellcome Trust Case Control Consortium, Jane Worthington

Heritability

Heritabilities were estimated to quantify the extent to which genetic variation contributes to the development of disease, independent of the disease prevalence. For the logistic regression model as described in appendix A, the population prevalence of the disease or disease subsets was fixed to the specific population prevalence as reported in the United Kingdom^{30, 31} or, if not available, in studies from other Caucasian populations^{11, 32, 33} (Table 3).

The overall heritability of RA is 66% with a 95% confidence interval (CI) of 44-75%. The same approach was used to model the outcome of ACPA-positive and ACPA-negative RA. As shown in Table 4, the heritability estimates for ACPA-positive and ACPA-negative RA are similar to the heritability of total RA. The heritability of ACPA-positive RA is 68% (95% CI: 55-79) and the heritability of ACPA-negative RA 66% (95% CI: 21-82%).

Table 4. Heritability

Disease (subset)	twin pairs (n)	Heritability
Total RA	148	66% (95% CI: 44-75%)
ACPA-positive RA	114	68% (95% CI: 55-79%)
ACPA-negative RA	34	66% (95% CI: 21-82%)

Contribution of HLA SE alleles

The HLA SE alleles have recently been shown to be specifically associated with ACPA-positive RA³⁴. Therefore we set out to investigate if there is a difference in the extent to which the HLA SE alleles contribute to the genetic variance of ACPA-positive versus ACPA-negative RA.

HLA-DRB1 typing was available for 142 twin pairs (96%). The HLA SE alleles were found to be preferentially associated with ACPA-positive RA. Specifically, 109 (109/123

= 89%) of patients with ACPA-positive RA carried one or two SE alleles, compared to 15 (15/32 = 47%) of patients with ACPA-negative RA. The contribution of the HLA SE to RA in general, as well as to ACPA-positive and to ACPA-negative RA was estimated by including this genetic risk factor in the logistic regression model. The prevalence for each genotype was fixed to the specific population prevalence as reported in the United Kingdom ^{30, 31} or, if not available, in studies from other Caucasian populations ^{11, 32, 33} (Table 3).

This analysis revealed that the contribution of HLA SE alleles to the total genetic variance of RA is 11% (CI 10-12%) (Table 5). In the ACPA-positive disease subset the HLA SE alleles contribute 18% (CI 16-19%), whereas they only contribute 2.4% (CI: 1.6-10%) to the heritability of ACPA-negative RA.

Table 5. Contribution of HLA SE alleles to genetic variance

Disease (subset)	twin pairs (n)	Contribution of HLA SE alleles
Total RA	142	11% (95% CI: 10-12%)
ACPA-positive RA	113	18% (95% CI: 16-19%)
ACPA-negative RA	29	2.4% (95% CI: 1.6-10%)

DISCUSSION

The present study investigated the heritability of ACPA-positive and ACPA-negative RA. Data from a large RA twin cohort revealed a heritability for both disease subsets of approximately 66%. Furthermore, the contribution of the predisposing HLA SE alleles to ACPA-positive and ACPA-negative RA was assessed. While the HLA SE alleles contribute 18% to the genetic variance of ACPA-positive RA, they contribute only 2.4% to the genetic variance of ACPA-negative RA.

The heritability of ACPA-positive and ACPA-negative RA is similar despite the fact that the concordance of these two disease subsets differs. Both among mono- and dizygotic twins the concordance of ACPA-negative RA is lower than that of ACPA-positive RA. This can be explained by the fact that the concordance, which indicates how often a twin brother or sister of a patient is affected as well, is influenced by the prevalence of the disease in the population, whereas heritability is not. Concordance therefore reflects the prevalence of the disease in the population. Due to the fact that ACPA-negative RA is less prevalent than ACPA-positive RA the concordance of this disease subset is lower, despite similar heritability.

The fact that the prevalence of the disease affects the concordance but not the heritability also serves to explain the seeming discrepancy between the apparently modest monozygotic concordance of 16% and the heritability of 66% for RA. The concordance figure of 16% needs to be interpreted in light of the prevalence of RA which is only 1% ³².

This indicates that genetic factors are important determinants for disease development and is compatible with a high heritability.

The estimate of 66% (95% CI: 44-75%) for the overall heritability of RA is consistent with the results of a previous study that reported an overall heritability of 65% (95% CI: 50-77%)²². The previous report used the same twin cohort but a different modeling approach. Both models quantify the genetic and environmental contribution to a dichotomous variable by assuming that there is a continuous underlying liability to disease and that a threshold of liability divides subjects in affected with RA and unaffected. The previous study used a normal distribution for the liability while we used a logistic distribution. The advantage of a logistic distribution is that this model corresponds to the logistic regression model which is most commonly used for binary outcomes in epidemiology.

In twin studies, the variance of continuous traits, such as the underlying liability to disease, is often divided into variance due to environmental and due to genetic effects. In this paper, we estimated only additive genetic variances. Variance components for dominance genetic effects or for shared environmental effects can be added to this model, though it must be kept in mind that in twins these two effects are statistically confounded and cannot be added simultaneously to the same model. When a dominance genetic effect or a shared environmental effect was added to the model, we found that this determined less than 1% of the total variance of RA and of the ACPA-positive and ACPA-negative disease subsets. According to these data, dominance genetic effects or shared environmental factors do not appear to have a substantial effect on the development of RA.

The modeling approach employed in this study uses the population prevalences of the genetic risk factors of interest to estimate their contribution to the genetic variance. To ensure the validity of our estimates we therefore used prevalence figures of the HLA SE alleles reported in large studies of RA patients. While the prevalence of HLA SE alleles in ACPA-positive RA as reported in the literature (84%) was in line with the number observed in the twin study population (89%), the prevalence of HLA SE alleles in the ACPA-negative twins was found to be lower than the figure observed in a large UK RA study (47% versus 59%). To assess the impact of this discrepancy, the modeling was also performed with other assumed population prevalences, all of which consistently revealed a minor contribution of the HLA SE alleles to ACPA-negative RA. The results provided by the current modeling approach therefore appear to be valid irrespective of small uncertainties of prevalence figures.

The HLA SE alleles are, to our current knowledge, the genetic risk factors which confer the highest risk for disease development¹³. In the past, HLA genes have been estimated to contribute 37% to the overall inherited risk of developing RA³⁵. Our result that the contribution of the HLA SE alleles to the genetic variance is only 11% (95% CI: 10-12%) for RA and 18% (95% CI: 16-19%) for ACPA-positive RA may therefore be considered surprising. This difference may be due to the fact that the methods used to calculate the

contribution of the HLA SE alleles may not be completely comparable. Assuming that they are measuring similar parameters, a possible explanation for the difference could be that the contribution of SE was overestimated in the past due to the use of selected literature data for the calculation and weaknesses of the method of Rotter & Landaw, as indicated by Deighton et al. in the original publication³⁵. On the other hand, we cannot exclude that our figure of 11% contribution of HLA SE alleles to RA may represent an underestimate of the true value, due to the fact that the genotype-specific population prevalences which were required for our modeling, may vary among populations as discussed in the previous paragraph. Probably the most important explanation is however, the fact that a substantial part of the contribution of HLA to the genetic variance could be due to protective genetic factors³⁶ in addition to the predisposing SE alleles. While we deliberately only considered the predisposing SE alleles, Deighton et al used HLA haplotype sharing for their calculation, and thereby included the effect of protective HLA alleles, which may have resulted in a higher estimate. It is likely that further characterization of genetic risk factors, in particular the protective HLA-DRB1 alleles, and elucidation of interactions will be necessary to fully understand the role of genetic risk factors in disease development.

Recent genome wide association studies and candidate gene approaches have identified several new genetic risk factors for RA^{2,3}. The majority of these genetic risk factors, such as PTPN22 and C5-TRAF1 have been shown to be predominantly associated with ACPA-positive RA just like the HLA SE alleles^{34,37}. This may partly be due to the fact that some of the larger studies have included mainly ACPA-positive patients. One cannot exclude that newer studies which incorporate a more extensive investigation of ACPA-negative RA will show that these risk factors also predispose to ACPA-negative RA¹⁷.

At this moment however, the number of risk factors described to be preferentially associated with ACPA-negative RA is smaller and to our knowledge restricted to the HLA-DR3 haplotype and IRF5^{14,18}. The latter risk factors however do not confer as high a risk for disease development as the HLA SE alleles. The observation that the heritability of ACPA-negative RA is similar to the heritability of ACPA-positive RA is therefore intriguing. This suggests that most genetic risk factors for ACPA-negative RA are still unknown and remain to be discovered. One reason why the description of genetic risk factors for ACPA-negative RA has not advanced at the same pace as for ACPA-positive RA, could be that ACPA-negative RA may be a more heterogeneous disease entity than ACPA-positive RA, with different phenotypes being associated with different genetic risk factors. Another explanation is that many of the cohorts which have been used for large-scale genetics studies have consisted solely of patients with ACPA-positive RA. Because ACPA-negative disease is less prevalent, it is also more challenging to find sufficient numbers of patients to perform a well-powered study.

For the current study, the limited number of ACPA-negative twins also presented a statistical restriction. This resulted in a larger confidence interval for the heritability estimate of

ACPA-negative RA as compared to that of ACPA-positive RA. Confirmation of these results would therefore be helpful to reach a definitive conclusion regarding the heritability of ACPA-negative RA.

In conclusion, ACPA-positive and ACPA-negative RA have a similar heritability of approximately 66%. This means that genetic predisposition also plays an important role in the pathogenesis of ACPA-negative RA, for which most individual genetic risk factors remain to be identified.

APPENDIX A

Logistic regression model used to compute heritability.

We used a logistic regression model with a random effect to model the outcomes of twin pairs. With u corresponding to the random effect which represents all genetic factors that contribute to the outcome and y being 1 if a twin is affected and 0 if it is healthy, the probability to be affected for a single individual is given by the following equation:

$$P(Y=1|u) = \frac{\exp(\alpha+u)}{1 + \exp(\alpha+u)} \quad (1)$$

Given the population prevalence p of the outcome and the genetic variance τ^2 (= the variance of the random effect u), the parameter α can be derived from the following equation :

$$p = \int \frac{\exp(\alpha+u)}{1 + \exp(\alpha+u)} dF_{\tau}(u) \quad (2)$$

with F the normal distribution of u with mean zero and variance τ^2 . Since u is not observed we integrate over its distribution F . Note that due to the non-linear relationship between the prevalence p and the genetic variance τ^2 , the absolute value of the parameter α increases with the genetic variance τ^2 . This attenuation effect is well known from regression models which take into account errors in variables ²⁸.

Now given τ^2 and α , the log likelihood of the data is computed. Here (y_1, y_2) and (u_1, u_2) represent the pairs of outcomes and of random effects of a twin pair. The correlation of the random effects (u_1, u_2) is fixed at 1 if the twins are monozygotic and 1/2 if they are dizygotic. The log likelihood is then given by

$$l(y_1, y_2 | \alpha, \tau^2) = \log \int \frac{\exp(y_1(\alpha+u_1))}{1 + \exp(\alpha+u_1)} \frac{\exp(y_2(\alpha+u_2))}{1 + \exp(\alpha+u_2)} dF_{\tau}(u_1, u_2), \quad (3)$$

with F the bivariate normal distribution of (u_1, u_2) . The above steps are carried out for a grid of values of τ^2 and the value τ^2 for which the curve has its maximum is the maximum likelihood estimate of the genetic variance. The heritability is now given by the genetic

variance (τ^2) divided by the total variance of the outcome ($\tau^2 + 3$). Here the value 3 corresponds to the variance of the logistic distribution ³⁸.

APPENDIX B

Model used to compute the contribution of HLA SE to the total genetic variance.

The contribution of the HLA SE alleles to the total genetic variance of RA, of ACPA-positive RA and of ACPA-negative RA is computed as follows. In an extension of the approach explained in appendix A, we used a logistic regression model which now not only estimates the genetic variance but also the amount of this variance which is explained by the HLA SE alleles. To take into account that the risk provided by the HLA SE alleles differs for the development of RA, ACPA-positive RA, and ACPA-negative RA, these varying genotype specific population prevalences were derived from Table 3 using Bayes' theorem. The prevalences were subsequently entered into equation 2 to obtain the genotype specific α (α_{SE+} and α_{SE-}) of the regression model. The variance of the contribution of SE to the outcome equals $(\alpha_{SE+} - \alpha_{SE-})^2 P_{SE} (1 - P_{SE})$, and this variance was then included in the logistic regression model alongside with the parameter for the genetic variance. In the logistic regression model including HLA SE, the variance of the random effect (v^2) now measures the contribution of all remaining genetic factors besides HLA SE. Hence the part of the genetic variance explained by SE is given by the variance of the contribution of SE divided by the total genetic variance (τ^2):

$$\frac{(\alpha_{SE+} - \alpha_{SE-})^2 P_{SE} (1 - P_{SE})}{(\alpha_{SE+} - \alpha_{SE-})^2 P_{SE} (1 - P_{SE}) + v^2} = \frac{(\alpha_{SE+} - \alpha_{SE-})^2 P_{SE} (1 - P_{SE})}{\tau^2}$$

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Chapter 4

The contribution of genetic risk factors other than the HLA shared epitope alleles to the genetic variance of rheumatoid arthritis

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ABSTRACT

Objective Genetic factors explain 66% of susceptibility to rheumatoid arthritis, but it is unclear how much of this genetic risk can be explained by the genetic risk factors known to date. In addition to the contribution of the HLA shared epitope (SE) alleles which we have quantified previously, we now investigated the contribution of the protective HLA-DRB1*13 alleles and of 12 replicated single nucleotide polymorphisms (SNPs), to the genetic variance of total, anti-citrullinated protein antibody (ACPA)-positive and ACPA-negative RA.

Methods A cohort of 148 twin pairs, of which at least one twin had RA, were tested for ACPA. The contribution of the HLA-DRB1 alleles and SNPs to the genetic variance was assessed by a logistic regression model with genotype-specific parameters and a random effect model representing the contribution of unobserved genetic factors.

Results In the ACPA-positive subset, the HLA SE alleles contributed 19.2% to the genetic variance, while the contribution of the protective HLA-DRB1*13 alleles was very small: 0.13%. Both SE and DRB1*13 alleles hardly contributed to ACPA-negative RA (1.52%). The contributions of the separate SNPs to the different subsets of RA were small with an average of below 1%.

Conclusion A modest amount of the genetic variance of ACPA-positive, but not ACPA-negative RA can be explained by the HLA SE alleles, while the contribution of the protective HLA-DRB1*13 alleles and non-HLA loci is very small. These results indicate that the largest part of the genetic contribution to RA remains unexplained.

INTRODUCTION

Rheumatoid arthritis (RA) affects approximately 0.5-1% of the population in western civilizations ¹ and investigations of factors which determine a person's risk of developing RA have been ongoing since decades. Several risk factors for RA are now known, and these can be broadly classified as genetic or environmental risk factors. The relative importance of genetic and environmental risk factors can be investigated by estimating the heritability: the amount of disease variance which can be explained by genetic factors. The heritability of RA has consistently been reported to be approximately 66% ^{2,3}.

The hypotheses concerning how risk factors determine the development of RA have changed dramatically since the discovery of anti-citrullinated protein antibodies (ACPA) ⁴. Many of the risk factors for RA have now been found to be specific for either ACPA-positive or ACPA-negative RA. For example, the genetic risk factors which confer the highest risk of disease, the human leucocyte antigen (HLA) DRB1 shared epitope (SE) alleles, have been described to specifically predispose to autoantibody-positive, and most prominently ACPA-positive, disease ^{5,6}. This predilection for ACPA-positive RA has also been reported for the majority of the other genetic risk factors known to date ⁷. It was therefore surprising to discover that the heritability of ACPA-positive and ACPA-negative disease does not differ, and is 66% for both subsets of disease, as we have recently described ³. This suggests that many genetic risk factors for ACPA-negative RA remain to be discovered. Furthermore, the effect of the HLA SE alleles explained a relatively modest 18% of the genetic variance of ACPA-positive RA, raising the question which other genetic risk factors could account for the "missing" 82% of the heritability.

A possible answer to this question may lie in genes which confer protection from developing RA. In a recent meta-analysis of HLA-DRB1 associations in 2800 RA patients and 3000 controls, the presence of HLA-DRB1*13 alleles was associated with a markedly lower risk of ACPA-positive disease ⁸. This protective effect was present in both SE-positive and SE-negative individuals, demonstrating that protection was not just due to the absence of predisposing effects, but was an independent phenomenon. Furthermore, the DRB1*13 alleles were the only alleles with a significant protective effect, and the protection which had in the past been ascribed to alleles harboring a DERA or D70, could be explained solely by the protective effect of the DRB1*13 alleles. Within the DRB1*13 alleles, especially DRB1*1301 was associated with protection and in light of the strength of this protective effect (OR= 0.24 - 0.50), it seems plausible that these protective alleles may contribute considerably to the heritability.

Another factor which may play a role in explaining the remaining genetic variance of RA, is the effect of non-HLA genes associated with single nucleotide polymorphisms (SNPs). As a result of recent genome-wide association studies (GWAS), several new SNPs have been described to be associated with RA ⁹ and although the effects of these SNPs by

themselves tend to be limited, the combined effect of several SNPs may nonetheless be substantial with regard to the genetic variance of the disease.

For these reasons, we wished to investigate to what extent protective HLA effects, in the form of DRB1*13, and the effect of 12 well-replicated SNPs associated with RA, contribute to the genetic variance of RA. Using genotype frequencies reported in large case-control studies, we applied quantitative genetic methods to data from a relatively large twin cohort to assess the effect of these genetic risk factors for RA in general, as well as for ACPA-positive and ACPA-negative disease.

METHODS

Study population

A nationwide study of twin pairs of which one or both had RA was performed in the United Kingdom in 1989 ¹⁰. Twin pairs were eligible for inclusion if at least one twin fulfilled the 1987 revised ACR criteria ¹¹. Blood samples were collected and zygosity status was verified by DNA fingerprinting on all same-sexed pairs using non-radioactive Southern blotting with a multilocus probe ¹². SNP genotyping was not available for this twin cohort.

ACPA (Anti-CCP2) assays

Total IgG anti-CCP2 was measured in serum samples by enzyme-linked immunosorbent assay (ELISA) (Immunoscan RA Mark 2; Eurodiagnostica, Arnhem, The Netherlands). Samples with a value above 25 units/ml were considered positive according to the manufacturer's instructions.

Statistical analysis

The contribution of the HLA genotypes and the 12 SNPs to the total genetic variance of RA, ACPA-positive and ACPA-negative RA was computed from the genotype frequencies available for Dutch and UK cohorts and the total genetic variance (τ^2) estimated previously ³.

When investigating the genetic contribution of DRB1*13, it is important to take both DRB1*13 and SE alleles into account because these alleles are mutually exclusive at the HLA-DRB1 locus. All six possible genotype combinations of DRB1*13 (denoted as DR13) and SE therefore need to be taken into consideration (SE/SE, SE/x, x/x, DR13/x, DR13/DR13 and SE/DR13, where x denotes genotypes which are neither SE nor DR13). In order to quantify the effect of SE and DRB1*13 alleles separately, the following dummy variables were defined:

X_1 to measure the effect of 1 SE allele (assuming the value 1 for genotypes SE/x and SE/DR13 and 0 for the other four genotypes),

X_2 measuring the effect of 2 SE alleles (value=1 for genotype SE/SE and 0 for the other five genotypes),

X_3 measuring the effect of 1 DRB1*13 allele (value=1 for genotypes DR13/x and SE/DR13 and 0 for the other four genotypes),

X_4 measuring the effect of 2 DRB1*13 alleles (value=1 for genotype DR13/DR13 and 0 for the other five genotypes).

We assumed the following logistic regression model for the relationship between the HLA genotypes and the risk for RA:

$$P(Y=1|\text{genotypes}, u) = \frac{\exp(\alpha^* + \beta_1^* X_1 + \beta_2^* X_2 + \beta_3^* X_3 + \beta_4^* X_4 + u_r)}{1 + \exp(\alpha^* + \beta_1^* X_1 + \beta_2^* X_2 + \beta_3^* X_3 + \beta_4^* X_4 + u_r)} \quad (1)$$

With $Y = 1$ for RA patients and $Y=0$ for controls, and u_r is a Gaussian random effect with zero mean and variance τ_r^2 which represents unmeasured genetic factors.

Previously we estimated the total genetic variance τ^2 by using model (1) without the genotypes (assuming $\beta_1, \beta_2, \beta_3, \beta_4 = 0$). τ^2 equals the sum of the genetic variance explained by HLA ($\text{var}(G)$) and the genetic variance explained by other genetic risk factors (τ_r^2):

$$\tau^2 = \text{var}(G) + \tau_r^2 \quad (2)$$

The contribution of HLA ($\text{var}(G)$) to the total genetic variance τ^2 can thus be measured by

$$\frac{\text{var}(G)}{\text{var}(G) + \tau_r^2} = \frac{\text{var}(G)}{\tau^2}$$

For the genetic effect of HLA we considered the effect of the six genotypes on RA as described above. $\text{var}(G)$ can be expressed as

$$\begin{aligned} \text{var}(G) = & \text{var}(\beta_1^* X_1 + \beta_2^* X_2 + \beta_3^* X_3 + \beta_4^* X_4) = \\ & \beta_1^{*2} \text{var}(X_1) + \beta_2^{*2} \text{var}(X_2) + \beta_3^{*2} \text{var}(X_3) + \beta_4^{*2} \text{var}(X_4) + 2\beta_1^* \beta_2^* \text{cov}(X_1 X_2) + 2\beta_1^* \beta_3^* \text{cov}(X_1 X_3) \\ & + 2\beta_1^* \beta_4^* \text{cov}(X_1 X_4) + 2\beta_2^* \beta_3^* \text{cov}(X_2 X_3) + 2\beta_2^* \beta_4^* \text{cov}(X_2 X_4) + 2\beta_3^* \beta_4^* \text{cov}(X_3 X_4) \end{aligned} \quad (3)$$

The variance terms in this equation can be computed from the genotype frequencies. The β^* are the parameters of the random effects model (1) and they are related to the β of a standard logistic regression model without random effects (marginal model) by

$$\beta \approx (0.346\tau_r^2 + 1)^{-1/2} \beta^* \quad (4)$$

¹³. The β can be computed from the genotype frequencies in cases and controls and from the prevalence of RA.

Given β and τ^2 and using the two equations given by formula (2) and (4), we can compute τ^2 , β^* and hence $\text{var}(G)$. Note that $\text{var}(G)$ in formula (2) is a function of β^* as given by formula (3).

The contribution of SE alleles was defined as

$$\text{var}(G) = \text{var}(\beta_1^* X_1 + \beta_2^* X_2) = \beta_1^{*2} \text{var}(X_1) + \beta_2^{*2} \text{var}(X_2) + 2\beta_1^* \beta_2^* \text{cov}(X_1 X_2) \quad (5)$$

Analogously the contribution of DRB1*13 alleles was defined as

$$\text{var}(G) = \text{var}(\beta_3^* X_3 + \beta_4^* X_4) = \beta_3^{*2} \text{var}(X_3) + \beta_4^{*2} \text{var}(X_4) + 2\beta_3^* \beta_4^* \text{cov}(X_3 X_4) \quad (6)$$

The calculation of the genetic contribution of the separate SNPs was performed in a similar manner with the logistic regression formula (1) containing only 1 β and 1 X . Now X assumed the value 1 for genotypes containing 1 or 2 minor alleles.

All computations were performed in the freely available software environment for statistical computing R.

RESULTS

Contribution of HLA-DRB1*13 and *1301 to the genetic variance

The study cohort consisted of 64 monozygotic and 84 dizygotic twin pairs from the United Kingdom (UK). The heritability modeling using twin pairs with at least one affected requires that the prevalence for each genotype be fixed to the specific population prevalence, in this case from the UK. Genotype data from 2914 RA patients, (1470 ACPA-positive, 723 ACPA-negative RA patients and 721 RA patients without ACPA-status) and 1348 controls were used to calculate the HLA population prevalences listed in Table 1 (unpublished data received from Ann Morgan). Since four-digit HLA typing was not available for British controls, the effect of the HLA-DRB1*1301 alleles, which have been shown to be associated with the strongest protective effect, could not be assessed. The analysis was therefore

Table 1. HLA population prevalences from the United Kingdom used for modeling.

Prevalence of	Population			
	All RA patients	ACPA-positive	ACPA-negative	Controls
ACPA	67%	100%	0%	1%
HLA-DRB1				
SE/SE	26.7%	32.6%	12.7%	8.6%
SE/x	44.0%	46.5%	38.5%	34.1%
x/x	18.2%	12.5%	31.5%	42.0%
*13/x	5.2%	3.0%	10.0%	9.6%
*13/*13	0.5%	0.3%	1.0%	0.7%
SE/*13	5.3%	5.0%	6.4%	5.0%

performed for the HLA-DRB1*13 alleles, which in meta-analysis were also associated with a significant protective effect.

Before performing the heritability analysis, we first investigated whether the protective effect of HLA-DRB1*13 on the risk of ACPA-positive RA was also present in the UK, based on the prevalences shown in Table 1. In order to correct for skewing due to the association between ACPA-positive RA and the SE alleles, the analysis was stratified for the SE alleles as described previously⁸. There was no effect of DRB1*13 on the development of ACPA-positive RA in the SE-negative stratum (OR: 1.08, 95% CI: 0.74-1.58), while in the SE-positive stratum there was a trend towards a protective effect (OR: 0.72, 95% CI: 0.50-1.04). As expected, there was no effect of DRB1*13 on the risk of developing ACPA-negative RA (OR: 1.15, 95% CI: 0.90-1.47).

Although the effect of HLA-DRB1*13 in the UK was smaller than observed in a large European meta-analysis, this did not exclude a contribution of the HLA-DRB1*13 alleles to the genetic variance of RA. Using the genotype frequencies from the UK listed in Table 1, we estimated the contribution of HLA-DRB1*13 to the genetic variance, while also taking into account the effect of the SE alleles. As shown in Table 2, the contribution of the HLA-DRB1*13 alleles to the genetic variance of total, ACPA-positive and ACPA-negative RA was very limited.

Table 2. Contribution of HLA SE alleles and HLA-DRB1*13 alleles to the genetic variance.

	SE	DRB1*13	Combined SE and DRB1*13
Total RA	12.9%	0.1%	12.8%
ACPA-positive RA	19.2%	0.1%	19.6%
ACPA-negative RA	1.4%	0.4%	1.5%

The fact that the combined contribution of SE and DRB1*13 is not equal to the sum of their separate contributions can be explained by the existence of covariance between the various genotypes, as described by the covariance term in formulas (4) and (5) in the Methods section. The figures for the contribution of the SE alleles differed slightly from the previously reported estimates³, which is most likely due several factors: the statistical methods differed as described in the methods section, the current approach took both heterozygote and homozygote effects into account, used another reference category (no carriers of SE or DRB1*13 alleles) and was based on slightly different population prevalences.

The observation that the DRB1*13 alleles were not associated with a significant protective effect with regard to the risk of ACPA-positive RA in the UK, raised the question whether the contribution of the DRB1*13 alleles might have been larger in a population in which there was a strong effect of these alleles. To test this hypothesis, the contribution of DRB1*13 to the genetic variance of ACPA-positive RA was also assessed using population

prevalences from the Netherlands where there is a strong protective effect of DRB1*13. Genotype prevalences shown in Table 3 were extracted from the Leiden Early Arthritis Clinic (EAC) and the BeSt trial, which together consisted of 844 RA patients, (492 ACPA-positive and 352 ACPA-negative RA patients), as well as from a cohort of 1213 random healthy controls ⁸.

Table 3. Population prevalences from the Netherlands (ref. 8) used for modeling.

Prevalence of	Population			
	All RA patients	ACPA-positive	ACPA-negative	Controls
ACPA	58%	100%	0%	1%
HLA-DRB1				
SE/SE	18.7%	25.2%	7.6%	6.0%
SE/x	42.1%	46.8%	34.9%	30.8%
x/x	22.7%	14.9%	35.2%	36.2%
*13/x	8.6%	6.3%	12.3%	17.5%
*13/*13	0.6%	0%	1.5%	2.6%
SE/*13	7.4%	6.7%	8.5%	6.9%

This however did not result in a larger contribution of DRB1*13 to the genetic variance. As shown in Table 4, the DRB1*13 alleles contributed merely 1.46% to the genetic variance of ACPA-positive RA. The increase in the combined estimate of the contribution of SE and DRB1*13 over the two separate contributions is due to the positive covariance inherently present between a predisposing (SE) effect and protective (DRB1*13) effect. For ACPA-negative RA, the amount of explained genetic variance by the HLA alleles was very small and hardly increased by taking the effect of DRB1*13 into account. Since DRB1*1301 is the allele which is mainly responsible for the protective effect of DRB1*13, the analysis was repeated using the population prevalences of DRB1*1301 from the Netherlands. The resulting estimates were similar (data not shown), demonstrating that neither the DRB1*13 nor the DRB1*1301 alleles explain a large part of the genetic variance of RA.

Table 4. Contribution of HLA SE alleles and HLA-DRB1*13 alleles to the genetic variance assuming population prevalences from the Netherlands.

	SE	DRB1*13	Combined SE and DRB1*13
Total RA	8.9%	0.9%	11.1%
ACPA-positive RA	13.5%	1.5%	17.0%
ACPA-negative RA	0.5%	0.3%	0.9%

Contribution of SNPs to the genetic variance

Recent GWAS and meta-analyses of GWAS have revealed numerous SNPs to be associated with RA ⁷. For the current analysis, we assessed the effect of 12 SNPs which have been

well-replicated, and of which data on the prevalence in the UK in RA patients and controls was available. The prevalence figures in the population of the United Kingdom which were used for the logistic regression model are listed in Table 5. If the prevalence figures were not directly reported in the literature, they were determined based on the reported odds ratio's and patient numbers for the different RA subsets. For some of the SNPs (e.g. TRAF1-C5, STAT4, IL2RB), the prevalence in the total RA patient population was not completely in line with the prevalence in the ACPA-positive and ACPA-negative subgroups. This is most likely due to the fact that the patient population used to determine the prevalence in all RA patients was much larger than the groups of ACPA-positive and ACPA-negative RA patients combined, thereby causing small discrepancies in the SNP prevalences.

Table 5. Minor allele frequencies reported in studies from the United Kingdom used for modeling.

Gene region of SNP (UK reference)	Population			
	All RA patients	ACPA-positive	ACPA-negative	Controls
PTPN22 (T allele) ²³	15.0%	16.7%	12.1%	10.3%
TRAF1-C5 ²⁴	45.6%	45.5%	45.0%	43.8%
STAT4 ²⁴	24.2%	24.7%	25.0%	21.7%
TNFAIP3-OLIG3 ²⁵	25.1%	26.6%	23.2%	21.5%
CTLA4 ²⁶	43%	-	-	43.8%
CD40 ²⁷	22.6%	21.5%	24.5%	25.2%
MMEL1-TNFRSF14 ¹⁷	29.4%	-	-	33.2%
KIF5A ²⁸	34.2%	34.4%	33.9%	37.1%
PRKCQ ²⁸	17.1%	16.6%	17.7%	19.5%
CCL21 ²⁷	36.5%	37.0%	33.6%	34.8%
IL2RA ²⁹	24.4%	-	-	28.6%
IL2RB ²⁸	28.4%	29.2%	29.1%	26.1%

Table 6 shows the calculated contribution of the SNPs to the genetic variance. The contributions associated with the separate SNPs were small and, with the exception of PTPN22, were all well below 1%. If the effects of these SNPs were to be completely independent without interaction, then the addition of the contribution of the separate SNPs would give an impression of the total contribution of these SNPs. Assuming this to be the case, the 12 SNPs would together explain 3.4% of the genetic variance of total RA. Although this calculation is not possible for the ACPA-positive and ACPA-negative subset due to the lack of subset-specific prevalences for some of the SNPs, it appears unlikely, based on Table 3, that these estimates would exceed 5% of the explained genetic variance of total RA.

Table 6. Contribution of SNPs to the genetic variance.

Prevalence of	Population		
	All RA patients	ACPA-positive	ACPA-negative
PTPN22 (T allele)	1.1%	1.5%	0.2%
TRAF1-C5	0.1%	0.1%	0.0%
STAT4	0.2%	0.2%	0.3%
TNFAIP3-OLIG3	0.4%	0.7%	0.1%
CTLA4	0.0%	-	-
CD40	0.2%	0.4%	0.0%
MMEL1-TNFRSF14	0.4%	-	-
KIF5A	0.2%	0.1%	0.2%
PRKCQ	0.2%	0.3%	0.1%
CCL21	0.1%	0.1%	0.0%
IL2RA	0.5%	-	-
IL2RB	0.2%	0.2%	0.2%

DISCUSSION

In the present study, we investigated to what extent the effects of protective HLA alleles and non-HLA SNPs contribute to the, thus far largely unexplained, genetic variance of RA. The DRB1*13 and *1301 alleles had only a small contribution to ACPA-positive RA, and together with the HLA SE alleles explained 19.6% of the genetic variance of this subset of disease. Neither the HLA SE alleles nor the DRB1*13 alleles contributed substantially to the genetic variance of ACPA-negative RA, which was to be expected based on the fact that these alleles have not been found to confer a strong predisposing or protective effect for ACPA-negative RA⁸. The effects of 12 well-replicated SNPs on the genetic variance of RA were very modest. Most SNPs explained far less than 1% of the genetic variance, and assuming independent effects, all SNPs combined would explain less than 5%.

The HLA-DRB1*13 alleles were not significantly associated with protection from ACPA-positive RA in the UK, in contrast to the findings of a recent meta-analysis of case-control cohorts from Norway, Sweden, the Netherlands and Spain. While the allele frequency of DRB1*13 was similar in RA patients in the UK and the four populations contributing to the meta-analysis, the prevalence of these alleles in the UK controls used in the current report was 8%, compared to at least 14% in the controls from the other cohorts. The lack of effect in the UK may thus be due to a difference in population structure and distribution of HLA alleles in the general British population.

In light of the non-significant effect of the HLA-DRB1*13 alleles in the UK, it was not surprising that this did not result in a large contribution of DRB1*13 to the genetic vari-

ance. To investigate whether the DRB1*13 alleles might explain a larger part of the genetic variance of RA if they were associated with a stronger protective effect, the analysis was repeated in a hypothetical fashion assuming the Dutch prevalences for DRB1*13. Unexpectedly, this also did not result in a marked contribution of the HLA-DRB1*13 alleles to the genetic variance of ACPA-positive RA.

The most likely explanation for the limited contribution of HLA-DRB1*13 is the low prevalence of these alleles. Despite the fact that the HLA-DRB1*13 alleles are more prevalent in the Netherlands than in the UK, an allele frequency of 14.8% in the healthy controls used in this study still places a considerable limit on the maximally achievable effect. The explanatory value of a genetic risk factor is ultimately dependent on both the associated effect size and the prevalence in the general population. In case of the other type of genetic risk factor investigated in the current study: the SNPs, the contribution to the genetic variance is most likely constrained by the modest effect sizes with which they are associated.

It is remarkable that there is a historic estimate of 37% contribution of HLA genes to the overall inherited risk of developing RA, as described by Deighton et al ¹⁴. Due to the haplotype sharing approach which was used in the study by Deighton, protective HLA effects were also taken into account and the resulting 37% reflects far more than the contribution of solely the SE alleles. A possible reason for the fact that this estimate is much larger than our figure could be that, besides the effect of SE and DRB1*1301 alleles, other effects may remain within the MHC region ^{15, 16}, which were, perhaps unconsciously, also taken into account by the haplotype sharing approach.

The observation that 12 well-replicated SNPs had a relatively minor contribution to the genetic variance is in line with the results of a previous study which estimated that all non-MHC genetic variants (known at the end of 2008) would explain just 3.6% of the total disease variance ¹⁷. These results justify the conclusion that SNPs associated with moderate odds ratio's probably have a very limited contribution to the genetic variance.

In order to calculate the contribution of the 12 SNPs to the total genetic variance, we used the genotypic specific population prevalences reported in the largest number of total RA, ACPA-positive and ACPA-negative patients. This resulted in slightly deviant estimates for STAT4 and IL2RB, since the reported prevalences for ACPA-positive and ACPA-negative RA patients were higher than for the total RA population. This inconsistency can be resolved as soon as data on larger cohorts become available, but the contribution of these SNPs to the genetic variance will nonetheless remain small.

The results of the current study improve our understanding of the relative importance of distinct genetic factors in developing RA. However, most of the genetic variance remains unexplained, and RA therefore still suffers from "missing heritability", as do many other diseases ¹⁸. Where should one look for this missing heritability? It appears unlikely that other common variants (SNPs) with increasingly smaller odds ratio's will have a large con-

tribution, although one cannot exclude that the true causal genetic variants, which may be incompletely tagged by the current SNPs, may have a much stronger effect. Another possibility is that rare variants, with a low minor allele frequency precluding identification by the GWAS, could play an important role^{19,20}. Such rare variants could very well have considerable odds ratio's (> 2), and if in addition some of these variants were found to be inherited in a Mendelian fashion, a small number of families could thereby explain a large part of the genetic component of RA²¹.

Structural genomic variation, such as copy number variants (CNV), may contribute to the heritability, although recent data indicate that the SNP genotyping arrays used for the GWAS have captured most of the variability due to this trait²². Another possibility is that interaction between different genetic factors may play an important role, although the latest reports indicate that there is no gene-gene interaction between the known genetic risk factors and the most recently identified SNPs¹⁷. Similarly, gene-environment interaction could be a contributing factor to the heritability, although it is unclear to what extent this would be reflected in the current heritability estimates. All of the factors listed above may play a part in determining an individual's risk for RA. Future studies including large numbers of patients and controls, and using increasingly advanced techniques to study genetic variation will be required to shed more light on the causes of missing heritability.

In conclusion, the current data show that the HLA-DRB1*13 alleles do not substantially contribute to the genetic variance of this RA subset. Similarly, the effect of 12 well-replicated SNPs on the genetic variance of RA is small. Despite a substantial contribution of HLA, the largest part of the genetic risk for both ACPA-positive and ACPA-negative RA remains unexplained.

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Chapter 5

Is alcohol consumption truly protective for developing rheumatoid arthritis?

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ABSTRACT

Objective To evaluate the association between alcohol consumption and the risk of developing rheumatoid arthritis (RA) and other forms of arthritis.

Methods Included were patients participating in the Leiden Early Arthritis Cohort with RA (n=651), other forms of arthritis (reactive arthritis, spondylarthropathy or psoriatic arthritis, n=273), osteoarthritis (OA, n=73), as well as 5877 general population controls. Odds ratios were calculated by logistic regression analysis. Subgroup analysis was performed, stratified for antibodies to citrullinated peptide antigens (ACPA) status (n=257) compared to patients with other forms of arthritis (n=273). Subset analyses were performed on remission data and radiographic progression.

Results Alcohol consumption was associated with a reduced risk of RA (OR 0.27 (95%CI 0.22-0.34)), of other forms of arthritis (OR 0.34 (95%CI 0.24-0.48)) and OA (OR 0.31 (95%CI 0.16-0.62)). The degree of systemic inflammation, reflected by the level of erythrocyte sedimentation rate (ESR), was inversely associated with alcohol consumption. There was no dose-response relationship between the amount of alcohol consumed and the risk of arthritis. ACPA-positive RA patients reported less alcohol consumption than patients with other forms of arthritis (OR 0.59 (95%CI 0.36-0.99)). Adjustment for the ESR-level slightly changed this risk (OR 0.63 (95%CI 0.38-1.07)). No association between alcohol consumption and rate of joint destruction or remission was seen.

Conclusion Arthritis patients report less alcohol consumption than controls, regardless of the type of arthritis. The largest part of this association is not specific for RA, but subgroup analysis shows that there may be a particularly protective effect of alcohol consumption towards ACPA-positive RA.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, inflammatory disease which is characterized by persistent inflammation and joint destruction. It has previously been shown that environmental risk factors such as smoking may interact with genetic factors in predisposing to the development of RA characterized by the presence of antibodies to citrullinated peptide antigens ¹⁻⁵. Smoking habits and drinking habits are associated. It is of interest to evaluate the possible effect of alcohol consumption on the risk of developing RA.

Several reports have suggested a compromising effect of ethanol on both innate and adaptive immunity ^{6,7} with downregulation of proinflammatory cytokines and increase of anti-inflammatory cytokines ⁸⁻¹¹. Moreover, experimental studies with collagen-induced arthritis in mice have shown an anti-inflammatory and anti-destructive effect of ethanol consumption by interaction with innate immune responsiveness ¹².

The association between alcohol consumption and RA has been evaluated in several previous reports ¹³⁻¹⁷, but no definite conclusions could be drawn. Recently, a report comprising Swedish and Danish data on alcohol consumption in reference to development of RA indicated that there may be a dose-dependent protective effect of alcohol ¹⁸. Our objective was to evaluate whether there is an inverse association between alcohol consumption and the risk of developing arthritis, and more specifically in relationship to the presence of RA with antibodies to citrullinated peptide antigens (ACPA). By inclusion of several arthritic case groups, we investigated whether the effect of alcohol is specific for RA. The latter has never been evaluated in previous studies.

METHODS

Patient and control population

We performed a case-control analysis in which cases were participants in the early arthritis cohort (EAC) from the Leiden University Medical Center, the Netherlands. This cohort consists of patients presenting with arthritis to the outpatient clinic, who were subsequently diagnosed with various different forms of arthritis. Cases that were included in the analyses were recruited between February 1993 and December 2008 and were older than 18 years of age. Patients were diagnosed with RA according to the 1987 criteria of the American College of Rheumatology ¹⁹. Patients with other forms of arthritis were selected from the EAC cohort based on a definite diagnosis of reactive arthritis, spondylarthropathy, psoriatic arthritis or osteoarthritis (OA).

A total of 5877 non-arthritic controls were selected from the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis study (MEGA trial). This group consisted of patients with a first diagnosis of venous thrombosis. Between March

1999 and September 2004 patients were selected from files of the anticoagulation clinics in Amsterdam, Amersfoort, the Hague, Leiden, Rotterdam and Utrecht. This group has been described in detail elsewhere ²⁰. A group of 3297 partners of patients with venous thrombosis were included in our study. From January 2002 to September 2004 an additional number of 3000 random control subjects were recruited in the MEGA study and used in our study. Only control subjects with no recent history of venous thrombosis were included. Patients with severe psychiatric problems or those unable to speak Dutch were considered ineligible. All control subjects selected were older than 18 years of age. Both cohorts were approved by the medical ethical committee.

Data collection

Alcohol consumption was recorded at baseline, at first presentation to the outpatient clinic. In the EAC cohort, information on alcohol and smoking was initially obtained by means of an interview by a trained research nurse when patients first presented to the outpatient clinic. After December 2002, data were collected by self-administered questionnaires. A modified Stanford Health Assessment Questionnaire (HAQ) was filled in and the disease activity score in 44 joints was assessed (DAS44) ²¹. The control subjects in the MEGA trial were sent questionnaires to fill in at home. Cases and controls with missing data on alcohol consumption were excluded in our analyses.

The questions asked about alcohol- and smoking habits in both cases and controls were comparable. Data on alcohol consumption were dichotomized as current or non-drinker. Additionally, the amount of alcohol consumption was categorized into four groups, based on the quartiles of percentages in the control population: non-drinkers; low consumption; moderate consumption and high consumption. The data on smoking were divided in current/previous or non-smokers.

Statistical analysis

Statistical analysis was performed using SPSS version 16.0 (SPSS Inc, Chicago, Ill). To compare the distribution of baseline characteristics in the groups, Kruskal-Wallis-, Chi-square- and t-tests were performed. The effect of alcohol consumption on the risk of developing disease was analyzed by univariate and multivariate logistic regression. Relative risks are expressed as odds ratios with 95% confidence intervals based on the standard errors derived from the model. The latter analysis was adjusted for age, sex and body mass index (BMI) and smoking since these were possible confounders. Separate analyses were executed based on diagnosis, ACPA-status and the type of healthy control (partners vs random controls). Pearson's chi-square test was used for analyzing the association between alcohol consumption and the level of erythrocyte sedimentation rate (ESR). P-values less than 0.05 were considered significant for all statistical tests. Subset analyses were performed on remission data and radiographic progression. Sustained

DMARD-free remission was defined as fulfilling the following criteria for at least one year: 1) no current DMARD-use, 2) no swollen joints and 3) classification as remission by the patient's rheumatologist²². For remission data a Cox-regression analysis was performed. Data on radiographic progression with a follow-up duration of 7 years, were evaluated using a repeated measurement (linear multivariate regression) model^{23, 24} corrected for smoking, ACPA-status, sex, age, HAQ, DAS44, delay (time between onset of symptoms and first visit to the outpatient clinic) and baseline Sharp-van der Heijde score.

RESULTS

Alcohol consumption and risk of RA and other forms of arthritis

A total of 651 RA patients, 346 patients with other forms of arthritis (273 patients with reactive arthritis, spondylarthropathy or psoriatic arthritis, 73 patients with osteoarthritis (OA)) and 5877 healthy controls were included. The baseline characteristics are shown in Table 1. Patients with RA or OA were older and more often female than patients with other forms of arthritis and the healthy controls. The BMI differed between the group of

Table 1. Baseline characteristics of patients and controls.

Characteristic	RA, n=651	OA, n=73	Other arthritis, n=273 *	Controls, n=5877	P-value (Kruskal- Wallis)
Age (in yrs), mean (SD)	56.5 (16)	64.0 (12)	44.4 (15)	47.3 (13)	<0.001
Female gender, n (%)	435 (67)	56 (77)	123 (45)	3158 (54)	<0.001
BMI (in kg/m ²), mean (SD)	25.9 (4)	26.4 (4)	25.7 (4)	25.6 (4)	0.018
Symptom duration (in months), mean (SD)	6.8 (10)	9.1 (17)	5.8 (11)	----	<0.001
ESR (in mm/h), mean (SD)	39 (26)	22 (21)	34 (31)	----	<0.001
ACPA-positive, n (%)	257 (54%)	3 (7%) #	15 (10%) #	----	<0.001

* Spondylarthropathy, psoriatic arthritis, reactive arthritis: P-values are listed for the comparison between two cohorts, using t-tests for normally distributed continuous variables and Chi-square tests for dichotomous variables.

Age of RA patients vs healthy controls: p-value <0.001

Age of osteoarthritis patients vs healthy controls: p-value <0.001

Age of other arthritis patients vs healthy controls: p-value <0.001

Sex of RA patients vs healthy controls: p-value <0.001

Sex of osteoarthritis patients vs healthy controls: p-value <0.001

Sex of other arthritis patients vs healthy controls: p-value 0.005

BMI of RA patients vs healthy controls: p-value 0.058

BMI of osteoarthritis patients vs healthy controls: p-value 0.230

BMI of other arthritis patients vs healthy controls: p-value 0.650

ACPA was measured in 43 of 73 osteoarthritis patients and 157 of the 273 "other arthritis" patients.

Abbreviations: RA: rheumatoid arthritis, OA: osteoarthritis, BMI: body mass index.

OA patients and the group of healthy subjects, therefore these possible confounders were included in subsequent multivariate analyses.

Table 2 lists the number of patients and controls that reported drinking alcohol. Odds ratios (OR) were adjusted for age, sex, BMI and smoking due to the known association between smoking and drinking habits. This analysis shows a substantially reduced risk of all forms of arthritis associated with alcohol consumption. A subanalysis performed within in the group of cases included after December 2002 (when patients were given self-administered questionnaires), showed that the cited amount of alcohol consumption was not influenced by how the data on alcohol consumption were collected.

Table 2. Number of individuals drinking alcohol by diagnosis versus healthy controls

Diagnosis	Cases, n (%)	Healthy controls n = 5877, n (%)	OR *	95% CI *	p
RA, n=651	362 (56)	4901 (83)	0.27	0.22-0.34	<0.001
Total other arthritis n=273	175 (64)	4901 (83)	0.34	0.24-0.48	<0.001
• SpA, n= 76	44 (58)	4901 (83)	0.34	0.17-0.67	0.002
• Arthr.Ps., n=130	89 (69)	4901 (83)	0.38	0.23-0.62	<0.001
• React. Arthr, n=67	42 (63)	4901 (83)	0.27	0.14-0.52	<0.001
Osteoarthritis, n=73	39 (53)	4901 (83)	0.31	0.16-0.62	0.001

* 95% Confidence interval adjusted for age, sex, BMI, smoking

RA= rheumatoid arthritis, SpA= spondylarthropathy, Arthr. Ps.= psoriatic arthritis, React. Arthr.= reactive arthritis, BMI= body mass index, OR= odds ratio.

Dose-response relationship

When alcohol consumption was stratified into different categories (non-drinkers; low consumption (more than 0 but less than 2 alcoholic beverages per week); moderate consumption (2-14 alcoholic beverages per week); high consumption (more than 14 alcoholic beverages per week)) no dose-response relationship in the odds ratios related to the amount of alcohol consumption was found. For rheumatoid arthritis, odds ratios by amount of alcohol consumption relative to non-drinkers, were: low consumption: OR 0.12 (95%CI 0.08-0.18), moderate OR 0.46 (95%CI 0.36-0.59), and high OR 0.17 (95%CI 0.12-0.25). For the other forms of arthritis the analyses yielded similar results (data not shown).

Effect of symptom duration on alcohol consumption

To investigate if a long symptom duration of arthritis prior to presentation might result in decreased alcohol consumption, we evaluated whether there was an association between symptom duration and alcohol consumption. To this end, the symptom duration at first presentation was divided into three categories based on cumulative tertiles (short (0-2.0 months), medium (2.1-6.0 months), and long (>6.0 months)). For this analysis, patients with all different forms of arthritis were combined. There was no association between

symptom duration and the percentage of arthritis patients that reported drinking alcohol (short 62%, medium 53%, and long 60%)

Systemic inflammation and alcohol consumption

To examine if the inverse association between alcohol consumption and arthritis was related to the degree of inflammation, the presence of alcohol consumption was related to the level of systemic inflammation, as reflected by the level of the ESR (within the group of cases), and ESR was stratified into four groups. The level of the ESR was inversely correlated with alcohol consumption ($p = <0.001$) (Figure 1).

We also evaluated whether there was an association between a subjective marker for disease activity, in the form of the visual analogue scale on general health and the reporting of drinking alcohol. In contrast to our findings with regard to ESR, there was no association between the level of general health and reporting of drinking alcohol.

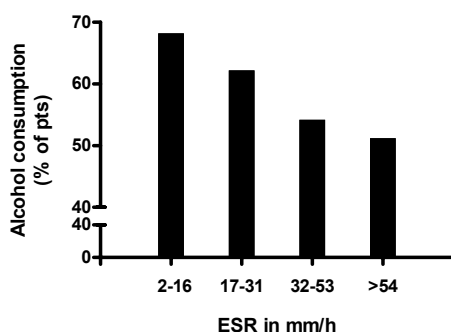


Figure 1: Alcohol consumption by ESR category.

Pearson test over all four categories <0.001

Comparison of ESR 2-16 vs ESR 17-31: p -value 0.090

ESR 2-16 vs ESR 32-53: p -value <0.001

ESR 2-16 vs ESR >54 : p -value <0.001

Reported alcohol consumption in ACPA-positive RA patients compared to other forms of arthritis

A recent population based case-control study of two Scandinavian cohorts showed that alcohol consumption is associated with attenuation of the effect of established risk factors for RA; smoking and HLA-DRB1 shared epitope with regard to ACPA-positive RA¹⁸.

To investigate whether reported alcohol consumption may be particularly negatively associated with the development of RA compared to other arthritides, a subgroup analysis was performed comparing the RA patients stratified for ACPA-status, with the patients with other forms of arthritis ($n=273$). This revealed a particularly negative association in

ACPA-positive RA patients ($n=257$) (OR 0.59, 95% CI: 0.36-0.99), compared to other forms of arthritis. Correction for the ESR-level slightly changed this risk (OR 0.63, 95% CI: 0.38-1.07). No differences were observed between alcohol consumption and the risk of ACPA-negative RA relative to the other forms of arthritis (data not shown).

Effect of alcohol consumption on radiographic progression and remission

To further investigate the negative association between the reported alcohol consumption and RA, we analyzed the effect of alcohol consumption on remission and radiographic progression. An increase in remission or decrease in radiographic progression would support the notion that alcohol might have an immunomodulatory effect in the process of inflammatory arthritis.

To compare the progression in radiographic destruction over 7 years, the rate of progression was plotted within the four alcohol consumption categories (none, low/little, moderate, high/substantial) (Figure 2). No significant association between alcohol consumption and rate of joint destruction over 7 years follow-up was found.

With regard to remission, there was no association between alcohol consumption and the frequency with which RA patients achieved remission (data not shown).

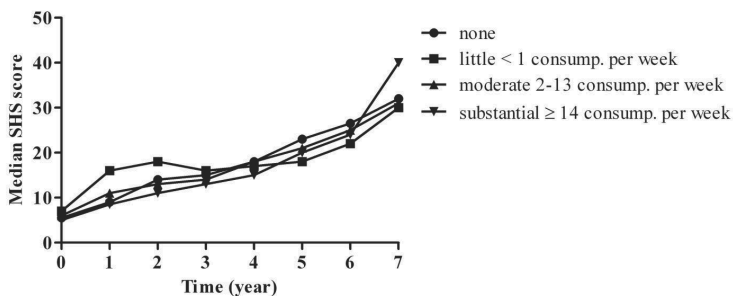


Figure 2: Median joint destruction over 7 years in RA patients in four alcohol categories.

DISCUSSION

This study shows that patients presenting with arthritis report less alcohol consumption than population controls, regardless of the type of arthritis. This may indicate a protective effect of alcohol on the development of arthritis, which is not specific for RA. Alternatively, it may be that patients stop drinking due to the presence of disease.

There was no dose-response relationship with the amount of alcohol consumed. Other studies reported varying dose-response relationships within categories with low or moder-

ate^{25, 26} respectively high¹⁸ alcohol consumption. Thus it is at present difficult to draw any definitive conclusions about a possible dose-effect of alcohol consumption on the risk of RA.

The inverse association between the ESR level and alcohol consumption shows that patients with more severe systemic inflammation report drinking less alcohol, or alternatively, that alcohol may protect against the development of more systemic inflammation. This last hypothesis is supported by reports that have shown a suppressive effect of alcohol on the level of systemic inflammation^{27, 28}. Reports on alcohol use in other diseases with an inflammatory background have also shown a protective effect of alcohol consumption, which supports a more general role of alcohol in suppressing systemic inflammation^{29, 30}. Thus, the association between alcohol consumption and arthritis might be due to a more general effect of alcohol on systemic inflammation rather than a specific protective effect in RA. It could be postulated that patients suffering from any illness tend to drink less due to compromised general health. We evaluated the visual analogue scale on general health, as a subjective marker for disease activity, in relationship to alcohol consumption. There was no correlation between the level of general health and reporting of drinking alcohol.

In the subgroup analysis investigating the association of ACPA with alcohol consumption, there was an inverse association, suggesting that alcohol consumption may be particularly negatively associated with ACPA-positive RA. In order to further analyze the effect of alcohol consumption specifically for ACPA-positive RA, we performed a combined analysis of our data and of previously published data from Sweden (the EIRA cohort) and Denmark (the COCORA cohort) comparing ACPA-positive to ACPA-negative patients. This small "meta"-analysis yielded an OR 0.31 (95% CI 0.23-0.40), with a consistent protective effect seen in all cohorts. The analysis of all available data showed that the effect of alcohol was consistent in both the Dutch, the Swedish and the Danish cohort when controls were used that suffered from arthritis but that do not harbour antibodies to citrullinated antigens. These data are very relevant because they provide evidence that in addition to smoking, alcohol consumption is a risk factor that differs between ACPA-positive and ACPA-negative RA. These data support the concept that ACPA-positive disease is a different disease than ACPA-negative disease³¹.

Källberg et al showed a more pronounced risk reduction with alcohol consumption among carriers of HLA-DRB1 shared epitope alleles than among non-carriers¹⁸. In our study no association was found between the effect of alcohol consumption and the presence of the shared epitope within the ACPA-positive RA patients.

If there was to be a real immunomodulatory effect of alcohol this would have effect on long-term phenotype such as rate of joint destruction or ability to achieve remission. Nissen et al²⁶ previously showed a J-shape relation between alcohol consumption and radiological progression; low alcohol consumption was most beneficial compared to no- and heavy consumption. We repeated this analysis (with similar adjustment variables) in our study, but did not find a significant association between alcohol consumption and rate

of joint destruction over 7 years of follow up. Although this could be due to lack of power in our analyses, no tendency toward reduced radiographic progression was observed. In our study merely baseline data on alcohol consumption were used in analyses addressing this topic, due to the strict advice to stop drinking alcohol in combination with the use of some DMARDs, such as methotrexate, thereby causing bias. In the study performed by Nissen et al both baseline and follow-up data on alcohol consumption were used in the analyses and subsequently have influenced the data on radiographic progression.

It is unknown how long and in what way the immunomodulatory effect of alcohol will affect disease manifestation and the long-term disease course. Jonsson et al ¹² showed that collagen induced arthritis remained non-destructive in mice that were provided with 10% ethanol in drinking water over a period of 6 weeks. The beneficial effects of ethanol might be mediated by up-regulation of testosterone production which in turn inhibits NF- κ B activation leading to decreased cytokine/chemokine production (IL-6, MIP-1 α , TNF- α) and decreased chemotactic activity of leukocytes.

In contrast to the inhibitory effects of acute alcohol, prolonged alcohol treatment in mice results in augmentation of macrophage TNF- α production ³², and in increased expression of co-stimulatory molecules CD80 and CD86 on TLR9-activated CD11b-positive splenocytes thereby contributing to systemic immunodysregulation including T-cell activation ^{33, 34}. Overall therefore, it is difficult to determine the immunomodulatory role of alcohol in the course of arthritis, based on the different outcomes of in vivo experiments in mice.

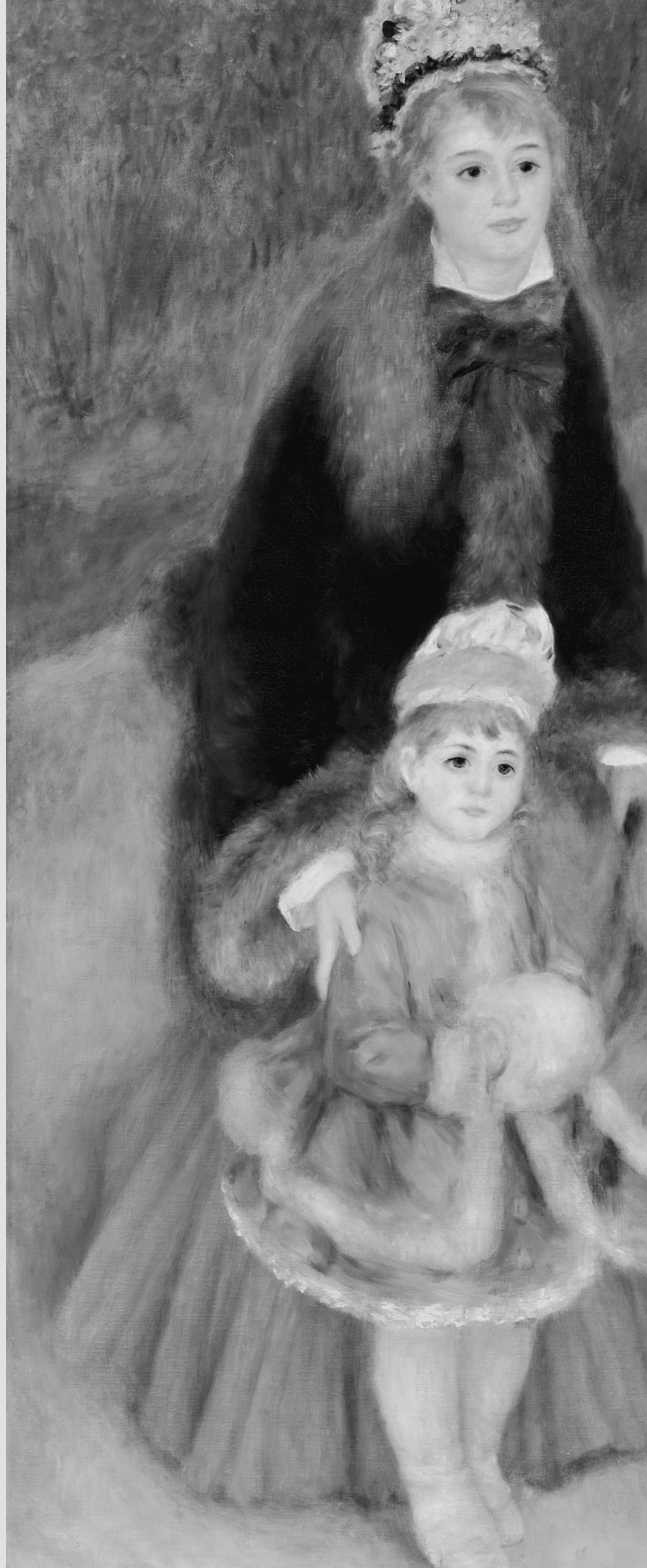
One of the main comments on our study could be that the effect seen is being caused by recall bias. This means that cases might not actually have been drinking less alcohol but reported to be drinking less when faced with a questionnaire in the outpatient clinic, while the drinking behaviour was in fact unchanged. The questions asked in cases and general population controls were similar therefore minimising a major effect of recall bias ³⁵. The assumption that drinking behaviour may be influenced by the disease duration was not confirmed by our data.

In summary, this study suggests that patients presenting with arthritis report less alcohol consumption, regardless of the form of arthritis. This observation could be due to the fact that compromised general health leads to decreased alcohol consumption. The inverse association between alcohol and ESR might be related to the effect of alcohol on systemic inflammation rather than a protective effect specific for RA although our data also suggest that alcohol consumption may be particularly protective against ACPA-positive RA.

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Part II

Characteristics of ACPA



Chapter 6

Glycan profiling of anti-citrullinated protein antibodies isolated from human serum and synovial fluid

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ABSTRACT

Objective Anti-citrullinated protein antibodies (ACPA) exhibit unique specificity for RA. Whether and how ACPA contribute to disease pathogenesis, however, is incompletely understood. The Fc part of human IgG carries two N-linked glycan moieties which are crucial for the structural stability of the antibody and modulate its binding affinity to Fc γ receptors and its ability to activate complement. We have purified ACPA from serum and synovial fluid and analyzed Fc glycosylation profiles in a specific manner.

Methods ACPA were isolated by affinity purification using cyclic citrullinated peptides as antigen. IgG₁ Fc glycosylation was analyzed by mass spectrometry. ACPA glycan profiles were compared to glycan profiles of total serum IgG₁ obtained from 85 well-characterised patients. Glycan profiles of paired synovial fluid and serum samples were available from 11 additional patients.

Results Compared to the pool of serum IgG₁, ACPA IgG₁ lack terminal sialic acid residues. In synovial fluid, ACPA are highly agalactosylated and lack sialic acid residues, a feature that was not detected for total IgG₁ in synovial fluid. Moreover, differential ACPA glycan profiles were detected in RF-positive versus -negative patients.

Conclusion ACPA IgG₁ exhibit a specific Fc-linked glycan profile which is distinct from total serum IgG₁. Moreover, Fc glycosylation of ACPA differs markedly between synovial fluid and serum. As Fc glycosylation directly affects the recruitment of Fc-mediated effector mechanisms, these data could further our understanding of the contribution of ACPA to disease pathogenesis.

INTRODUCTION

Antibodies relevant to tissue pathology in autoimmune diseases are identified based on antigen binding specificity of the variable region. Only very few autoantibodies, however, mediate pathology by direct interaction with the antigen. In most other cases, the constant region (Fc part) determines antibody-mediated effector functions such as complement activation, antibody-dependent cellular cytotoxicity (ADCC) and engagement of activating or inhibitory Fc receptors. These Fc-mediated effects are influenced by the Fc part's amino acid sequence (i.e. antibody isotype and sub-class) and by Fc-linked carbohydrate structures. The latter are located in the C γ 2 domain of the heavy chain in close vicinity to amino acids that interact with Fc receptors and the complement system. Accordingly, Fc-linked carbohydrate structures have recently received increasing attention, as modification of Fc-linked glycan residues of therapeutic antibodies has been shown to strongly influence the antibodies' therapeutic potential¹⁻⁶.

The Fc part of human immunoglobulin G (IgG) carries two N-linked, highly heterogeneous glycan moieties interposed between the heavy chains. These carbohydrate structures are crucial for the stability of the antibody and influence its biological activity⁷⁻⁹. Attached to amino acid Asn297 of each heavy chain is a conserved, biantennary glycan backbone of N-acetylglucosamine (GlcNAc) and mannose residues, which is modified by core fucose, additional N-acetylglucosamine, galactose and terminating sialic acid residues to yield a large variety of glycoforms (Figure 1). The most prevalent glycoforms are characterized by

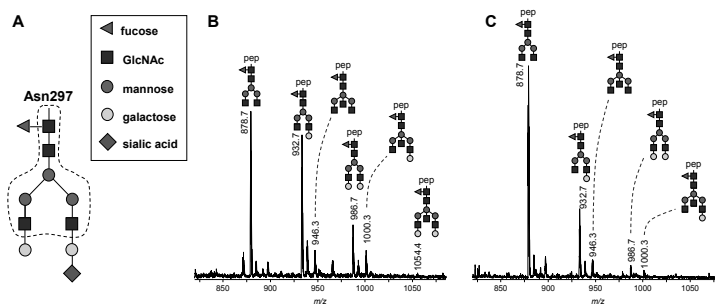


Figure 1A: Schematic depiction of a monosialylated glycan chain linked to Asn297 of the IgG heavy chain and legend for symbols used in this manuscript.

The dotted line indicates the conserved sequence of glycan residues. An additional sialic acid residue can be attached to the second galactose residue, and an additional N-acetylglucosamine residue ('bisecting GlcNAc') to the central mannose (not depicted).

Figure 1B, 1C: Typical mass-spectrometry profiles of IgG₁ tryptic glycopeptides obtained after isolation of ACPA from serum (B) and synovial fluid (C). Sum mass spectra of the elution range of the IgG₁ glycopeptides with neutral glycans are shown³⁴. pep = peptide moiety; m/z = mass/charge.

presence or absence of galactoses, so called G0 (no galactose), G1 (one galactose) and G2 (two galactoses) glycoforms, which make up around 20-35%, 35% and 16% of all IgG Fc-linked glycoforms in healthy subjects, respectively ⁷. Lack of galactose residues results in a concomitant lack of terminal sialic acid residues and increases the affinity for activating Fcγ receptors (FcγR) ^{10, 11}. In contrast, presence of the fully processed glycan structure terminating in sialic acid residues has anti-inflammatory effects ^{1, 3}. Presence of an additional, bisecting GlcNAc residue and absence of core fucose residues was found to enhance ADCC through high-avidity interaction with FcγRIIIa ^{5, 12-14}. Hydrolysis of the entire glycan backbone by glycosidase treatment or mutational deletion of the entire Asn297 N-linked glycosylation site leads to a change of the Fc part's quaternary structure. This yields a non-immunogenic antibody unable to interact with FcγR and, consequently, to elicit significant cytokine release ^{2, 6, 8}. Of interest, immune complexes containing such aglycosylated IgG fail to be eliminated rapidly from the circulation ¹⁵.

In line with these structural observations, both human and murine studies indicate a strong impact of Fc-linked glycan variants on the pathogenic potential of IgG antibodies in inflammatory disorders such as rheumatoid arthritis. Early studies have demonstrated a predominance of IgG-G0 glycoforms in sera of RA patients that correlates with disease activity and reverses to normal levels in patients who undergo (spontaneous) remission ¹⁶⁻¹⁹. Removal of galactose residues from murine IgG by β-galactosidase treatment resulted in increased arthritogenicity of transferred IgG in a murine arthritis serum transfer model ²⁰. Likewise, pregnancy-induced remission in the pristane-induced arthritis model was paralleled by a decrease of IgG-G0 glycoforms, while the postpartum period was accompanied by a respective increase in agalactosylated glycoforms ²¹. While these studies have narrowed the focus on the presence or absence of galactose residues, more recent studies indicate that not a lack of galactose residues itself, but rather the concomitant absence of terminal sialic acid residues may be responsible for the enhanced inflammatory activity exerted by G0 glycoforms ^{1, 3}. Indeed, a receptor specifically recognizing IgG Fc-linked sialic acid residues has newly been described on a population of murine regulatory splenic macrophages, a cellular subset that may be involved in downregulation of inflammatory responses ²². While these latter findings await confirmation in the human system, relevance of sialic acid residues for modulating immune responses is demonstrated by the finding that intravenous immunoglobulin preparations (IVIg) completely lose their immunosuppressive capacity upon removal of sialic acid residues by neuraminidase treatment ^{1, 3}. Together, these findings underline the importance of IgG Fc-linked glycan structures for antibody-mediated inflammatory responses.

Anti-citrullinated protein antibodies (ACPA) are highly specific autoantibodies for a subgroup of rheumatoid arthritis patients that suffer from severe erosive disease. A body of evidence points to a crucial role for ACPA in disease pathogenesis, but the pathogenic potential of ACPA and the mechanism by which ACPA could cause tissue pathology are

ill-defined. ACPA are present in serum years before the onset of clinical symptoms, ACPA production has been detected in synovial membrane explants, and increased ACPA levels were found in synovial fluid as compared to serum²³⁻²⁵. ACPA-positive RA patients suffer from more severe disease than patients without a citrulline-specific immune response, and presence of ACPA favours the development of overt RA in patients with undifferentiated arthritis²⁶⁻²⁹. Intriguingly, however, ACPA levels do not or only moderately correlate with disease activity, and depletion of CD20⁺ B cells improves clinical symptoms while ACPA levels are only moderately affected^{30, 31}. The observation that ACPA can be present without signs of inflammation, both before and during disease, raises the possibility that the quality rather than the quantity of the ACPA immune response influences disease. So far, studies have focussed on ACPA fine specificity and isotype usage, but data on ACPA Fc-linked glycan residues have not been obtained. In light of the strong influence of Fc-linked glycans on the inflammatory potential of antibodies, we have recently developed a technique that allows analysis of Fc-linked glycans in an antigen specific manner³². We have now used this technique to analyze Fc glycosylation of IgG₁-ACPA in serum and synovial fluid of early arthritis patients in order to further characterize the immune response to citrullinated antigens.

PATIENTS AND METHODS

Serum and synovial fluid samples

Serum samples of 85 clinically well-defined, ACPA-positive arthritis patients participating in the Leiden Early Arthritis Clinic (EAC) were collected following informed consent of study participants and study-approval by the local institutional review board. The Leiden EAC is a population-based inception cohort that includes patients with self-reported symptom duration of ≤ 2 years³³.

Paired serum and synovial fluid samples were collected from 11 additional ACPA-positive rheumatoid arthritis patients. 7 samples originated from the outpatient clinic of the Department of Rheumatology and Clinical Immunology at Charité Hospital in Berlin (Germany), 4 samples were collected at the respective outpatient clinic of the Department of Rheumatology of Leiden University Medical Center (The Netherlands).

ACPA positivity was determined based on reactivity of sera against a second generation cyclic citrullinated peptide (CCP2) in a commercially available ELISA system (Immunoscan RA Mark 2; Euro-Diagnostica, Arnhem, The Netherlands). Serum and synovial fluid samples were aliquoted and stored at -80°C until further use.

Isolation of ACPA and total IgG from serum and synovial fluid samples

ACPA were isolated from total human serum and synovial fluid as previously described³². In brief, sera and synovial fluid samples were incubated in CCP2-coated ELISA plates (Immunoscan RA Mark 2; Euro-Diagnostica, Arnhem, The Netherlands) for 1 hour at 37°C. Supernatants were discarded and plates were washed thoroughly. Bound antibodies were eluted from the plates by adding 100mM formic acid (pro analysi for mass spectroscopy; Merck Darmstadt, Germany) for 15 minutes at room temperature. Eluates were collected in 96-well V-bottom plates, dried in a vacuum centrifuge and subjected to tryptic digest by adding 200ng trypsin (sequencing grade; Promega, Leiden, The Netherlands) in 40µl ammonium bicarbonate to each well followed by incubation at 37°C overnight. Digested samples were stored at -20°C until further use. Purity of eluted ACPA was verified by subjecting ACPA-negative control sera to the same isolation procedure. Non-trypsinized eluates of ACPA-negative samples were tested for the presence of human IgG by ELISA. None of the ACPA-negative eluates contained detectable amounts of IgG, indicating that only citrulline-specific IgG-molecules were eluted from the CCP2-plates (data not shown; for methodological details see³²).

Purification of total IgG was achieved by incubating serum or synovial fluid samples with Protein A-sepharose beads (GE Healthcare, Eindhoven, The Netherlands) in 96-well filter plates (Multiscreen Solvinert, 0.45 µm pore-size low-binding hydrophilic PTFE; Millipore, Billerica, MA) on a shaker for 1 hour. Beads were thoroughly washed and bound IgG-molecules (IgG₁, IgG₂ and IgG₄) were eluted into a 96-well V-bottom plate using 100mM formic acid. Samples were dried by vacuum centrifugation, digested with trypsin and stored at -20°C until further use^{32, 34}.

Fc glycosylation analysis

Analysis of Fc-linked N-glycans was performed as previously described^{32, 34}. In brief, trypsinized samples obtained from the ACPA and total serum IgG isolation procedures were applied to an RP column (C18 PepMap 100 Å, 3 µm, 75 µm x 150 mm; Dionex/LC Packings, Amsterdam, The Netherlands) using an Ultimate3000 nanoLC (Dionex/LC Packings). The LC system was coupled via an online nanospray source to an Esquire HCTultra ESI-IT-mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electron transfer dissociation module (PTM Discovery System™) and was operated in the positive ion mode. Ions from mass/charge (*m/z*) 600 to *m/z* 1800 were registered. The high-performance liquid chromatography method resulted in resolution of the glycopeptides based on the peptide moiety, with IgG₁ glycopeptides eluting first, followed by IgG₄ and IgG₂ glycopeptides. Glycopeptides with neutral glycan moieties tended to elute earlier than glycopeptides with antenna sialylation. Average mass spectra were generated over a 1-minute elution range for both the neutral and the acidic glycopeptides of each IgG subclass.

As the most frequent isotype in ACPA-positive RA patients is IgG₁, only IgG₁ Fc-linked N-glycans were analyzed in this study. Furthermore, it is important to note that also the Fab-fragment of IgG-molecules can carry N-glycans³⁵. Most previous studies have analyzed N-glycans released enzymatically from total human serum IgG, which results in a mixture of both Fc-linked and Fab-linked N-glycans. However, Fab-linked glycoforms can differ considerably from Fc-linked N-glycans, and vary between IgG subclasses³⁶. In our approach, the specific mass of the Fc peptide portion obtained after tryptic cleavage of the IgG molecule allows to exclusively analyze Fc-linked N-glycans, whilst Fab-linked glycopeptides exhibit a heterogeneous group of signals that do not interfere with Fc glycosylation analysis at the glycopeptide level. Due to the exclusive analysis of Fc-linked N-glycans, the total number of glycoforms detected was lower than that of previous reports, and some glycoforms were not detected at all.

Statistical analysis

In order to account for measurement variability, the relative peak intensity of the glycoform containing one galactose residue (G1) identified by mass spectrometry was deliberately set at 1, and peak intensities of all other glycoforms were normalized accordingly. Frequencies of individual glycoforms are reported as percent of the total number of detectable glycoforms based on this normalization procedure. Differences in frequencies of glycoforms obtained from different samples were evaluated for statistical significance using the non-parametric Kruskal-Wallis test (in case of unpaired samples) followed by Dunn's post hoc test to adjust for errors introduced by multiple testing. Dunn's test generalizes the Bonferroni adjustment procedure for multiple testing to maintain the probability of a type-I error at $\leq 5\%$. For glycoforms of paired serum/synovial fluid samples, groups were compared using the non-parametric Mann-Whitney rank-sum test. This is indicated where appropriate. P-values <0.05 were considered statistically significant. Data were analyzed using SPSS version 16.0.2 and GraphPad Prism Software 4.0.

RESULTS

Fc-linked galactosylation of ACPA IgG₁ isolated from human serum

Specific Fc glycosylation profiles of ACPA IgG₁ and respective profiles of total serum IgG₁ were obtained from 85 ACPA-positive Dutch early arthritis patients (mean age 52.6 ± 14.4 years; 69.4% female). Based on previous observations reporting a predominance of agalactosylated glycoforms in patients with rheumatoid arthritis, we first analyzed frequencies of IgG₁ Fc-linked galactose residues (Figure 2). A median of 25.4% (IQR 19.8-32.3) of serum IgG₁ molecules were found to lack galactose residues, consistent with previous reports (Figure 2A)¹⁷. ACPA IgG₁ contained slightly higher frequencies of G0-glycoforms

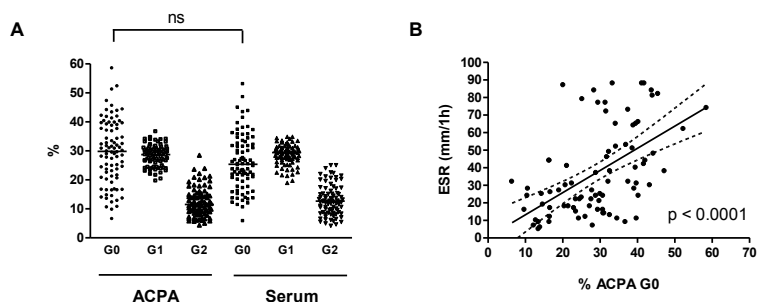


Figure 2: Fc galactosylation of ACPA IgG₁ and serum IgG₁.

Figure 2A: Frequencies of G0 (no galactose), G1 (1 galactose) and G2 (2 galactoses) glycoforms for ACPA IgG₁ and serum IgG₁ as the percentage of all detected glycoforms. Horizontal lines indicate the medians.

Figure 2B: Correlation between the frequency of ACPA-G0 glycoforms and erythrocyte sedimentation rate (ESR). Dashed lines represent the 95% confidence interval of the linear regression (solid line). ns = not significant.

as compared to total serum IgG₁ (29.8% (IQR 20.9-38.8)), a nominal difference that did not remain significant after correction for multiple testing. Almost two thirds (62.4%) of patients carried ACPA IgG₁ that exhibited higher G0-frequencies than their respective serum IgG₁. Patients of whom these ACPA were isolated did not differ significantly in age (data not shown), but did have slightly higher ESR than those with ACPA G0-frequencies below those of serum IgG₁ (median ESR 32.0 mm/1h (IQR 21.5 - 65.0) vs. 24.5 (IQR 15.5 - 40), $p = 0.06$). This is in line with a linear correlation between the frequency of ACPA G0-glycoforms and ESR (Spearman $\rho = 0.54$; $p < 0.0001$; Figure 2B). A similar correlation, although weaker, was found for G0-glycoforms of serum IgG₁ (Spearman $\rho = 0.38$; $p < 0.0003$) and has been reported previously¹⁸. Importantly, the frequency of ACPA G0-glycoforms did not correlate with ACPA-titres ($p = 0.28$; data not shown). Furthermore, no significant difference was noted between ACPA IgG₁ and total serum IgG₁ with respect to the presence of G1- or G2-glycoforms. Taken together, we observed a trend towards more agalactosylated glycoforms on ACPA IgG₁ than on serum IgG₁ that did not reach statistical significance at the available sample size.

ACPA-specific Fc-linked sialylation and fucosylation patterns

Given the reported impact of sialic acid and core fucose residues on the biological activity of antibodies, we next determined the frequency of IgG₁ Fc-linked terminal sialic acid and core fucose residues (Figures 3 & 4). Absence of sialic acid residues increases the affinity for activating FcγR, while presence of sialic acid accounts for anti-inflammatory effects, as has been shown for IVIG^{3,11}. The frequency of sialic acid residues attached to ACPA IgG₁ was found to be significantly reduced as compared to serum IgG₁ for both G1- (Figure 3A; ACPA median 1.73% (IQR 1.36-2.16), serum median 2.16% (IQR 1.94-2.53); $p < 0.001$) and G2-glycoforms (Figure 3B; ACPA median 5.47% (IQR 3.94-8.96), serum median

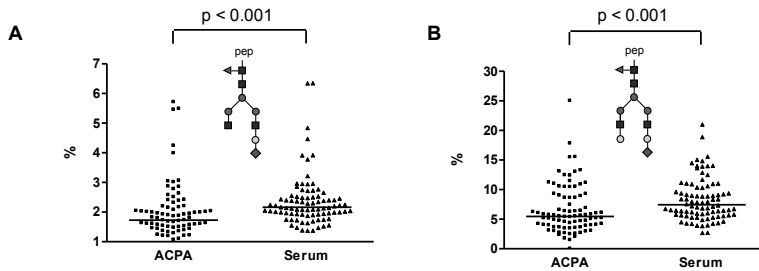


Figure 3: ACPA-specific Fc-linked sialylation patterns. Frequencies of monosialylated G1- (A) and G2- glycoforms (B) for both ACPA IgG₁ and serum IgG₁. No Fc-linked disialylated glycoforms were detected. Horizontal lines indicate the medians. pep = peptide moiety (see Figure 1 for other definitions).

7.43% (5.82-10.44); $p < 0.001$). As terminal sialic acid residues require galactose residues for linkage, sialic acid can only be detected on G1- or G2-, but not on G0-glycoforms. We did not detect glycoforms carrying two sialic acid residues in our samples.

Fucosylation strongly influences ADCC by modulating the Fc part's binding avidity to FcγRIIIa. In the absence of core fucose residues ADCC is significantly increased^{13, 14, 37}. The vast majority of IgG₁-antibodies analyzed here contained core fucose residues with no significant overall difference between ACPA IgG₁ and total serum IgG₁ (Figure 4; ACPA median 93.9% (IQR 92.5-95.3); serum median 94.0% (IQR 92.1-94.9), $p = 0.66$).

Overall, these data indicate that the glycosylation profile of ACPA IgG₁ differs considerably from total serum IgG₁ in the degree of sialylation.

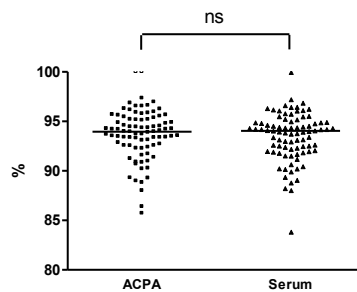


Figure 4: ACPA-specific Fc-linked fucosylation patterns. Overall frequency of fucosylated ACPA IgG₁ glycoforms (G0+G1+G2) versus fucosylated serum IgG₁ glycoforms (G0+G1+G2). Horizontal lines indicate the medians.

Fc-linked glycosylation of ACPA isolated from synovial fluid

RA is primarily characterized by synovial inflammation and progressive joint destruction. ACPA are thought to be involved in this process. However, the origin of ACPA circulating in serum is still unknown, and it remains to be determined to what extent serum ACPA contribute to synovial inflammation in the joint. In fact, ACPA titres in synovial fluid (SF) exceed those in serum, and accumulating evidence suggests that ACPA can also be gener-

ated locally by plasma cells within the synovial membrane ²⁴. So far, however, qualitative differences between serum ACPA and ACPA in SF have not been determined. In order to investigate whether Fc glycosylation differs between ACPA in SF and ACPA circulating in serum, we isolated ACPA from serum and synovial fluid of 11 paired samples. ACPA IgG₁ glycosylation profiles were compared to profiles of total IgG₁ isolated from the same compartments. In contrast to ACPA in serum, ACPA isolated from SF were found to be highly agalactosylated (Figure 5A; serum-ACPA: G0 median 27.4% (IQR 21.7-37.6), SF-ACPA: G0 median 47.0% (IQR 34.9-60.1), Mann-Whitney $p = 0.008$). This finding was specific for ACPA IgG₁, as no such predominance of G0-glycoforms was observed for the pool of total synovial fluid IgG₁ (Figure 5B; serum IgG₁: G0 median 27.5% (IQR 15.6-35.0); SF-IgG₁: G0 median 31.4% (IQR 17.6-43.2), Mann-Whitney $p = 0.5$). Increased frequencies of ACPA G0-glycoforms in synovial fluid as compared to serum were observed in

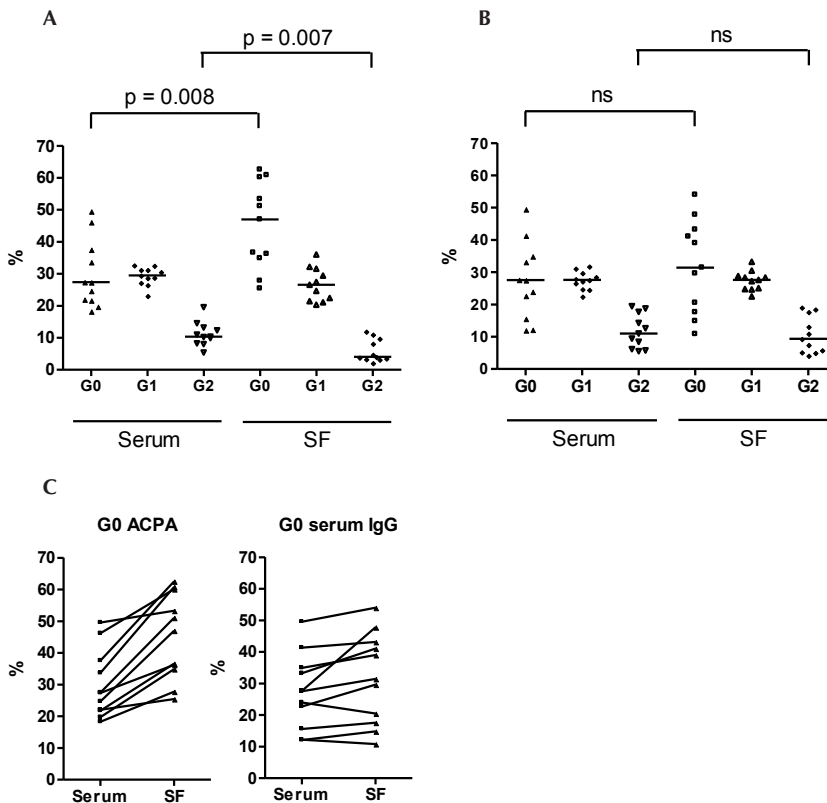


Figure 5: ACPA-specific Fc glycosylation in serum versus synovial fluid.

Figure 5A and 5B: G0-, G1- and G2-glycoforms of ACPA IgG₁ (A) and total serum IgG₁ (B) isolated from 11 paired samples of serum and synovial fluid of. Horizontal lines indicate the medians.

Figure 5C: ACPA IgG₁ and total serum IgG₁ G0-frequencies in serum and synovial fluid from each individual patient. Significance levels were determined by comparing individual groups using the non-parametric Mann-Whitney rank sum test.

all 11 patients analyzed (Figure 5C). In contrast, G0-frequencies of total synovial fluid IgG₁ were higher than in serum for some patients, but lower in others. Due to the lack of galactose residues, SF-ACPA also exhibited a significantly lower degree of sialylation (data not shown). All glycoforms examined in SF exhibited a high degree of fucosylation (up to 100%) with no significant difference between ACPA and total IgG₁ (data not shown).

In summary, ACPA IgG₁ in synovial fluid differ from ACPA IgG₁ in serum in terms of Fc glycosylation. Synovial fluid ACPA are highly agalactosylated and lack terminal sialic acid residues. This differential glycan profile appears to be a specific feature of ACPA, as no significant difference in Fc glycosylation was observed for total IgG₁ isolated from the same compartments.

Differential ACPA IgG₁ Fc glycosylation profile in RF-positive and -negative patients
Previous studies indicate that the specificity and positive predictive value of ACPA for RA are increased in the presence of rheumatoid factors (RF) ²⁵. Individuals with both ACPA and IgM-RF are significantly more likely to develop RA than those who test positive for ACPA only, suggesting that ACPA and RF directly or indirectly interact ^{38, 39}. Intriguingly, RF binding sites include the Cγ2 domain of the Fc part of human IgG which is in close proximity to amino acid Asn297 to which the carbohydrate chains are attached. Therefore, it has been suggested that Fc glycosylation could influence RF-binding to IgG-Fc, and indeed fractions of RF exhibiting high affinity for agalactosyl IgG have been identified in RA patients ^{40, 41}. Based on these observations, we were interested in investigating whether ACPA Fc glycosylation differs between IgM-RF-positive and -negative patients (Figure 6). We observed a significant predominance of ACPA IgG₁ G0-glycoforms in RF-positive (n = 61; G0 median 31.6% (IQR 24.4-39.3)) as compared to RF-negative patients (n = 24; G0 median 19.1% (IQR 13.7-31.3), Figure 6A). This RF-dependent difference was only observed for ACPA, but not for total serum IgG₁ (Figure 6B). In order to exclude that the

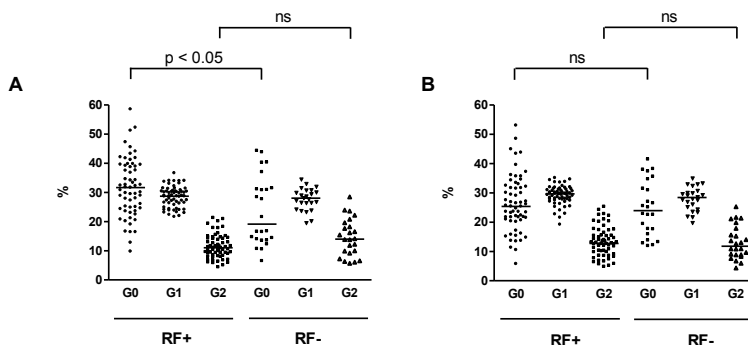


Figure 6: Differential ACPA Fc glycosylation in rheumatoid factor (RF)-positive and RF-negative patients. Galactosylation profiles for ACPA IgG₁ (A) and total serum IgG₁ (B) molecules in IgM-RF-positive and -negative patients. Horizontal lines indicate the medians.

observed difference could be due to the co-elution of agalactosylated RF bound to ACPA during the ACPA isolation procedure, we also isolated ACPA from RF-negative/ACPA-positive sera after mixing them with RF-positive/ACPA-negative sera. Fc glycan profiles of ACPA isolated from these mixing experiments did not differ from the profiles obtained without additional RF (data not shown), making it unlikely that our results are confounded by RF bound to ACPA.

We also noted a significant lack of the sialylated ACPA-linked G2-glycoform in RF-positive patients (RF-positive: median 5.2% (IQR 3.6-6.5), RF-negative: median 8.6% (4.4-11.1), $p = 0.038$), while the sialylated G1-glycoform was equally frequent in both subgroups (data not shown). No difference within these subgroups was observed for core fucosylation. Of note, RF-positive and -negative subgroups did not differ significantly in age or the degree of ESR-/C-reactive protein elevation (data not shown).

DISCUSSION

N-glycans are crucial determinants of IgG Fc-mediated antibody effector functions. Modification of the Fc-linked carbohydrate backbone by addition of galactose, sialic acid, fucose and N-acetylglucosamine residues has a differential impact on Fc-mediated immune responses both in-vivo and in-vitro^{10-14, 20}. Serum IgG molecules of RA patients have long been known to lack Fc-linked galactose residues when compared to serum IgG molecules of age-matched healthy controls, but whether antibodies of defined antigenic specificity differ in their degree of Fc glycosylation remained undetermined in these patients. Given recent insights in RA pathogenesis based on the identification of anti-citrullinated protein antibodies, we sought to determine whether these RA-specific autoantibodies exhibit specific Fc glycosylation profiles that would help to elucidate their role in disease pathogenesis.

Comparing Fc-linked glycosylation profiles of ACPA IgG₁ to the pool of total serum IgG₁ in early arthritis patients, we found a significant lack of sialic acid residues on ACPA IgG₁ molecules. Furthermore, we observed a non-significant trend towards a lower degree of ACPA-galactosylation which could result in an additional reduction in sialic acid residues. This finding points to an increased inflammatory potential of ACPA IgG₁ and is in line with the recent observation that sialic acid residues rather than galactose residues determine the anti-inflammatory activity of human IgG³. Sialylated IgG molecules have reduced affinity to activating FcγR and, at least in the mouse, have been described to bind to a specific receptor on regulatory macrophages in the spleen²². Likewise, a complete lack of Fc-linked sialic acid residues favours the pathogenic potential of IgG as demonstrated by the loss of anti-inflammatory activity of IVIG upon treatment with neuraminidase³. Thus, ACPA IgG₁ circulating in human serum exhibit, based on functional data from murine

studies, a more pro-inflammatory Fc glycosylation profile than the pool of total serum IgG₁.

With regard to core-fucosylation, we found that ACPA IgG₁ G0-glycoforms were highly fucosylated, an observation previously reported for total serum IgG G0-glycoforms of RA patients when compared to healthy controls^{42, 43}. Based on in-vitro studies this high core fucose content is associated with a low potential to induce ADCC¹³, but the functional relevance for RA pathogenesis is currently unclear and warrants further study.

Extending our ACPA-specific glycan analysis to synovial fluid we observed both a reduction in ACPA IgG₁-linked galactose residues as well as a concomitant absence of sialic acid residues. This lack of galactose and sialic acid residues was specific for ACPA, as no such difference in Fc glycosylation was observed for the pool of total IgG₁. Although this finding is suggestive of an increased inflammatory potential of SF-ACPA as compared to serum ACPA, we cannot conclude that this ACPA Fc glycosylation profile is cause rather than consequence of inflammation. Despite this limitation, our observations demonstrate for the first time that ACPA in serum and in synovial fluid are not only quantitatively but also qualitatively different. Fc-linked glycan residues are unlikely to be enzymatically modified post-secretion in SF, as such modifications would be independent of antigen specificity and also affect the pool of total IgG in SF. Therefore, differential ACPA Fc glycosylation in SF as compared to serum suggests that SF-ACPA originate predominantly from specific B cell subsets in synovial tissue. Such ACPA-specific B cells could be under the influence of local cytokines that regulate glycan processing in plasma cells susceptible to the respective cytokine signal. Local ACPA-production has been postulated previously and is in line with the histological presence of germinal centers in synovial tissue of RA patients^{23, 24, 44}. RA B and T cells are known to exhibit reduced enzymatic activity of β 1, 4-galactosyltransferase, the enzyme responsible for adding galactose residues to terminal GlcNAc residues in N-glycans^{45, 46}. Whether even more complex differences in N-glycan processing exist among B cell subsets in different compartments in RA is unknown. In the context of RA and other autoimmune disorders such as SLE, it would be of particular interest to investigate whether newly generated and long-lived plasma cells differentially process Fc-linked N-glycans.

We noted differential ACPA Fc glycosylation profiles in IgM-RF-positive versus -negative patients. Serum-ACPA of RF-positive patients lacked galactose residues when compared to ACPA isolated from RF-negative patients. This differential Fc glycosylation profile was only observed for ACPA but not for total serum IgG, the latter being in line with previous results⁴⁷. Our finding is intriguing, as it fuels the hypothesis of an interaction between ACPA and RF. ACPA associate with RA independently of RF, but the risk to develop RA increases markedly (>30 fold) if RF are additionally present. In contrast, IgM-RF alone do not seem to associate with RA in the absence of ACPA³⁹. RF binding sites have been mapped to the C γ 2 and C γ 3 domains of human IgG. Not all RF recognize the same antigenic

determinant, but at least one binding site involves Asn297 which carries the Fc-linked N-glycans ⁴¹. Thus, it has been postulated that Fc glycosylation can influence RF-binding affinity, and indeed RF with high affinity for agalactosylated IgG were repeatedly identified in RA patients ^{40, 41}. Moreover, IgG aggregates in synovial fluid of RA patients were found to contain high amounts of agalactosylated IgG, suggesting that a lack of galactoses facilitates association of IgG molecules ⁴⁸. It is conceivable that lack of galactose residues leads to a conformational change in IgG-Fc structure ⁹ which could favour high affinity RF binding and even reveal novel epitopes that promote the generation of high affinity RF. So far, binding activity of IgG-RF was found to increase with decreasing RF Fc galactosylation (and -sialylation), compatible with the idea that Fc-glycosylation influences “antigenicity” of the Fc-tail ⁴⁹. Whether RF directly or even preferentially also bind to ACPA that lack galactose residues cannot be concluded from our data, but our data suggest that the IgG recognized by RF exhibits a similar glycosylation profile to the one detected on ACPA. Thus, it is intriguing to speculate that ACPA G0-glycoforms help in the generation of RF with high affinity for agalactosylated IgG, thereby facilitating immune complex formation and complement activation.

Finally, as others before, we observed a strong correlation between the frequency of serum IgG-G0 glycoforms and ESR as well as age (data not shown). This association was even more pronounced for ACPA-G0 glycoforms (Figure 2). Of note, we accounted for these correlations in our subgroup analyses, rendering it unlikely that our results are influenced by these confounding factors.

In summary, we describe a detailed analysis of Fc-linked glycosylation profiles specific for ACPA. Fc glycosylation profiles differ between antibodies of different antigenic specificities and between antibodies isolated from different compartments. These data enhance our understanding of the citrulline-specific immune response and may open up novel strategies for therapeutic interventions.

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Chapter 7

The ACPA isotype profile reflects long-term radiographic progression in rheumatoid arthritis

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ABSTRACT

Objectives The presence of anti-citrullinated protein antibodies (ACPA) is a powerful predictive factor for the development and progression of rheumatoid arthritis (RA). The ACPA response has been shown to consist of various isotypes, but the consequences of differences in isotype-distribution have not been extensively investigated. In this study, we investigated the relationship between ACPA isotypes, disease progression and radiological outcome.

Methods ACPA isotypes were determined in sera of anti-CCP2-positive patients by enzyme-linked immunosorbent assay (ELISA). To investigate if the ACPA response continues to evolve during disease development, we studied the ACPA isotype profile during progression of undifferentiated arthritis (UA) to RA. The association of disease progression with ACPA isotype use was assessed using long-term radiographic follow-up data from RA patients in two independent cohorts.

Results The ACPA isotype distribution did not expand during disease progression from UA to RA, but was relatively stable over time. In both RA cohorts, the baseline ACPA isotype profile was a significant predictor of disease severity, with more isotypes indicating a higher risk of radiographic damage (odds ratio for every additional isotype: 1.4 (95% CI: 1.1-1.9) $p < 0.001$). ACPA isotypes supplied additional prognostic information to ACPA-status alone, even after correction for other predictive factors.

Conclusions The magnitude of the ACPA isotype profile at baseline reflects the risk of future radiographic damage. These results indicate that not only the presence, but also the constitution of the ACPA response, is relevant for the disease course of RA.

INTRODUCTION

Anti-citrullinated protein antibodies (ACPA) have emerged as a very distinctive feature of rheumatoid arthritis (RA) patients. The presence of these antibodies, which is commonly assessed by reactivity against cyclic citrullinated peptides (CCP), has been shown to potentially predict both the development of RA and the extent of associated joint destruction¹⁻³. Recent evidence indicates that well-known risk factors for RA such as the HLA-DRB1 shared epitope alleles and smoking predominantly predispose to ACPA-positive RA^{4,5}. These differences in clinical phenotype and underlying risk factors have led to the concept that ACPA-positive and ACPA-negative RA constitute distinct entities with different pathophysiological mechanisms of disease^{6,7}.

Studies using animal models to investigate the biologic processes underlying RA have shown that ACPA can exacerbate arthritis in mice^{8,9}. These findings suggest that anti-citrulline immunity may play a role in the pathogenesis of autoantibody-positive RA. Possibly, isotype switching may be one of the events required in order for autoantibodies to contribute to disease pathogenesis.

Isotype switching during the maturation of an immune response leads to an increased diversity of antibody structure. The generated antibody isotypes differ substantially with regard to their ability to mediate effector mechanisms. For example, IgM and IgG3 are much more potent activators of the complement system than other isotypes. Furthermore, the various isotypes can bind to specific Fc receptors on the surface of different cell-types, which in the case of IgG1 and IgG3 can lead to the activation of macrophages with concomitant cytokine production. In light of these well-known differences between antibody isotypes, a detailed investigation of the isotype distribution of an autoantibody response such as ACPA may provide important clues about the role of these antibodies in the pathogenesis of the disease¹⁰.

The isotype use of the ACPA response has been studied using several tests, some of which were derived from the commercially available CCP2 assays, which were modified in order to be able to measure IgG subclass antibodies, as well as IgM and IgA ACPA¹¹⁻¹⁴. The fact that ACPA of the IgM isotype could be detected at various stages of the disease indicates that new B cells are continuously being recruited into the ACPA response¹⁴. This means that the anti-citrullinated protein immune response is constantly being (re)activated during the course of ACPA-positive arthritis. It is unclear however, if during this process of continuous reactivation throughout disease progression, there is also further maturation of the ACPA response in the sense of isotype switching. In addition, the reported findings of the differences in ACPA isotype use between patients¹³, raise the question to what extent the exact features of the ACPA response, such as isotype use, are associated with disease progression.

In this study, we examined the relationship between ACPA isotypes and disease development and progression. The use of two large, independent cohorts with long-term follow-up data enabled us to investigate the association between the ACPA isotype profile and progression of radiographic damage.

METHODS

Patient populations

The Leiden EAC is an inception cohort of patients with recent-onset arthritis (less than 2 years of complaints) that was initiated in 1993 ¹⁵. At standardized follow-up visits two weeks after the first presentation and yearly thereafter, diagnoses were recorded for all patients. Patients were classified as having RA when they fulfilled the 1987 revised ACR criteria.

In order to investigate the association of ACPA isotypes with radiographic damage, baseline serum samples of all ACPA-positive RA patients in the EAC ($n=171$) were analyzed for ACPA isotypes. Annual radiographs of hands and feet of all RA patients were assessed for radiographic damage according to the Sharp-van der Heijde score (SHS) ¹⁶.

To determine if the findings in the Leiden EAC cohort could be replicated in a second cohort, ACPA isotypes were measured in a Norwegian cohort which was part of the European Research on Incapacitating Disease and Social Support (EURIDISS) project ¹⁷. Consecutive RA patients ($n=238$) with a maximum disease duration of 4 years at baseline were enrolled in this study and followed longitudinally for 10 years. 125 patients had X-rays of the hands available at baseline and after 5 and 10 years of follow-up for scoring with the SHS method. There were no significant differences between the patients with X-rays available at follow-up ($n=125$) and the whole cohort ($n=238$) ¹. ACPA isotype measurements were performed on baseline serum samples. The protocols of both cohorts were approved by the local ethics committees and all participants provided written informed consent.

Anti-CCP2 assays

Total IgG anti-CCP2 was measured in baseline serum samples from patients with UA or RA by a second-generation ELISA (for the EAC cohort: Immunoscan RA Mark 2; Eurodiagnostica, Arnhem, The Netherlands, for the EURIDISS cohort: INOVA diagnostics, San Diego, CA, USA). Samples with a value above the cut-off as specified by the manufacturer were considered positive.

Measurement of ACPA isotypes

ACPA IgG1, IgG2, IgG3, IgG4, IgA and IgM were determined using a sandwich ELISA technique as described previously ¹⁴. Briefly, assays were performed using anti-CCP2

ELISA plates (Eurodiagnostica) with isotype-specific detection antibodies. A series of successive dilutions of pooled patient sera that were positive for all isotypes was included as a standard on all plates, along with a positive control to verify comparability between plates. Sera from 8 representative healthy controls were included on each plate to determine the cut-off for positivity. Cut-off values were defined as the mean plus 2 standard deviations of the measurements of the healthy control sera.

Statistical analysis

To assess changes in ACPA isotype usage between baseline and follow-up patients who presented with undifferentiated arthritis (UA) and subsequently developed RA, McNemar's test was used. Changes in the levels of ACPA isotypes between baseline and follow-up samples were evaluated using Wilcoxon signed ranks test. Correlation between changes in the levels of the various ACPA-isotypes were investigated using Spearman's rank correlation coefficient, due to the non-normal distribution of the data.

To investigate the association between ACPA isotypes and radiographic progression, differences in radiographic damage between groups were assessed using Mann-Whitney U-tests and logistic regression analysis. The radiographic progression (dependent variable) was calculated as the difference in SHS between baseline and the last time point of follow-up (4 years in the EAC, 10 years in the EURIDISS). This value was subsequently dichotomized into minor and major radiographic progression based on the median values per cohort (10 in the EAC, 17 (hands only) in the EURIDISS). Patients with a change in SHS greater than or equal to the median were classified as having major progression, while the other patients were classified as having minor progression. Multivariate logistic regression analysis was performed to adjust for additional variables (age, gender, CRP, IgM rheumatoid factor and SHS at baseline). The positive likelihood ratio was calculated as the sensitivity divided by (1- specificity). Likewise, the negative likelihood ratio was calculated as (1- sensitivity) divided by the specificity. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) 16. P-values below 0.05 were considered to be statistically significant.

RESULTS

The ACPA isotype distribution does not expand during disease progression

Previously, we have shown that in patients with longstanding RA, the ACPA isotype profile at baseline did not markedly differ from the ACPA isotype use after 7 years of follow-up on the population level ¹⁴. In order to extend these findings to an earlier stage of the disease, and to investigate if the ACPA response continues to evolve during disease progression, we studied the ACPA isotype profile in baseline and follow-up serum samples of 67 ACPA-

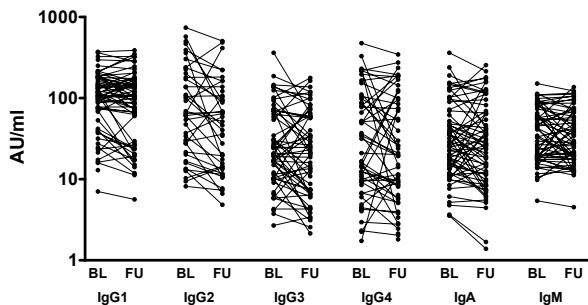


Figure 1: ACPA isotype levels at baseline and after 1 year of follow-up of 67 UA-RA patients. All patients had UA at baseline and had developed RA at the time of follow-up. Dots depict the levels of individual patients in arbitrary units per milliliter (AU/ml) at baseline and follow-up. The number of arbitrary units/ml for one isotype is not comparable to the number of arbitrary units /ml for another isotype. Differences in the levels of ACPA isotypes between baseline and follow-up samples were assessed using Wilcoxon signed ranks test.

positive patients from the Leiden EAC. All patients had undifferentiated arthritis (UA) at baseline, meaning they did not fulfill the ACR criteria for RA and their clinical presentation was not compatible with any other well-defined rheumatologic disease. For the present study 579 UA patients were analyzed of whom 142 were ACPA-positive as measured by the CCP2 ELISA. Of all anti-CCP2-positive UA patients who had developed RA after one year, serum samples collected at baseline and after one year of follow-up were examined (n=67 samples available). Measurements of isotypes were restricted to patients who were positive for ACPA, since the occurrence of ACPA isotypes has been shown to be confined to ACPA-positive patients^{13, 14}.

As shown in Figure 1, ACPA isotype levels varied considerably during disease progression, with some patients showing an increase and others showing a decrease in levels. Most of the changes in the levels of the different isotypes were significantly correlated with each other. Especially changes in IgG3- and IgA-ACPA-levels were accompanied by similar changes in levels of the other isotypes (correlation coefficient: 0.23-0.66).

Table 1. ACPA isotype use in patients who experienced disease progression from undifferentiated arthritis (UA) to RA

ACPA Isotype	BL (UA) n=67	FU (RA) n=67	lost	gained	p-value McNemar
IgG1	67 (100%)	66 (99%)	1	0	1
IgG2	51 (76%)	44 (66%)	12	5	0.14
IgG3	34 (51%)	35 (52%)	7	8	1
IgG4	64 (96%)	65 (97%)	0	1	1
IgA	45 (67%)	41 (61%)	9	5	0.42
IgM	39 (58%)	40 (60%)	7	8	1

The isotype profile at baseline (BL (UA)) compared to the isotype profile after 1 year of follow-up (FU (RA)).

Both the paired-samples analysis and the comparison of the median levels at baseline and follow-up revealed that there was no significant difference between baseline and follow-up levels for any of the isotypes. Despite the fluctuation in levels, most patients remained either positive or negative for a certain isotype at both baseline and follow-up. Thus, the number of patients which was positive for a certain ACPA isotype did not change (Table 1). Although some patients lost or acquired certain ACPA isotypes, there was no overall trend towards an increase in ACPA isotype use. The median number of ACPA isotypes present at baseline and follow-up was also the same: 5.

On the basis of these results, we can conclude that despite a worsening of the clinical phenotype, the ACPA isotype use does not expand during disease progression from UA to RA.

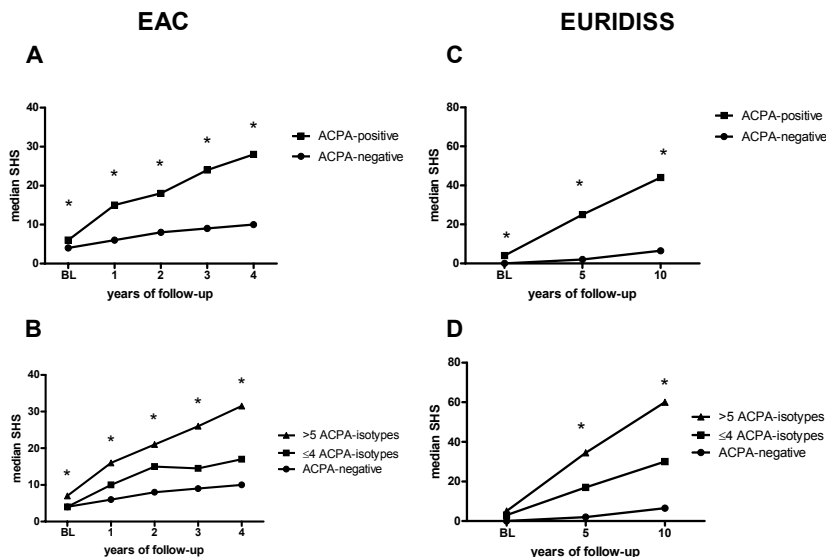


Figure 2: Effect of ACPA isotypes on radiographic damage in EAC and EURIDISS.

Depicted are the median Sharp-van der Heijde radiographic scores (SHS) over time.

Panels A and B present the results of the EAC and contain information on 251 RA patients of whom 113 were ACPA-positive with ACPA isotype data.

Panels C and D depict the results of the EURIDISS cohort and contain information on 125 RA patients of whom 74 were ACPA-positive with ACPA isotype data.

Due to the non-normal distribution of SHS, medians were compared by Mann-Whitney U-test.

* in panels A and C indicates a significant difference with a p-value < 0.05 between ACPA-positive and ACPA-negative patients.

* in panels B and D indicates a significant difference with a p-value < 0.05 between the upper two groups: patients with less than 5 ACPA isotypes and patients with 5 or more ACPA isotypes.

ACPA isotypes at baseline predict future radiographic progression in patients with ACPA-positive RA

In view of the finding that the presence of ACPA isotypes in individual patients is relatively constant over time, we next investigated if the baseline ACPA isotype profile is associated with radiographic progression in the two independent cohorts: the Leiden EAC and in the Norwegian EURIDISS cohort. As has been described previously ^{1, 2, 18}, ACPA-positive patients had substantially more radiographic damage than ACPA-negative patients in both cohorts (Figure 2A and 2C).

Within the ACPA-positive patients, a high number of ACPA isotypes present at baseline was associated with significantly more radiographic damage during the disease course (Figure 2B and D).

A more detailed investigation of the effect of the ACPA isotype profile on radiographic progression was performed using logistic regression analysis. As shown in Table 2 for the EAC and in Table 3 for the EURIDISS, several ACPA isotypes were significantly more prevalent in patients with major as compared to minor radiographic progression. By logistic regression analysis, these isotypes, which were more prevalent in patient with major radiographic progression, were significant predictors of radiographic progression within the ACPA-positive group of patients (Tables 2 and 3). The significant odds ratio ($p \leq 0.02$) for every additional ACPA isotype which was present, reflects the effect of the number of ACPA isotypes (Tables 2 and 3).

The predictive effect of ACPA isotypes remained significant even after adjustment in multivariate logistic regression analysis for other variables known to influence radiographic progression (age, gender, disease activity, RF status and SHS at baseline) (Tables 2 and 3). The presence of five or more isotypes increased the odds of progression by 3.53 (95% CI 1.4-9.3) and 6.76 (95%CI 1.60, 28.7) in the EAC and EURIDISS cohorts, respectively.

Patients who used a total of 5 ACPA isotypes or more, also had significantly higher levels of total IgG anti-CCP2 ($p < 0.01$) which is known to be associated with radiographic progression ¹. Due to this strong correlation, the level of anti-CCP2 and the number of ACPA isotypes cannot be entered into the same multivariate model as explanatory variables, as this results in annulment of the effect. We therefore used a matching approach to assess the effect of ACPA isotypes independently of the effect of anti-CCP2 level. Patients with ≤ 4 and with ≥ 5 ACPA isotypes were matched for anti-CCP2 levels, resulting in 18 pairs in the EAC and 15 pairs in the EURIDISS (Figure 3A and 3C). The median SHS over time among these pairs with equivalent anti-CCP2 levels is depicted in Figure 3B and 3D. In the EAC, patients positive for 5 or more ACPA isotypes had significantly more radiographic damage at the first four timepoints, and in the EURIDISS, there was a trend towards more radiographic damage in patients using 5 or more ACPA isotypes ($p = 0.10$ after 10 years of follow-up). These findings suggest that ACPA isotypes and the total anti-CCP2 IgG level

Table 2. Predictive value of ACPA isotypes for 4-year radiographic progression in the EAC

	Minor progression* n = 122	Major progression* n = 129	Positive Likelihood Ratio	Negative Likelihood Ratio	Odds Ratio	p-value	Adjusted Odds Ratio**	adjusted p-value**
ACPA-positive [^]	35 (37%)	96 (83%)	2.24	0.27	8.23 (4.4-15.6)	<0.01	3.91 (1.7-8.8)	<0.01
<i>Analysis within</i>								
ACPA-positive pts, n=	32	81						
IgA ACPA-positive	15 (47%)	58 (72%)	1.53	0.53	2.86 (1.2-6.7)	0.02	3.71 (1.4-9.8)	<0.01
IgM ACPA-positive	19 (59%)	54 (67%)	1.14	0.80	1.37 (0.6-3.2)	0.47	1.59 (0.6-4.1)	0.34
IgG1 ACPA-positive #	32 (100%)	81 (100%)	1.00	0.00	-	-	-	-
IgG2 ACPA-positive	20 (63%)	69 (85%)	1.35	0.56	3.45 (1.4-8.9)	0.01	4.08 (1.5-11.3)	<0.01
IgG3 ACPA-positive	13 (41%)	55 (68%)	1.66	0.54	3.09 (1.3-7.2)	<0.01	3.92 (1.6-9.9)	<0.01
IgG4 ACPA-positive	29 (91%)	80 (99%)	1.09	0.11	8.28 (0.8-82.8)	0.07	16.2 (1.0-258)	0.05
Number of ACPA isotypes								
(median, IQR)	4 (2-6)	5 (4-6)	-	-	1.45 (1.1-1.9)	<0.01	1.61 (1.2-2.2)	<0.01
5 or more ACPA isotypes	15 (47%)	57 (70%)	1.49	0.57	2.69 (1.2-6.2)	0.02	3.53 (1.4-9.3)	0.01

*Patients with a change in SHS greater than or equal to the median were classified as having major progression, while the other patients were classified as having minor progression.
[^] ACPA status was available for 95 patients with minor progression and 116 patients with major progression. Percentages were calculated relative to the number of patients with available measurements.
 ** multivariate logistic regression with adjustment for age, gender, CRP, IgM RF and SHS at baseline.
 # logistic regression analysis could not be performed to investigate the effect of IgG1 ACPA, because 100% of patients in the major progression group were positive for this isotype.

Printed in bold are p-values < 0.05.

Table 3. Predictive value of ACPA isotypes for 10-year radiographic progression in the EURIDISS

	Minor progression* n= 62	Major progression* N= 63	Positive Likelihood Ratio	Negative Likelihood Ratio	Odds Ratio	p-value	Adjusted Odds Ratio**	adjusted p-value**
ACPA-positive	22 (36%)	52 (83%)	2.31	0.27	8.60 (3.7-19.8)	<0.01	4.63 (1.7-12.6)	<0.01
<i>Analysis within</i>								
ACPA-positive pts, n=	22	52						
IgA ACPA-positive	12 (55%)	42 (81%)	1.47	0.42	3.50 (1.2-10.4)	0.02	5.28 (1.2-22.6)	0.03
IgM ACPA-positive	5 (23%)	24 (46%)	2.00	0.70	2.91 (0.9-9.1)	0.07	4.32 (1.1-16.9)	0.04
IgG1 ACPA-positive #	21 (96%)	52 (100%)	1.04	0.00	-	-	-	-
IgG2 ACPA-positive	7 (32%)	31 (60%)	1.88	0.59	3.16 (1.1-9.1)	0.03	5.81 (1.5-22.8)	0.01
IgG3 ACPA-positive	13 (59%)	40 (77%)	1.31	0.56	2.31 (0.8-6.7)	0.13	3.48 (0.9-13.3)	0.07
IgG4 ACPA-positive	17 (77%)	44 (85%)	1.10	0.65	1.62 (0.5-5.6)	0.45	1.62 (0.4-6.5)	0.49
Number of ACPA isotypes								
(median, IQR)	3.0 (2.0-5.3)	5.0 (3.3-6.0)	-	-	1.41 (1.1-1.9)	0.02	1.62 (1.1-2.4)	0.01
5 or more ACPA isotypes	6 (27%)	29 (56%)	2.07	0.60	3.36 (1.1-9.9)	0.03	6.76 (1.6-28.7)	0.01

*Patients with a change in SHS greater than or equal to the median were classified as having major progression, while the other patients were classified as having minor progression.

** multivariate logistic regression with correction for age, gender, CRP, IgM RF and SHS at baseline.

logistic regression analysis could not be performed to investigate the effect of IgG1 ACPA, because 100% of patients in the major progression group were positive for this isotype.

Printed in bold are p-values < 0.05.

are not interchangeable, and that determination of ACPA isotypes may supply additional information to the total ACPA IgG level.

In summary, these results demonstrate that in addition to the presence of ACPA, the constitution of the anti-citrullinated protein autoantibody response is significantly associated with the disease course of RA. Within ACPA-positive patients, an expanded ACPA isotype profile indicates a high risk for severe radiographic damage.

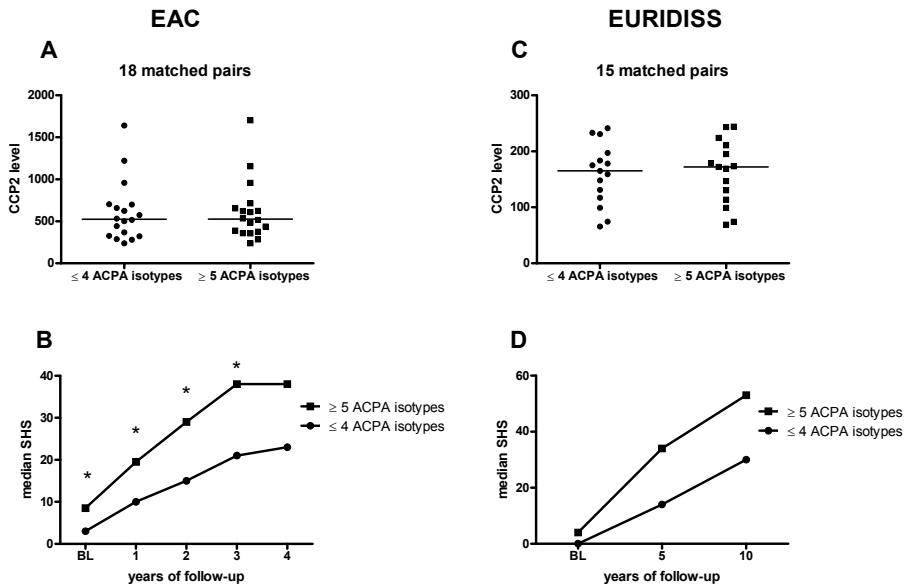


Figure 3: Effect of ACPA isotypes after matching for total anti-CCP2 levels.

Patients with ≤ 4 ACPA isotypes were matched to patients with ≥ 5 ACPA isotypes with equivalent anti-CCP2 levels.

Panel A depicts the anti-CCP2 levels of the 18 matched patient pairs in the EAC.

Panel B shows the median Sharp-van der Heijde radiographic scores (SHS) over time for the 18 patients with ≥ 5 ACPA isotypes compared to the 18 matched patients with ≤ 4 ACPA isotypes in the EAC.

* indicates a significant difference with a p-value < 0.05 .

Panel C depicts the anti-CCP2 levels of the 15 matched patient pairs in the EURIDISS.

Panel D shows the median Sharp-van der Heijde radiographic scores (SHS) over time for the 15 patients with ≥ 5 ACPA isotypes compared to the 15 matched patients with ≤ 4 ACPA isotypes in the EURIDISS.

DISCUSSION

In this study, we investigated the relationship between the ACPA isotype distribution and the progression and severity of RA. A more extensive use of ACPA isotypes at baseline was associated with more radiographic damage in RA patients in both cohorts. However, disease development from UA to RA was not accompanied by an expansion of the ACPA isotype profile.

The continued presence of IgM ACPA in patients with longstanding RA is an especially noteworthy feature of the ACPA response. IgM antibodies have a half-life of approximately 1 day¹⁹ and since long-lived plasma cells producing IgM antibodies against T cell-dependent antigens have not been described, the presence of IgM most likely indicates that the ACPA response is continuously reactivated during the course of the disease.

In previous studies, patients who were positive for IgA ACPA were found to have higher disease activity scores (DAS), even after taking into account the higher total ACPA-levels in this group of patients¹³. We have shown here that the presence of IgA ACPA and other isotypes also affects radiographic progression. The measurement of one solitary ACPA isotype (e.g. IgA) in addition to the standard anti-CCP assay, may not have a very high diagnostic yield, in view of the relatively modest likelihood ratio statistics associated with the single isotypes. However, the risk of radiological damage increased with every additional isotype, demonstrating that the breadth of the isotype response (i.e. the total number of isotypes present) may be of particular importance.

Previous reports have revealed that ACPA can be detected years before disease manifestation²⁰⁻²² and that they tend to persist in the vast majority of patients in whom they have developed²³. The fact that most patients are ACPA-positive for a certain period of time before they develop disease, indicates that not all ACPA are pathogenic. A potential underlying mechanism could be that the anti-citrullinated protein immune response may need to fully evolve and develop certain characteristics which then enable ACPA to cause tissue damage. Possibly, isotype switching and a resulting larger number of antibody isotypes, may be one of the features required for autoantibodies to contribute to disease pathogenesis.

The current study does not allow us to draw any conclusions about the events taking place before disease onset, when isotype switching could perhaps be involved in disease development. We therefore decided to study isotype switching in a very early phase after the onset of arthritis: during disease progression from UA to RA. Although UA-RA patients have been shown to have more ACPA isotypes at baseline than UA-nonRA patients¹⁴, the size of the ACPA isotype profile did not increase during the first year of follow-up, despite the development of RA in these patients during that period. Previous studies examining the ACPA response at baseline and follow-up in RA patients without overt disease progression, have reported that anti-CCP2-titers and levels of ACPA isotypes can even decrease with time^{14, 23}. A possible explanation for these observations could be the effect of immunosuppressive treatment. The current first-line treatment for patients with RA and patients with severe, progressive UA²⁴ consists of disease-modifying antirheumatic drugs (DMARDs), which all have more or less immunosuppressive functions. This makes it difficult to discern if the previously reported decrease in ACPA isotype levels is due to the natural course of the immune response or to the influence of medication.

Another possible reason why disease progression from UA to RA is not accompanied by a further expansion of the ACPA isotype use, could be that the clinical phenotype may lag behind the features of the autoantibody response. Analogous to observations in systemic lupus erythematosus (SLE), where a rise in anti-dsDNA antibodies is followed by a disease flare ^{25, 26}, the extended isotype distribution of UA-RA patients at baseline may be reflected by the disease course throughout the first year of the disease. The elucidation of the precise temporal relationship between changes in ACPA characteristics and disease phenotype will require further comprehensive follow-up studies.

The presence of 5 or more ACPA isotypes was significantly associated with higher levels of total IgG anti-CCP2 ($p < 0.01$) which is known to be associated with radiographic progression ¹. Nonetheless, when patients were matched for anti-CCP2-levels, the presence of 5 or more ACPA isotypes was still associated with more radiographic progression, indicating that the presence of ACPA isotypes supplies additional information compared to the total anti-CCP2 IgG level. However, the number of matched patient pairs in which the independent effect of ACPA isotypes could be determined was limited, and more data will be required to validate these observations.

These findings are particularly important for our comprehension of the development of the ACPA response. As the anti-citrullinated protein immune response evolves and matures, it is conceivable that all features of the ACPA response, including the antibody level and the isotype use, will expand. Despite the fact that both total ACPA IgG level and the breadth of the ACPA isotype profile may, in this respect, be reflections of the same underlying immunological process, they can independently affect disease outcome. The association of radiographic outcome with these markers of ACPA maturity supports the theory that within ACPA-positive patients, the evolution of the ACPA response may be of pathophysiological importance.

A possible pathway by which differences in the ACPA isotype distribution may influence disease severity is through selective activation of the complement system. ACPA can activate the complement system *in vitro* and it is conceivable that a more diverse isotype profile would lead to an increase in complement activation. Furthermore, the differential effects of ACPA isotypes on disease severity may also be due to the recruitment of other effector mechanisms such as binding to Fc receptors. Ligation of Fc receptors by *in vitro* generated fibrinogen-ACPA immune complexes has been shown to result in cell activation and cytokine secretion ²⁷, both of which are of prime importance for tissue damage in RA.

The hypothesis that the nature and characteristics of the ACPA response may determine disease development and progression can certainly not be proven by the current study which was designed to investigate associations. Nonetheless, the present findings are consistent with this hypothesis and add to a growing body of evidence for the role of ACPA in the pathogenesis of the disease.

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Chapter 8

Epitope spreading of the ACPA response occurs before disease onset and is associated with the disease course of early arthritis

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ABSTRACT

Objective Antibodies against citrullinated proteins (ACPA) are the most predictive factor for the development of rheumatoid arthritis (RA). To investigate if the recognition of citrullinated epitopes changes during disease onset or progression, we studied the fine specificity of ACPA in serum samples collected throughout the disease course, from before the onset of arthritis to long-standing RA.

Methods Antibodies recognizing five distinct citrullinated antigens were determined by enzyme-linked immunosorbent assay. Sera from 36 individuals who had donated blood before and after disease manifestation were used to investigate the development of citrullinated antigen recognition before disease onset. The association of ACPA reactivities with disease outcome was studied using sera of anti-cyclic citrullinated peptide 2 (CCP)-positive patients with undifferentiated arthritis (UA) who did or did not progress to RA (UA-RA n=81, or UA-UA n=35). To investigate the ACPA recognition profile in RA patients over a prolonged period of time, baseline serum samples from 68 RA patients were compared to samples obtained 7 years later.

Results The number of recognized citrullinated peptides increased in the period preceding disease onset. At the time of disease manifestation, UA patients who later developed RA recognized significantly more peptides than UA-UA patients. At later stages of the disease course, the ACPA fine specificity did not change.

Conclusion Epitope spreading with an increase in the recognition of citrullinated antigens occurs before onset of RA. Immunological differences in ACPA fine specificity are present at baseline between UA-UA patients and UA-RA patients and are associated with the future disease course.

INTRODUCTION

Anti-citrullinated protein antibodies (ACPA) are a very distinctive feature of rheumatoid arthritis (RA) patients. The presence of these antibodies, which is most commonly assessed by reactivity against cyclic citrullinated peptide-2 (CCP), has been shown to be highly predictive for both the development of RA and the extent of associated joint destruction ¹. Recent evidence indicates that well-known genetic risk factors for RA: the HLA-DRB1 shared epitope (SE) alleles and the PTPN22 T-allele are predominantly associated with ACPA-positive RA ^{3, 4}. These reports, together with the finding that ACPA can exacerbate arthritis in mice ⁵⁻⁷, suggest that anti-peptidylcitrulline immunity plays an important role in the pathogenesis of the disease. To elucidate the biological mechanisms underlying RA, and possibly identify targets for intervention, it is therefore very important to gain more insight into the development of ACPA.

Studies investigating at which point in time ACPA first appear, have revealed that these antibodies can often be detected several years before disease onset ⁸⁻¹⁰. The mere presence of ACPA therefore does not appear to be sufficient to precipitate disease. An explanation for this observation could be that the anti-citrullinated protein immune response first needs to mature more fully, in the course of which, ACPA could acquire distinct characteristics which are instrumental in mediating tissue damage.

An antibody characteristic which has been shown to be crucial for the pathogenicity of autoantibodies, is the fine specificity of antigen recognition ¹¹. An increase or shift in antigen recognition during the course of an immune response (a phenomenon known as epitope spreading), can have very important pathophysiological consequences as has been described in, for example, systemic lupus erythematosus (SLE) ¹².

Taking into consideration that ACPA can be detected before the clinical diagnosis of RA, and that the presence of ACPA is strongly associated with disease progression, we hypothesized that epitope spreading of the ACPA response may play a role in the evolution of the disease. In the present study we therefore investigated the reactivity pattern of ACPA before disease onset and during disease progression. In order to cover the entire spectrum of the disease course ranging from ACPA-positive healthy individuals to patients with long-standing RA, we made use of several different cohorts, which together provide an overview of the ACPA fine specificity development over time.

MATERIALS AND METHODS

Study population

In a previous study, individuals who had donated blood before the onset of arthritis were identified among RA patients treated at the Department of Rheumatology of the Umeå University Hospital ⁸. The time period between collection of the pre-disease sera and disease onset ranged from 43 days to 10.8 years with a median of 2.5 years.

Patients with UA or RA were selected from the Leiden Early Arthritis Clinic (EAC), an inception cohort of patients with recent-onset arthritis (less than 2 years of complaints) that was initiated at the Department of Rheumatology of the Leiden University Medical Center in 1993 ¹³. Diagnoses were recorded for all patients at annual follow-up visits and RA was diagnosed according to the 1987 revised ACR criteria. Patients who did not fulfill the diagnosis criteria and whose clinical presentation was not compatible with any other well-defined rheumatologic disease entity, were classified as having UA. For the present study we analyzed 116 anti-CCP positive UA patients and 68 anti-CCP-positive RA patients.

Informed consent was obtained and the study was approved by the local medical ethics review board.

Anti-CCP2 assays

Total IgG anti-CCP2 was measured by enzyme-linked immunosorbent assay (ELISA) (Immunoscan RA Mark 2; Eurodiagnostica, Arnhem, The Netherlands). Samples with a value above 25 units/ml were considered positive according to the manufacturer's instructions.

Anti-citrullinated peptide assays

ELISA assays were developed against peptides derived from vimentin, fibrinogen and alpha-enolase. Although there are also other targets for ACPA ^{14, 15}, we chose to primarily investigate epitopes from these proteins because they have been most consistently identified as citrullinated autoantigens ¹⁶⁻¹⁸.

Antibodies against both the citrullinated (Cit) and the uncitrullinated form of 2 linear peptides derived from vimentin (Vim 1-16: STCitS VSSS SYCitCit MFGG and Vim 59-74: VYAT CitSSA VCitLCit SSVp), 2 linear peptides derived from fibrinogen (Fib α 27-43: FLAE GGGV CitGPRVVER H and Fib β 36-52: NEEG FFSA CitGHR PLDK K) and 1 linear peptide derived from alpha-enolase (Eno 5-20: KIHA CitEIF DSCitG NPTV) were determined by ELISA. All peptides were synthesized with a C-terminal spacer and biotin tag. The vimentin and fibrinogen epitopes which were used for the present study were selected based on the fact that they were most frequently recognized by sera from ACPA-positive patients with long-standing RA. A linear alpha-enolase peptide was used with a small difference in sequence compared with the peptide used by Lundberg et al ¹⁹. Because antibodies from

different RA patients have been shown to recognize distinct epitopes of the same protein, an approach using citrullinated peptides rather than whole proteins was used in order to attain optimal discriminative ability ²⁰.

Fine specificity ELISA assays were performed as described previously ²¹. Briefly, streptavidin-coated pre-blocked microtiter plates were coated with the different peptides, followed by incubation with the serum samples. After washing, antibodies were detected with a rabbit anti-human IgG HRP-conjugated antibody and tetramethylbenzidine (TMB) as the coloring substrate. Baseline and follow-up samples were always analyzed on the same day and on the same plate. The inter-assay variability of the assays was very low ($\leq 2\%$ of samples with conflicting positive/negative results).

Definition of cut-off values and citrulline-specificity

Cut-off values on the citrullinated and arginine-containing peptides were defined as the mean plus two times the standard deviation of the values of 30 control subjects. A patient sample was considered to recognize a particular peptide in a citrulline-specific manner when it fulfilled all three of the following requirements: 1) an OD value on the citrullinated peptide above the citrulline cut-off and 2) an OD value on the arginine-variant below the arginine cut-off and 3) an OD difference ($=\text{OD for citrullinated peptide} - \text{OD for arginine-containing peptide}$) of at least 0.1. The number of patients that recognized both the citrullinated and the arginine-containing peptide above cut-off levels and thus did not bind to the peptide in a citrulline-specific way was small (approximately 3%).

Statistical analysis

To assess changes in the frequency of recognition of the citrullinated peptides between baseline and follow-up samples, McNemar's test was used. Differences in the number of epitopes recognized, and in the levels of antibody reactivity between baseline and follow-up samples were evaluated using Wilcoxon signed ranks test. When two independent patient groups were compared (UA-UA versus UA-RA patients), Mann Whitney U tests were used to analyze differences between median optical density (OD-)values and the median number of recognized peptides. Relative risks (RR) with 95% confidence intervals were calculated for the development of RA from UA, based on the presence of a certain fine specificity. Logistic regression analysis was performed to calculate the risk associated with recognizing one additional citrullinated peptide for the outcome UA or RA after one year of follow-up. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) 14.0 and StatCalc 2.0.

RESULTS

Epitope spreading occurs before disease onset

In order to assess the recognition of citrullinated epitopes before disease onset, we made use of a unique serum collection of 36 individuals who had donated blood before the onset of RA and shortly after disease manifestation (on average 7.5 months post-disease onset)⁸. As shown in Figure 1A, the sera collected before disease onset (pre-RA) had a

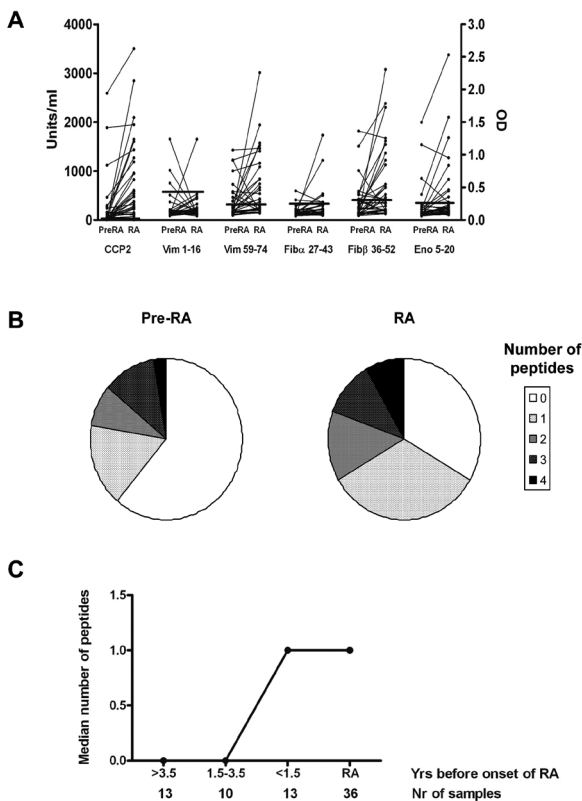


Figure 1: Reactivity of sera collected before disease onset (pre-RA) and after disease onset (RA).

Figure 1A: The number of units/ml against CCP2 and the raw optical density (OD-) values against the five fine specificity peptides are shown for the 36 individuals of whom serum was collected before and after disease onset. Differences between the median levels at the pre-RA and RA timepoints against each of the five peptides were investigated by Wilcoxon signed rank tests.

Figure 1B: Differences in peptide recognition between samples obtained before disease onset (pre-RA) and after disease onset (RA). Shown are the percentages of pre-RA and RA sera (n=36) that bound to the indicated number of peptides.

Figure 1C: Development of epitope recognition prior to the onset of arthritis. Depicted is the median number of peptides recognized by serum samples available from the specified time period antedating the symptoms of RA (n=36) and at the time RA was diagnosed (n=36).

significantly lower reactivity against all of the peptides, except for vimentin 1-16, than the sera collected after disease manifestation. For individual patients, the changes in reactivity to the different peptides over time were often correlated, but this correlation was not absolute.

Most peptides, with the exception of vimentin 1-16, were recognized substantially more frequently by the RA samples than by the pre-RA sera as listed in Table 1. The presence of (one or more isotypes of) RF also increased in the period before disease onset (data not shown). While many of the sera demonstrating reactivity to one of the fine specificity peptides also contained IgA- or IgM-RF, this was not always the case. Recognition of the fine specificity peptides was limited to the 29 individuals who were anti-CCP2-positive after disease onset.

Table 1. Recognition of individual citrullinated peptides before and after disease onset.

Peptide	Pre-RA n=36	RA n=36	lost reactivity	gained reactivity	p-value McNemar
CCP2	21 (58%)	29 (81%)	2	10	0.039
Vimentin 1-16	3 (8.1%)	1 (2.7%)	3	1	0.63
Vimentin 59-74	10 (27%)	14 (38%)	4	8	0.39
Fibrinogen α 27-43	2 (5.6%)	7 (19%)	0	5	0.062
Fibrinogen β 36-52	9 (25%)	15 (42%)	4	10	0.18
Enolase 5-20	4 (11%)	9 (25%)	1	6	0.13
Recognition of ≥ 1 peptide	14 (38%)	24 (66%)	2	12	0.013

The overall gain in peptide recognition resulted in a significant increase in the number of patients who recognized 1 or more peptides (38% versus 66%, $p=0.01$) (Figure 1B). The median number of recognized peptides was 0 before disease development compared to 1 in the RA samples ($p=0.08$). As the period between pre-RA sample collection and disease manifestation varied among patients, the expansion of epitope recognition over time is shown in more detail in Figure 1C. This figure illustrates that the recognition of citrullinated epitopes increases before disease onset.

Taken as a whole, the analysis of the ACPA fine specificity profile before and after disease onset reveals that there is epitope spreading prior to the onset of RA.

The baseline ACPA reactivity profile differs between UA patients who progress to RA (UA-RA) and those who do not (UA-UA)

To investigate if a distinct recognition pattern of citrullinated peptides is associated with disease progression, we made use of serum samples from a different cohort: the Leiden EAC. ACPA reactivities were determined in baseline serum samples from UA patients who had not been treated with disease modifying antirheumatic drugs (DMARDs). Of

116 anti-CCP2 positive UA patients, 81 fulfilled the ACR criteria for RA after one year of follow-up (UA-RA), whereas the remaining 35 patients did not develop RA (UA-UA). In Figure 2A, the baseline reactivity is depicted in the form of the raw optical density (OD-) values. At baseline, the reactivity of the UA-RA sera was significantly higher than that of the UA-UA sera against two of the five peptides. When the OD-values were translated into negative/positive recognition by applying cut-off values based on 30 control subjects,

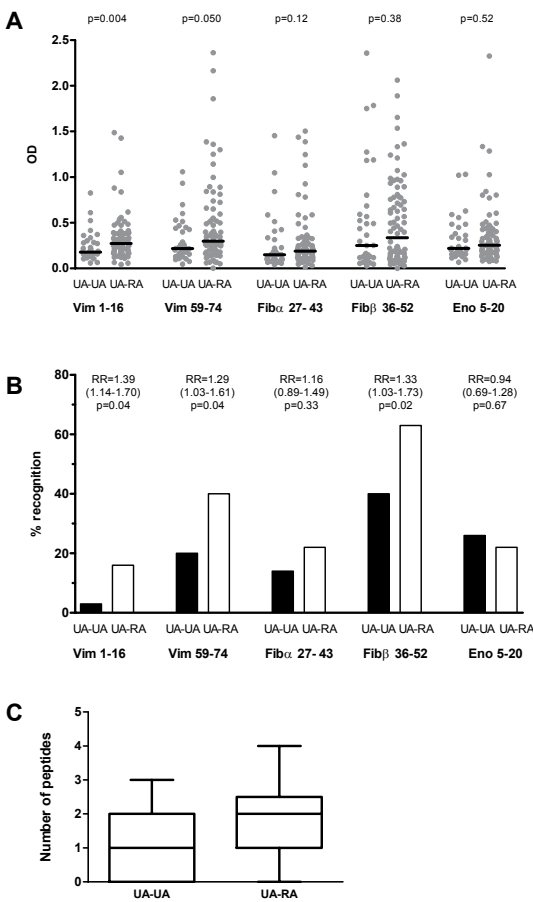


Figure 2: Baseline reactivity of UA-UA versus UA-RA sera.

Figure 2A: Depicted are the raw optical density (OD-) values for each of the UA-UA (n=35) and UA-RA (n=81) patients. Lines indicate the median OD. Differences between the median OD-values of the UA-UA and UA-RA patients for each of the five peptides were investigated by Mann-Whitney U tests.

Figure 2B: Recognition of individual citrullinated peptides at baseline by UA-UA and UA-RA patients. Depicted are the percentages of patients per group that recognized the different peptides in a citrulline-specific manner. Relative risks (RR) with 95% confidence intervals were calculated for the development of RA from UA, based on the presence of a certain fine specificity.

Figure 2C: Number of peptides recognized by UA-UA and UA-RA patients. Error bars indicate the minimum and maximum number of peptides recognized per patient. Boxes designate the 25th and 75th percentile, while the lines in the boxes indicate the median number of recognized peptides.

the prevalence of recognition of both vimentin peptides and fibrinogen β 36-52 was significantly higher among UA-RA than among UA-UA patients (Figure 2B). As shown in Figure 2C, the median number of recognized peptides per patient was larger in UA-RA patients (2 peptides) than in UA-UA patients (1 peptide) ($p=0.02$). Regression analysis revealed that for every additional epitope which was recognized, the risk of developing RA increased by a factor of 1.55 ($p=0.02$).

In summary, these results indicate that ACPA-positive UA-UA and UA-RA patients are immunologically distinct at baseline with regards to their ACPA reactivity profile. The UA-RA patients displayed reactivity against a significantly larger number of citrullinated epitopes.

Recognition of citrullinated peptides does not change during disease progression

To investigate whether the reactivity against citrullinated antigens would further increase during disease development from UA to RA, we performed fine specificity assays with serum samples obtained at baseline and after approximately 1.5 year of follow-up of the same UA-RA patients as described above. All patients had progressed to RA at this point in time. Serum samples were available for 67 of the previously tested 81 patients. As shown in Figure 3A, the recognition of all five fine specificity peptides did not markedly change. At follow-up, a smaller number of patients recognized the individual peptides, but the difference was not statistically significant. The number of citrullinated peptides recognized per patient was also lower at follow-up as compared to baseline (Figure 3B).

On the basis of these results, we can conclude that the ACPA reactivity with regard to the five fine specificity peptides we measured, did not further increase during the progression of UA to RA.

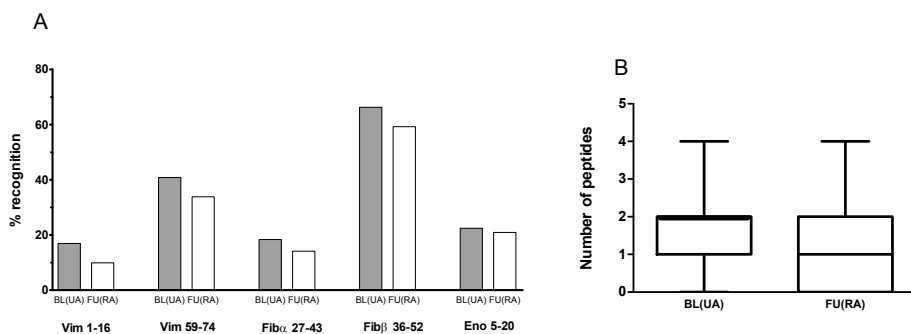


Figure 3: Recognition of individual citrullinated peptides by UA-RA patients at baseline and after 1.5 years of follow-up. **Figure 3A:** Depicted are the percentages of patients ($n=67$) which recognized the peptides in a citrulline-specific manner at baseline (when all patients had UA), and after 1.5 years of follow-up when all patients had developed RA.

Figure 3B: Number of peptides recognized by UA-RA patients at baseline and after 1.5 years of follow-up. Error bars indicate the minimum and maximum number of peptides recognized per patient. Boxes designate the 25th and 75th percentile, while the lines in the boxes indicate the median number of recognized peptides.

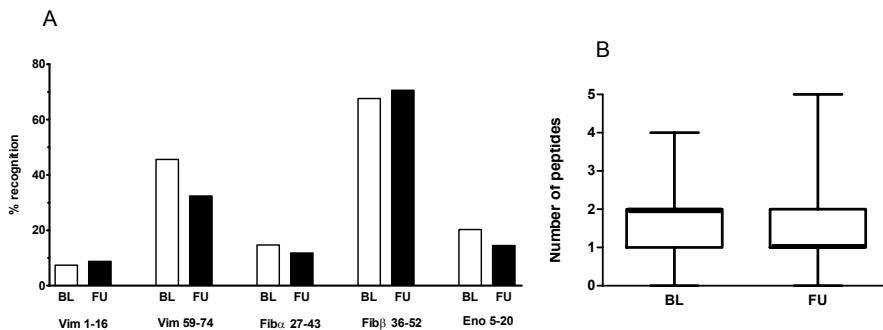


Figure 4: Citrulline-specific recognition of the different peptides at baseline and after seven years of follow-up in 68 RA patients. Figure 4A: The graph shows the percentage of patient samples which bound to the selected peptides citrulline-specifically at baseline and after a median of seven years of follow-up. **Figure 4B:** Number of peptides recognized by 68 RA patients at baseline and after seven years of follow-up. Error bars indicate the minimum and maximum number of peptides recognized per patient. Boxes designate the 25th and 75th percentile, while the lines in the boxes indicate the median number of recognized peptides.

No changes in recognition of anti-citrullinated peptides by RA patients after seven years of follow-up

To further substantiate the finding that the ACPA recognition pattern does not change during the course of the disease, we examined a group of 68 patients who presented with RA and of whom serum samples were available at baseline and after a median follow-up time of 7 years (interquartile range: 6.2-7.9 years). As depicted in Figure 4A, the percentage of patients that recognized the five peptides did not change after 7 years of follow-up. With regard to the absolute number of peptides that was recognized per patient, the findings were similar to the observations described above for UA-RA patients. The number of recognized peptides was slightly lower at the time of follow-up, as can be seen in Figure 4B, but this difference was not statistically significant ($p=0.25$).

Overall, these results reveal that with regard to these five fine specificity peptides, the ACPA recognition pattern does not change during the course of RA and that the reactivity profile does not expand after disease onset.

DISCUSSION

In this study, we analyzed the recognition pattern of ACPA throughout the disease course of RA. The results reveal that the recognition of citrullinated epitopes expands before disease onset. Furthermore, at the time of disease onset, an extensive recognition pattern of citrullinated peptides by patients who present with UA, is associated with rapid disease progression to RA in the first year of follow-up. After disease development, we could not detect a further increase in reactivity to the five fine specificity peptides used in this study.

The accumulation of autoantibody reactivities before disease manifestation which we observed in RA patients, has also been reported in other studies of human disease, such as SLE¹² and pemphigus²², as well as in animal models such as the collagen-induced arthritis model (CIA)²³. Together, these findings suggest that epitope spreading before disease onset may be a commonly occurring phenomenon which, as shown for the first time by the data presented here, may also take place before the onset of RA.

It is intriguing that at the time of disease onset, ACPA-positive UA patients who will subsequently develop RA and those who will not, are already immunologically distinct with regards to their ACPA response. The fact that none of the tested fine specificity peptides was exclusively recognized by UA-RA patients, indicates that the measurement of these ACPA reactivities may not have immediate clinical utility for individual patients. Rather, the results indicate that on the population level, the “footprint” of past epitope spreading (being an expanded epitope recognition repertoire) is associated with disease progression. These differences in the ACPA fine specificity repertoire are not an isolated immunological phenomenon, since UA patients who rapidly progress to RA also exhibit a broader use of ACPA isotypes²⁴. The characteristics of the ACPA response at baseline thus reflect the progression of the disease in UA patients. These findings are consistent with the hypothesis that the nature and characteristics of the ACPA response may determine disease development and progression, but additional studies will be required to fully elucidate the role of ACPA in the pathogenesis of the disease.

Although baseline sera from UA-RA patients recognized more peptides than sera from UA-UA patients, we could not detect a further increase in reactivity after disease onset. A very similar observation has been reported in SLE, where the accrual of new types of autoantibodies gradually increased up to the time of diagnosis and then virtually stopped¹². These findings are also in line with previous reports which have shown that anti-CCP2-titers and levels of ACPA isotypes decrease with time^{24, 25}. A possible explanation for these observations is the effect of immunosuppressive treatment such as DMARDs, which are commonly prescribed once the diagnosis of RA has been established. This makes it difficult to discern if the observed decrease in ACPA reactivities over time is due to the natural course of the immune response or to the influence of medication.

The patients studied in the current investigation were generally treated with monotherapy with conventional DMARDs. Consistent with the severe phenotype known to be associated with ACPA-positive disease, medication was frequently changed in all patients, which hampered a thorough analysis of clinical subgroups stratified for treatment. Further studies will be required to definitively answer the question whether specific therapies do or do not affect epitope recognition profiles.

Although we could not observe a further expansion of the ACPA reactivity profile after disease manifestation, it should be noted that the number of citrullinated epitopes investigated in the current study is limited. Therefore, one cannot rule out that epitope spreading

after disease onset was in fact present, but could not be detected. However, the data of the cohort of individuals who had donated blood before disease onset, reveal that there is clearly epitope spreading of the ACPA response before disease onset, suggesting that the five peptides had enough discriminative potential to allow the detection of diversification of epitope recognition. Nonetheless, further studies using a larger set of citrullinated antigens will be required to fully explore the possibility of epitope spreading after disease onset in RA.

When investigating epitope spreading, two other immunological phenomena must be taken into account: antibody cross-reactivity and affinity maturation. With regard to the peptides used in the current study, a previous report described that the HLA SE alleles are strongly associated with the recognition of citrullinated vimentin 59-74, but not with the recognition of citrullinated fibrinogen β 36-52, indicating that there was little cross-reactivity between the different epitopes²¹. The fine specificity of the ACPA response as investigated in the current study, was also associated with the presence of HLA SE alleles (data not shown). Furthermore, in the current study several patients were single-positive for each specific peptide, which rules out a large extent of antibody cross-reactivity. Affinity maturation, which leads to a higher antibody binding affinity and thus makes antibodies easier to detect by ELISA, could in this manner, have contributed to the increase in epitope recognition which we measured using this method. With regard to our understanding of the immune response *in vivo*, however, the distinction between epitope spreading and affinity maturation is, in this case, largely inconsequential, as the effect would be the same: increased antibody reactivity to a certain citrullinated epitope.

The *in vitro* methodology employed in this study cannot mimic the way in which antigen is presented *in vivo* and was therefore not intended as a technique by which to identify a specific ACPA fine specificity as the culprit of disease onset or exacerbation. Instead, the study was designed to assess the extent to which epitope spreading occurs in association with disease progression by using a selected set of peptides as a model for ACPA diversification. It was not possible to discern clusters of peptides which were preferentially recognized together, nor was it possible to identify a specific sequence in which the ACPA reactivities developed over time.

In summary, our findings indicate that the recognition of citrullinated epitopes expands before the development of arthritis. Furthermore, at the time of clinical presentation, UA patients who will later develop RA already recognize more citrullinated antigens than UA patients who will not develop RA. During disease progression from UA to RA, as well as during the further disease course of RA, we did not observe additional changes in anti-citrulline reactivity. Together, these data indicate that an important part of the evolution of the ACPA response may take place before disease onset.

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Chapter 9

Distinct ACPA fine specificities, formed under the influence of HLA shared epitope alleles, have no effect on radiographic joint damage in rheumatoid arthritis

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ABSTRACT

Objectives HLA shared epitope (SE) alleles are associated with joint destruction, the presence of anti-citrullinated protein antibodies (ACPA) and the ACPA fine specificity repertoire in rheumatoid arthritis (RA). A large variation in joint destruction is seen within the ACPA-positive patient population, and it is conceivable that certain ACPA reactivities contribute to radiological damage. Here, we investigated whether ACPA fine specificities, which are formed under the influence of SE alleles, associate with the extent of radiographic joint damage.

Methods Antibodies recognizing six citrullinated epitopes were determined in sera of 330 ACPA-positive RA patients genotyped for SE alleles. The association between SE alleles, ACPA fine specificity and radiographic joint damage was assessed using radiographic follow-up data. A second cohort of 154 RA patients with 5- and 10-year radiographic follow-up was used for replication.

Results SE alleles predisposed to the recognition of certain citrullinated epitopes. However, none of the ACPA fine specificities studied influenced radiographic joint damage. Importantly, although SE alleles associated with radiographic damage in the total RA population, this association was no longer detectable after stratification for the presence of ACPA.

Conclusions SE alleles are instrumental in shaping the ACPA repertoire. However, ACPA fine- specificities formed under the influence of SE alleles do not seem to affect joint destruction.

INTRODUCTION

HLA shared epitope (SE) encoding genes are the most prominent genetic risk factor for rheumatoid arthritis (RA). SE alleles exert their risk effect by predisposing for anti-citrullinated protein antibody (ACPA)-positive RA ¹. This and other findings have led to the concept that RA represents at least two distinct disease entities (ACPA-positive and -negative RA) with different pathogenetic mechanisms ^{2,3}.

The strength of the SE effect on susceptibility to ACPA-positive disease depends on the number of SE alleles present, with two alleles conferring a higher risk of disease than one allele. The fact that ACPA are also found in SE-negative patients, albeit in lower frequency, indicates that SE alleles are not absolutely required for the development of ACPA ¹. It has been postulated that SE alleles primarily facilitate priming and activation of T helper cells, which can then provide help to ACPA-producing B cells. Such help is required for isotype switching and generation of a potent and long-lasting antibody response.

It is conceivable that certain citrullinated antigens are more potent than others in activating T cells in the context of SE alleles. Only very few T-cell epitopes have so far been found, and no skewing of T-cell responses has been described. However, SE alleles were found to associate with antibodies targeting peptides from citrullinated vimentin, but not with the presence of antibodies recognizing citrullinated fibrinogen ⁴. This differential modulation of the ACPA response by SE alleles, and the fact that disease phenotypes vary greatly among ACPA-positive patients, has raised the question whether certain ACPA fine specificities might associate with a more severe disease phenotype. If so, designing assays that test for these specificities would be of prognostic value and could influence treatment decisions in clinic.

In the present study, we analysed the relation between SE alleles, different ACPA fine specificities and their independent effects on disease outcome.

METHODS

Patients and Radiographs

ACPA fine specificity was determined on baseline serum samples of RA patients participating in the Leiden Early Arthritis Clinic (EAC) ⁵. The present study included patients who presented between March 1993 and November 2006 and who fulfilled the 1987 revised ACR criteria for RA within the first year of follow-up. Annual radiographs of hands and feet were assessed as previously described ⁶.

Replication of the association between SE alleles and radiographic progression in relation to ACPA status was performed using data of Norwegian RA patients with a maximum disease duration of 4 years included in 1992-1993 in the European Research on Incapaci-

tating Disease and Social Support (EURIDISS) project ^{7, 8}. Radiographs of the hands were available for 154 patients at baseline and assessed according to the Sharp-van der Heijde method ⁹ by one experienced reader with known time order.

Anti-CCP2 assays

Anti-CCP2 antibody levels were measured by ELISA (EAC: Immunoscan RA Mark 2; Eurodiagnostica, Arnhem, The Netherlands; EURIDISS: INOVA Diagnostics, San Diego, CA, USA).

ACPA fine specificity assays

Antibodies against the citrullinated (Cit) and the arginine-containing form of two peptides derived from vimentin (Vim1-16; Vim59-74), two peptides derived from fibrinogen (Fib α 27-43; Fib β 36-52), one peptide derived from alpha-enolase (Eno 5-20) and against citrullinated myelin basic protein (MBP) were determined by in-house ELISA as previously described ^{4, 10}.

Cut-off values were defined as the mean plus three times the standard deviation of the values of 30 control subjects (anti-CCP-negative EAC patients diagnosed with gout). Recognition was deemed to be citrulline-specific when the following requirements were met: 1) OD value citrullinated peptide > cut-off, and 2) OD difference (=OD citrullinated peptide – OD arginine-containing peptide) $\geq$ 0.1. The number of patients recognizing both the citrullinated and the arginine-variant above cut-off levels was small (~ 3%).

Statistical analysis

Association between fine specificity recognition and SE alleles was assessed using chi-square tests. Association of fine specificities with the rate of joint destruction was assessed using a repeated measurement analysis on log-transformed radiological data. Adjustments were made for age, gender and treatment strategy used at the time of inclusion as previously described ^{5, 6}. No association between inclusion period and recognition of separate ACPA fine specificities was found (data not shown). For the EURIDISS cohort, non-parametric Mann-Whitney U test was used because of non-normal distribution of the data despite log-transformation. For comparison, Mann-Whitney U test was also applied to the Leiden EAC where indicated.

RESULTS

HLA SE alleles associate with several ACPA fine specificities

SE alleles associate with the presence of certain ACPA fine specificities ⁴. We first extended these findings by analyzing reactivity to more citrullinated epitopes, by increasing the

number of patients studied, and by analyzing the effect of the SE gene dose on ACPA fine-specificity. Three out of six citrullinated epitopes studied associated with the presence of SE alleles (Table 1). Reactivity to citrullinated vimentin 59-74, citrullinated alpha-enolase 5-20 and citrullinated MBP was found in significantly increased frequency in SE-positive patients, whereas no such effect was observed for the other three antigens. The effect was independent of the number of SE alleles, as patients with one or two SE alleles displayed a comparable profile of ACPA epitope recognition. These data indicate that one SE allele is sufficient to facilitate the development of certain ACPA fine specificities, and that presence of a second SE allele does not further skew the ACPA profile towards more frequent recognition of a specific epitope.

Table 1: Association of HLA SE alleles with ACPA fine specificity.

Recognition of citrullinated	Number of HLA SE alleles			Total n=330
	0 n=65	1 n=192	2 n=73	
Vimentin 1-16	8/63 (13%)	25/188 (13%)	8/73 (11%)	41/324 (13%)
Vimentin 59-74	16/64 (25%)	100/190 (53%)	46/73 (63%)	162/327 (50%)
Fibrinogen-α 27-43	20/64 (31%)	51/190 (27%)	15/73 (21%)	86/327 (26%)
Fibrinogen-β 36-52	43/63 (68%)	128/181 (71%)	52/73 (71%)	223/317 (70%)
α-enolase 5-20	12/64 (19%)	70/190 (37%)	27/73 (37%)	109/327 (33%)
MBP	30/64 (47%)	128/190 (67%)	51/73 (64%)	209/327 (64%)

Displayed is the number of patients within the ACPA-positive patient population who harbor ACPA recognizing defined citrullinated antigens. Chi-squared test over all 3 allelic groups (Vim 1-16: $p = 0.88$; Vim 59-74: $p < 0.001$; Fib- α : $p = 0.35$; Fib- β : $p = 0.92$; α -enolase: $p = 0.022$; MBP: $p = 0.006$).

The ACPA fine specificity repertoire does not predict future radiographic progression. ACPA-positive patients suffer from more severe disease than ACPA-negative patients. However, it is unknown whether distinct ACPA fine specificities are pathogenetically driving the inflammatory process and thus lead to a more severe disease outcome. Considering radiographic joint damage as the most objective sign of disease severity in RA, we analyzed the association between baseline recognition of specific citrullinated antigens and progression of radiographic joint damage in ACPA-positive patients over the course of 5 years (Figure 1, panels A and B). The ACPA fine specificity repertoire in individual patients did not change during this time period ¹¹. We did not detect a difference in radiographic outcome in patients who harbour ACPA of one of the fine specificities tested. Also, patients with ACPA that recognized more citrullinated peptides did not suffer from a higher degree of progression of joint damage than patients with a limited ACPA repertoire (Figure 1, panel C). These data indicate that harbouring ACPA of any of the specificities tested has no direct influence on the progression of radiographic joint damage.

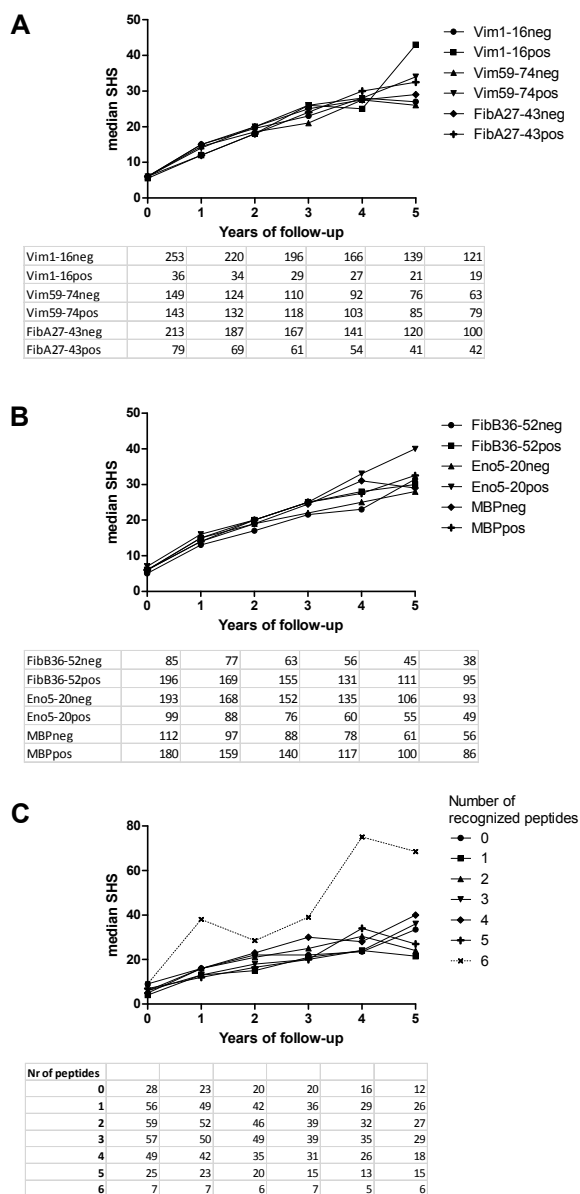


Figure 1: Association between different ACPA fine specificities and radiographic progression.
Figure 1A, B: Median Sharp-van der Heijde scores (SHS) during 5 year follow-up of ACPA-positive patients from the Leiden EAC testing positive or negative for six different ACPA fine specificities at baseline.
Figure 1C: Median SHS in ACPA-positive patients grouped by the number of ACPA fine specificities recognized. All statistical comparisons (repeated measurement analysis) were non-significant ($p>0.05$). Numbers of patients in each group are given in the table below each figure. The line representing the group of patients recognizing six fine specificities diverts from the rest due to the low number of patients in this group. In order to highlight this, this group is depicted with a dotted line. Radiographic data was available for $n=292$ ACPA-positive patients at baseline and $n=142$ at year 5.

Stratification for ACPA status abolishes the effect of HLA SE alleles on radiographic joint damage

None of the fine specificities tested was found to associate with radiographic progression. A drawback of this analysis is that lack of association does not exclude that the investigation of other citrullinated peptides would have revealed a positive association with long-term joint damage. As this limitation can persist in case more (non-associating) reactivities would be analyzed, we reasoned that an additional way to investigate the relationship between the ACPA recognition profile and radiographic outcome is to study the effect of SE alleles on joint damage in ACPA-positive disease only. Since SE alleles affect the recognition of certain citrullinated epitopes (Table 1), SE alleles can be interpreted as a surrogate marker for the constitution of the ACPA response.

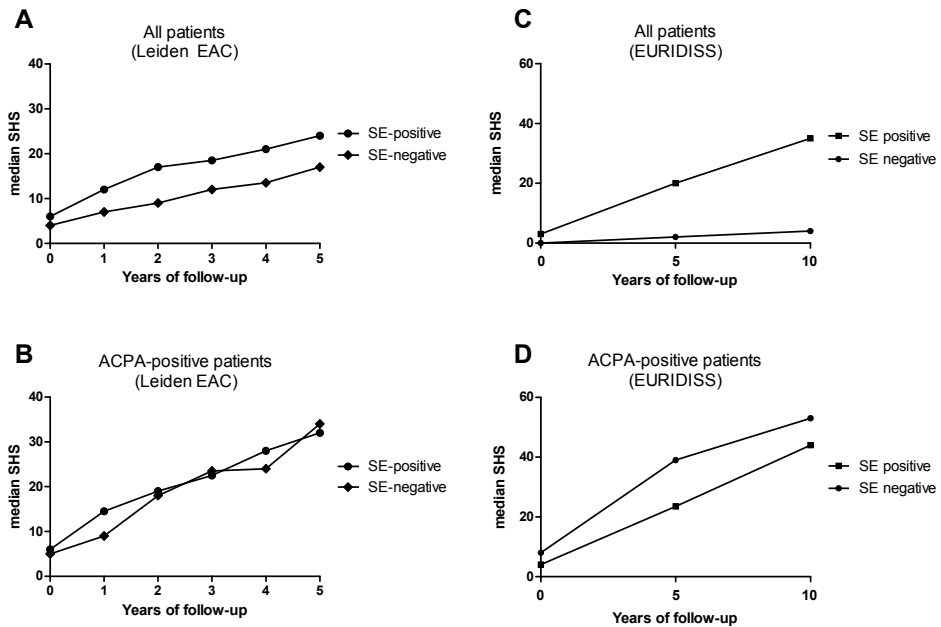


Figure 2: Shared epitope (SE) alleles exert no effect on radiographic progression after stratification for ACPA.

Median Sharp-van der Heijde scores (SHS) in relation to SE positivity in the entire RA population and after stratification for ACPA-positive disease in patients of the Leiden Early Arthritis Clinic (panels A and B) and of the EURIDISS cohort (panels C and D).

For the Leiden EAC: radiographic data was available for $n=481$ patients at baseline and $n=214$ at year 5, of which $n=266$ were ACPA-positive (data for $n=132$ available at year 5). Figure 2A: Repeated measurement analysis: $p = 0.013$; Mann-Whitney U test $p < 0.001$ at year 1, $p < 0.001$ at year 2, $p = 0.007$ at year 3, $p = 0.005$ at year 4, $p = 0.012$ at year 5; Figure 2B: Repeated measurement analysis: $p > 0.05$; all Mann-Whitney U test p -values > 0.05 .

For the EURIDISS cohort: radiographic data was available for $n=154$ patients at baseline and $n=142$ at year 10, of which $n=94$ were ACPA-positive (data for $n=83$ available at year 10). Figure 2C: Mann-Whitney U test $p = 0.029$ at baseline, $p < 0.001$ at year 5, $p < 0.001$ at year 10; Figure 2D: Mann-Whitney U test $p = 0.36$ at baseline, $p = 0.93$ at year 5, $p = 0.97$ at year 10.

While SE alleles associated with the degree of radiographic joint damage in the total RA population (Figure 2A), they no longer contributed to radiographic joint damage in ACPA-positive disease (Figure 2B). We sought replication of this observation in the Norwegian EURIDISS cohort. The findings in this cohort with 5- and 10-year radiographic follow-up confirmed our results: there was no association between the presence of SE alleles and radiographic damage in ACPA-positive disease (Figure 2C and D).

DISCUSSION

Recent findings on ACPA fine specificity and its association with SE alleles have fuelled a hypothesis in which (a) ACPA are pathogenetically driving the disease and in which (b) certain ACPA fine specificities (developing preferentially under the influence of SE alleles) could be more pathogenic than others, leading to more severe joint destruction over time^{4, 12}. Further insights into which ACPA fine specificities might be associated with disease severity could therefore have prognostic value and contribute to our understanding of disease pathogenesis.

Here, we could not detect an association between ACPA fine specificities and radiographic joint damage. An anti-peptidylcitrulline immune response to 3 out of 6 of the epitopes studied developed preferentially in patients harboring SE alleles, but this did not translate into more severe radiographic outcome. Also, the number of citrullinated epitopes recognized by an individual patient did not influence the degree of joint destruction. Although we accounted for baseline treatment strategy in this analysis, and although no association between year of inclusion and recognition of the separate ACPA fine specificities was found, we cannot fully exclude that treatment effects later in the disease course could have influenced our results. The number of citrullinated epitopes also limits our study, and it cannot be excluded that other epitopes would have been more useful for this purpose. We addressed this issue by using SE alleles as a surrogate marker for those ACPA fine specificities that develop under the influence of SE alleles. After stratification for ACPA, SE alleles no longer contributed to joint damage. Based on this finding, we consider it unlikely that a SE-associated ACPA fine specificity can be identified that predicts disease course in RA. If such a predictive recognition profile exists, antibodies recognizing this epitope are likely to be generated independent of SE alleles.

Our findings are relevant for strategies aimed at identifying patients that are at risk for rapidly progressive disease and provide evidence that the recognition profile of the ACPA response is unlikely to have a relevant impact on radiographic progression.

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Chapter 10

**Gene-environment interaction
influences the reactivity of
autoantibodies to citrullinated
antigens in rheumatoid arthritis**

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In a recent publication in *Nature Genetics* ¹, Mahdi et al. investigated gene-environment interactions and the specific association with autoantibodies to citrullinated α -enolase, in order to get an impression of the etiologic importance of this antigen in susceptibility to rheumatoid arthritis (RA). Mahdi et al report that a specific interaction is present between genotype, smoking and autoimmunity to a citrullinated α -enolase peptide (CEP-1), suggesting that the major genetic and environmental susceptibility factors for rheumatoid arthritis are associated mainly with the development of anti-CEP1-positive disease.

As referenced by Mahdi et al ¹, pivotal pathophysiological insight has been obtained in the past decade by the identification of anti-citrullinated protein antibodies (ACPA). ACPA are highly specific for RA and have been implicated in disease etiology ^{2, 3}. It has now become clear that RA represents two main syndromes, ACPA-positive- and ACPA-negative disease. These are most likely etiologically distinct as the most prominent genetic risk factor, the HLA-DRB1 shared epitope alleles (HLA-DRB1 SE alleles), as well as the most important environmental risk factor, smoking, are predominantly restricted to ACPA-positive RA ^{4, 5}.

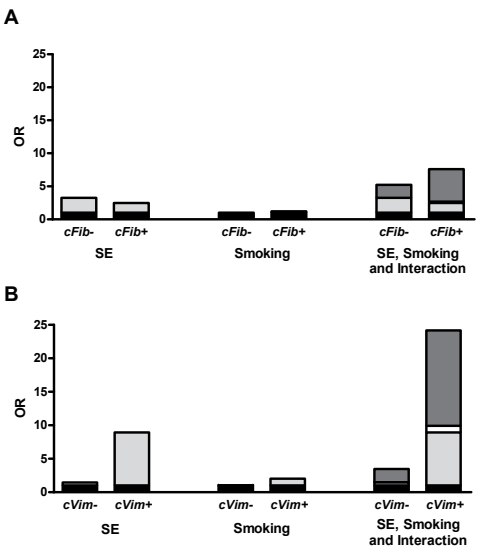


Figure 1: Risk of autoantibody-positive disease conferred by HLA-DRB1 SE alleles, smoking and their interaction.

Bars are colored black from OR 0 to 1, light-grey to indicate the risk conferred by the presence (1 or 2 alleles) of HLA-DRB1 SE alleles, white for the risk conferred by smoking, and dark-grey for the interaction. The effect the HLA-DRB1 SE alleles was corrected for the effect of smoking and vice versa, therefore the depicted numbers differ slightly from the figures listed in Table 1.

Anti-CCP-negative RA patients were used as the reference category for all comparisons.

Figure 1A: Odds ratio's for anti-CCP-positive RA patients who were either negative of positive for the citrullinated fibrinogen peptide (cFib- and cFib+).

Figure 1B: Odds ratio's for anti-CCP-positive RA patients who were either negative of positive for the citrullinated vimentin peptide (cVim- and cVim+).

Previously, we have shown that the HLA-DRB1 SE alleles influence the specificity of the antibody response against citrullinated antigens, using peptides derived from citrullinated fibrinogen (cFib) and citrullinated vimentin (cVim) ⁶. These observations indicated that at least part of the associations reported by Mahdi et al. may also exist for other citrullinated antigens. We have therefore performed further studies to investigate whether similar interactions between genetic and environmental risk factors could also be found for reactivity against other citrullinated peptides.

Samples from 701 patients participating in the Leiden Early Arthritis Clinic (EAC) were investigated in this case-only analysis ⁷. All patients fulfilled the 1987 criteria for RA of the American College of Rheumatology. Anti-CCP2-antibodies were determined by enzyme-linked immunosorbent assay (ELISA) (Immunoscan RA Mark 2; Eurodiagnostica, Arnhem, The Netherlands). Antibodies against both the citrullinated and the uncitrullinated form (arginine-variant) of linear peptides derived from vimentin (vimentin 59-74) and fibrinogen (fibrinogen β 36-52) were determined by ELISA as described previously ⁶. 361 RA patients were anti-CCP-negative and of the 340 anti-CCP-positive RA patients, 247 (73%) recognized cFib, and 167 (49%) recognized cVim.

For all analyses, a case-only study design was used with ACPA-negative rheumatoid arthritis patients from the EAC as the reference group. Logistic regression analysis was used to determine the separate effects of the HLA-DRB1 SE alleles and smoking on the specificity of the ACPA response in individuals with rheumatoid arthritis. The association of HLA-DRB1 SE alleles was the strongest in the subset of individuals who were anti-cVim-positive (Table 1 and Figure 1)

Interaction analyses were performed investigating both departure from additivity and departure from multiplicativity using logistic regression analysis ⁸. The combined effect of the HLA-DRB1 SE alleles and smoking in susceptibility to rheumatoid arthritis was the largest in the anti-cVim-positive subset (Figure 1). For this subset, a very high OR of 58 (95%CI 19.6-174.9) was present for smokers who were homozygous for the HLA-DRB1 SE alleles, compared to an OR of 5.4 (95% CI 2.1-14.2) for the anti-cVim-negative subset (Table 1). This OR of 58 is very similar to the OR of 53 reported by Mahdi et al. in the anti-CEP1-positive patients with the same combination of risk factors (homozygous HLA-DRB1 SE alleles and smoking).

Biological interaction, defined as departure from additivity, can be quantified using three measures: the relative excess risk due to interaction (RERI); the attributable proportion (AP) due to interaction; and the synergy index (S). While all measures of biological and multiplicative interaction were significant for anti-CCP-positive RA patients who also recognized cFib, there was no significant biological interaction for anti-CCP-positive patients who did not recognize cFib (Table 1). With regard to the recognition of citrullinated vimentin, the interaction analysis revealed even larger differences. While the RERI and the AP indicated some degree of biological interaction for the anti-CCP-positive anti-cVim-

Table 1, Part 1: The effect of the HLA-DRB1 SE alleles, smoking and the interaction between these two risk factors on the specificity of the ACPA response.

Group	HLA SE-positive, n / total n (%)	OR (95% CI)	Smokers, n / total n (%)	OR (95% CI)	HLA SE-positive smokers/ HLA SE-negative nonsmokers n / n	OR (95% CI)
RA: CCP-	182/361 (50%)	ref	141/405 (35%)	ref	56/117	ref
RA: CCP+ cFib-	72/91 (79%)	3.73 (2.16-6.43)	37/90 (41%)	1.31 (0.82-2.09)	30/12	5.22 (2.49-10.96)
RA: CCP+ cFib+	196/247 (79%)	3.78 (2.61-5.48)	130/240 (5.4%)	2.21 (1.60-3.07)	109/30	7.59 (4.54-12.70)
RA: CCP+ cVim-	115/173 (67%)	1.95 (1.34-2.84)	78/168 (46%)	1.62 (1.13-2.34)	58/93	3.46 (2.05-5.86)
RA: CCP+ cVim+	153/167 (92%)	10.75 (5.99-19.29)	89/162 (55%)	2.28 (1.58-3.31)	81/88	24.17 (10.48-55.72)

Table 1, Part 2: The effect of the HLA-DRB1 SE alleles, smoking and the interaction between these two risk factors on the specificity of the ACPA response.

Group	HLA SE-double positive smokers/ HLA SE-negative nonsmokers n / n	OR (95% CI)	Additive interaction measures			Multiplicative interaction p-value
			RERI (95% CI)	AP (95% CI)	S (95% CI)	
RA: CCP-	8/117	ref	ref	ref	ref	ref
RA: CCP+ cFib-	8/12	9.75 (3.10-30.67)	1.96 (-0.82-4.75)	0.38 (-0.03-0.78)	1.87 (0.74-4.71)	0.44
RA: CCP+ cFib+	32/30	15.60 (6.52-37.33)	4.91 (1.85-7.97)	0.65 (0.47-0.83)	3.92 (1.77-8.70)	0.02
RA: CCP+ cVim-	13/35	5.43 (2.08-14.16)	1.98 (0.47-3.49)	0.57 (0.29-0.85)	5.07 (0.62-41.70)	0.05
RA: CCP+ cVim+	28/7	58.50 (19.57-174.86)	14.23 (0.84-27.63)	0.59 (0.40-0.78)	2.59 (1.55-4.35)	0.63

Listed are the number of patients per group which were positive for HLA-DRB1 SE alleles, smoking or both of these risk factors. The odds ratios (OR) are the result of univariate analysis. The interaction between the HLA-DRB1 SE alleles and smoking is quantified by the additive and multiplicative measures of interaction. The assay for citrullinated fibrinogen yielded ambiguous results in 2 patients, therefore the table contains the results of 340 anti-CCP2-positive patients tested for anti-citrullinated vimentin (cVim)-reactivity, and 338 anti-CCP2-positive patients tested for anti-citrullinated fibrinogen (cFib)-reactivity. Smoking information was available for 44 anti-CCP2-negative patients of whom HLA SE information was lacking, and for a total of 330 anti-CCP2-positive patients.

negative subgroup, all three measures were significant for the anti-cVim-positive cases. The multiplicative interaction measure showed borderline significance in the anti-CCP-positive anti-cVim-negative subgroup, resembling the significant multiplicative interaction Mahdi et al. found in the anti-CCP-positive anti-CEP-1-negative subgroup. In agreement with Mahdi et al., we considered departure from additivity as the more appropriate statistical method for these analyses.

Our data show that the presence of established genetic and environmental risk factors predispose to the recognition of cVim and to a lesser extent cFib, autoantigens which have also been implicated in rheumatoid arthritis ⁹. These data complement the findings reported by Mahdi et al. ¹ by demonstrating that the interaction between genotype, smoking and autoimmunity to citrullinated antigens is not confined to citrullinated α -enolase. Together, these observations imply that the combination of genetic and environmental risk factors predispose to a broader citrullinated epitope recognition profile, possibly by influencing the magnitude of the anti-citrullinated protein response.

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Chapter 11

**The interaction between
HLA SE alleles and smoking
and its contribution to
autoimmunity against several
citrullinated antigens**

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ABSTRACT

Objective Recent data suggest that a gene-environment interaction exists between smoking and the HLA SE alleles in shaping the autoimmune reaction towards specific citrullinated antigens. Here, we determined the effects of HLA SE alleles and TE (Tobacco Exposure) on the immune response against various citrullinated antigens. These associations were analyzed in the ACPA-positive stratum to control for the possibility that found associations are explained by the known interaction between HLA SE alleles and TE with regard to ACPA status.

Methods The reactivity of 661 RA patients against several citrullinated antigens from vimentin, fibrinogen, enolase and myelin basic protein was determined by ELISA. The effects of the HLA SE alleles and TE were assessed by logistic regression analysis. Biologic interaction was analyzed by investigating if the effects of the risk factors combined exhibited departure from additivity.

Results A significant interaction between TE and HLA SE alleles was present for the presence of ACPA as reported previously. When these interaction effects were studied for several ACPA fine specificities, significant interactions were noted for several citrullinated peptides. However these interactions were not present after stratification for ACPA status, indicating that the interaction between TE and HLA SE alleles does not influence autoimmunity towards specific citrullinated antigens, but rather to ACPA development.

Conclusion Our data indicate that the gene-environment interaction between HLA SE alleles and smoking does not appear to shape the reactivity of the ACPA response. These data suggest that smoking promotes non-specific citrullination rather than citrullination of specific antigens.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease involving inflammation of the joints. The detection of anti-citrullinated protein antibodies (ACPA), often measured by an anti-citrullinated antibodies assay (anti-CCP2), is an accepted diagnostic tool for RA ¹. Anti-CCP antibodies are highly specific for RA, can be detected years before the first clinical manifestation of RA ^{2,3} and are reported to be a good predictor for the development of RA ⁴. Anti-CCP antibodies recognise multiple citrullinated peptides and proteins and are thus a collection of ACPA ⁵. Likewise ACPA have been shown to be able to initiate and enhance arthritis in murine models of arthritis ^{6,7} and human ACPA are able to activate both FcR-positive cells ^{8,9} and the complement system arguing that they could play a role in disease pathogenesis ¹⁰.

ACPA-positive and ACPA-negative disease have been shown to be associated with different genetic and environmental risk factors, fuelling the hypothesis that different pathophysiological mechanisms are underlying these two separate disease subsets ^{11,12}. Some types of human leukocyte antigens (HLA alleles), particularly HLA-DRB1 alleles encoding an amino acid sequence named Shared Epitope (SE), are known to be associated with RA susceptibility ¹³, more specifically susceptibility to ACPA-positive RA ¹⁴.

It has been shown that ACPAs can recognize a variety of citrullinated antigens, including citrullinated fibrinogen (cFib) and citrullinated vimentin (cVim), which is also known as the Sa antigen ¹⁵. However not all ACPA-positive sera will recognize all citrullinated antigens, as has been shown by analyzing the reactivity against different citrullinated peptide antigens ^{5,16}. In ACPA-positive patients, HLA SE alleles have been shown to predispose for the development of antibodies against a peptide from cVim, but not for the development of antibodies recognizing a peptide from cFib. These data indicate that HLA SE alleles influence not only the magnitude, but also the specificity of the ACPA response ¹⁷.

Like the HLA SE alleles, the environmental factor Tobacco Exposure (TE) is also known to predispose mainly to autoantibody-positive disease ¹⁸. The combined effect of HLA SE alleles and TE has been shown to exceed the sum of their single effects: a phenomenon known as biological interaction ¹⁹⁻²¹. A gene-environment interaction thus exists between the HLA SE alleles and TE with regard to the development of ACPA-positive RA. This interaction is displaying a dose-dependent effect as there is an even stronger gene-environment interaction present for heavy cigarette smoking ²².

Recently, it has been reported that a specific interaction exists between the presence of HLA-DRB1*04, TE and the specific recognition of a peptide derived from citrullinated enolase (CEP1) ²³. Such epidemiological studies might be of relevance as they link established genetic and environmental risk factors to an autoimmune response against specific citrullinated antigens that are expressed in the inflamed joint of RA patients. Therefore, such findings might provide relevant clues to aid the understanding of the pathological

mechanism underlying RA-development and progression. More recently however, we have reported a similar interaction for two peptides derived from two other citrullinated proteins, cVim and cFib²⁴. These latter data indicate that the observed interaction might not be confined to an autoimmune response against a specific citrullinated antigen. Rather, these findings indicate that these interaction effects extend to several citrullinated autoantigens, which might even be explained by a 'general' predisposition for ACPA development. Here, we wished to analyze in greater detail the extent of the interaction between HLA SE alleles and TE on the antigen-recognition profile of ACPA, especially in ACPA-positive individuals to exclude the possibility that the association is caused by the presence of ACPA rather than by the presence of an autoimmune reaction towards specific citrullinated epitopes.

MATERIALS AND METHODS

Patient population

The patients who were analyzed in this study were derived from the Leiden Early Arthritis clinic (EAC) cohort (n=661). The Leiden EAC is an inception cohort of patients with recent-onset arthritis (symptoms duration <2 years) that was started at the Department of Rheumatology of the Leiden University Medical Center in 1993 and is described in detail by de Rooy et al²⁵. All patients fulfilled the American College of Rheumatology (formerly the American Rheumatism Association) 1987 revised criteria for the classification of RA²⁶ within 1 year of follow up (EAC cohort). At baseline, patients were asked by trained research nurses if they had ever or never smoked. Cumulative smoking data were not included in these analyses due to insufficient power. The protocols were approved by the relevant local ethics committee and all participants provided informed consent.

Genotyping

DNA was collected at baseline for genotyping of the HLA-DRB1 region by polymerase chain reaction and hybridization to sequence-specific oligonucleotides as described previously²⁷. The HLA-DRB1*01:01, *01:02, *04:01, *04:04, *04:05, *04:08, *10:01 and *14:02 alleles were classified as the SE alleles.

Anti-CCP2 assays

Total IgG anti-CCP2 was measured in sera collected at baseline by enzyme-linked immunosorbent assay (ELISA) (Immunoscan RA Mark 2; Eurodiagnostica, Arnhem, The Netherlands). Samples with a value above 25 units/ml were considered positive according to the manufacturer's instructions. Individuals with antibodies against CCP2 were considered ACPA-positive.

ACPA fine specificity assays

ELISA assays were developed, as previously described ¹⁷, against peptides derived from cVim, cFib and citrullinated α -enolase 5-20 (cEno5-20). Antibody reactivity against both citrullinated (Cit) and the uncitrullinated form of two linear peptides derived from vimentin (Vim 1-16: STCitS VSSS SYCitCit MFGG and Vim 59-74: VYAT CitSSA VCitLCit SSVp), two linear peptides derived from fibrinogen (Fib α 27-43: FLAE GGGV Cit GPR VVER H and Fib β 36-52: NEEG FFSA CitGHR PLDK K) and one linear peptide derived from α -enolase (Eno 5-20: KIHA CitEIF DSCitG NPTV) were determined by ELISA ²⁸.

Briefly, streptavidin-coated pre-blocked microtiter plates (Thermo Scientific) were coated with the different Fib, Vim and Eno peptides, followed by incubation with the serum samples. After washing, antibodies were detected with a rabbit anti-human IgG HRP-conjugated antibody (P0214; DAKO, the Netherlands) and 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) as the coloring substrate, according to the recommendations of the manufacturer (Sigma-Aldrich).

Citrullination of MBP (Sigma-Aldrich) and the specific ELISA was performed as previously described ²⁹. Antibodies reactive with citrullinated MBP were determined in 1:100 diluted sera.

Although there are also other targets for ACPA ^{30,31} we chose primarily to investigate epitopes from these proteins because they have been most consistently identified as citrullinated autoantigens ^{15,32,33}. We elected to use citrullinated myelin basic protein (MBP) as a citrullinated protein antigen as its ELISA has been previously standardized to be the clinical equivalent (positive versus negative) of the original anti-Sa (cit-Vimentin) western blot assay ²⁹.

Cut-off values for the citrulline-specific responses were calculated as previously described in each assay and were defined as 3 standard deviations above the value of the mean concentration of the serum samples obtained from 41 gout controls. In fine specificity studies, citrulline-specific signals were defined as a positive signal, above the cut-off based on gout, against the citrullinated antigen and a minimum difference of 0.1 in delta OD values compared to the non-citrullinated antigen ³⁴.

The fine specificity reactivity was confined to the ACPA-positive subset. More than 98% of the individuals recognizing a specific fine specificity epitope were also CCP2-positive; therefore we did not analyze all the CCP2-negative patients for their fine specificity repertoire.

IgM-RF was determined by enzyme-linked immunosorbent assay (ELISA). Anti-CCP3 autoantibodies (IgA and IgG subforms) and anti-MCV autoantibodies were also measured by ELISA (Quanta Lite CCP version 3.1 for IgG/IgA from Inova Diagnostics San Diego, USA as well as ELISAs from Orgentec Diagnostika, Mainz, Germany). The cutoff level for both the anti-CCP3 test and the anti-MCV test was 20 arbitrary units, according to the manufacturers' instructions.

Sera were tested for ANF (anti-nuclear factor) using indirect immunofluorescence at a 1:40 dilution on Hep-2000 cells (Biomedical diagnostics).

Statistical analysis

Logistic regression analysis was performed to determine the odds ratio's (ORs) and 95% confidence intervals (95% CI) of developing autoantibody-positive relative to autoantibody-negative RA for the risk factors HLA SE alleles and smoking. The association between the recognition of different citrullinated epitopes and a gene-environment interaction was analysed using the same method. To take into account the effect of the known association of HLA SE alleles and TE with ACPA status, stratified analyses were performed within the ACPA-positive stratum.

Biological interaction, defined as the deviation from additivity, was evaluated by three measures: RERI, relative excess risk due to interaction; AP, the attributable proportion due to interaction; and S, the synergy index. These measures indicate significant biological interaction if they differ from 0 (RERI and AP) or 1 (S). RERI can be interpreted as the excess risk due to interaction relative to the risk without exposure. AP is interpreted as the attributable proportion of disease which is due to interaction among persons with both exposures. The synergy index can be interpreted as the excess risk from exposures (to both exposures) when there is interaction relative to the excess risk from exposure (to both exposures) without interaction³⁵. For a more detailed definition of these measures please refer to Rothman³⁶. To obtain the parameter estimates needed for calculating these three measures, a logistic regression model was fitted²¹. Data were analyzed using the Statistical Package for the Social Sciences (SPSS) 17.0 and p-values below 0.05 were considered to be statistically significant.

RESULTS

Effect of single risk factors on ACPA fine specificity

To investigate whether HLA SE alleles and TE are associated with specific reactivities within the total ACPA response, the CCP2 status was determined in 661 RA patients, of whom 348 were found to be anti-CCP2-positive (Table 1). In baseline sera of these 348 ACPA-positive as well as in 90 ACPA-negative RA patients we determined the reactivity

Table 1. Baseline characteristics.

	RA (max n=661)	Anti-CCP2-positive (max n=348)	Anti-CCP2-negative (max n=313)
Gender (female)	450/661 (68.1%)	228/348 (65.5%)	222/313 (70.9%)
Mean age at inclusion (mean ± SD)	55.8± 16	54.7± 15	57.1± 16
Anti-CCP2-positive	348/661 (52.6%)	348/348 (100%)	0/313 (0%)
RF-positive	321/537 (59.8%)	267/305 (87.5%)	54/232 (23.3%)

against two citrullinated vimentin peptides (cVim1-15 and cVim59-74), two citrullinated fibrinogen peptides (cFib α and cFib β), citrullinated α -enolase peptide and cMBP. Over 98% of the individuals positive for a specific citrullinated epitope, were also ACPA-positive (Table 2). Among ACPA-positive RA patients, 50% displayed antibodies against cVim 59-74 and 12% against cVim1-15. The highest percentage of reactivity was found for cFib β , for which 70% of the ACPA-positive RA patients displayed reactivity. The cEno peptide was recognized by 34% of the patients and cMBP by 65%. Almost all ACPA-positive individuals were also positive for MCV and CCP3 as described before ³⁷.

Table 2. Percentage of individuals positive for different fine specificity epitopes.

	RA (max n=658)	Anti-CCP2-positive (max n=345)	Anti-CCP2-negative (max n=92)
cVim 1-16+	42/652 (6%)	42/342 (12%)	0/89 (0%)
cVim 59-74+	172/658 (26%)	171/345 (50%)	1/92 (1%)
cFib α+	90/658 (14%)	90/345 (26%)	0/92 (0%)
cFib β+	235/644 (37%)	234/334 (70%)	1/89 (1%)
cEnolase+	118/658 (18%)	116/345 (34%)	2/92 (2%)
cMBP+	224/658 (34%)	223/345 (65%)	1/92 (1%)
ANF+	237/386 (61%)	195/298 (65%)	42/88 (48%)
0-3 cit reactivities #	396/597 (66%)	84/284 (30%)	91/92 (99%)
4-8 cit reactivities #	201/597 (33%)	200/284 (70%)	1/92 (1%)

An association between the presence of HLA SE alleles and the recognition of virtually all citrullinated peptides was found when analyzing all RA patients, ACPA-positive and ACPA-negative (Table 3). The strongest association for HLA SE alleles was found for the recognition of the Vim59-74 peptide (OR=8.4 (95% CI 4.8-14.8)). Similar associations were found for other citrullinated reactivities. To determine whether the presence of the HLA SE alleles influences the recognition of specific citrullinated epitopes, we performed similar analyses in the ACPA-positive stratum only. In doing so, we control for the possibility that the results described above are explained by the known association between HLA SE alleles and ACPA status, rather than by an influence of the HLA SE alleles on specific epitope recognition. Within the ACPA-positive subset we still found a significant association between HLA SE alleles and some, but not all citrullinated epitopes (Table 4). This suggests that HLA SE alleles are involved in shaping the immune response against specific citrullinated antigens.

Likewise, we performed a similar analysis to investigate the association between TE and the recognition of citrullinated peptides. This analysis revealed smaller, but similar associations (Table 3). The strongest association was found between cEno-recognition and TE, with an OR of 3.0 (95% CI 1.9-4.9). To analyze whether the observed association between TE and the recognition of citrullinated epitopes was also specific for the

Table 3. Odds ratio for developing antibodies against citrullinated epitopes in the presence of HLA SE alleles or TE.

	SE-positive N		N total		%	OR	95% CI	Smoking N	N Total	%	OR	95% CI
CCP2-	153	293	52.2	1	(ref)			117	300	39.0	1	(ref)
CCP2+	270	335	80.6	3.80*	2.67 – 5.42			170	309	55.0	2.08*	1.51 – 2.88
CCP-	151	290	52.1	1	(ref)			116	297	39.1	1	(ref)
CCP+cVim1-16+	33	41	80.5	3.80*	1.70 – 8.50			23	37	62.2	2.79*	1.38 – 5.65
CCP+cVim1-16-	233	288	80.9	3.90*	2.68 – 5.67			145	268	54.1	2.00*	1.43 – 2.81
CCP-	153	292	52.4	1	(ref)			116	299	38.8	1	(ref)
CCP+cVim59-74+	148	164	90.2	8.40*	4.78 – 14.78			86	150	57.3	2.31*	1.55 – 3.44
CCP+cVim59-74-	120	168	71.4	2.27*	1.51 – 3.41			83	157	52.9	1.93*	1.30 – 2.85
CCP-	153	293	52.2	1	(ref)			117	300	39.0	1	(ref)
CCP+cFib α+	67	87	77.0	3.07*	1.77 – 5.31			39	78	50.0	1.70*	1.03 – 2.81
CCP+cFib α-	201	245	82.0	4.18*	2.81 – 6.23			130	229	56.8	2.24*	1.57 – 3.18
CCP-	150	289	51.2	1	(ref)			116	296	39.2	1	(ref)
CCP+cFib β+	184	227	81.1	3.97*	2.65 – 5.94			115	205	56.1	2.16*	1.50 – 3.11
CCP+cFib β-	75	95	78.9	3.48*	2.02 – 5.99			49	92	53.3	1.93*	1.20 – 3.09
CCP-	152	291	52.2	1	(ref)			116	298	38.9	1	(ref)
CCP+cEno+	98	110	89.1	7.47*	3.93 – 14.19			64	100	64.0	3.04*	1.90 – 4.87
CCP+cEno-	170	222	76.6	2.99*	2.03 – 4.40			105	207	50.7	1.76*	1.23 – 2.52
CCP-	153	292	52.4	1	(ref)			116	299	38.8	1	(ref)
CCP+cMBP+	183	213	85.9	5.54*	3.54 – 8.68			116	199	58.3	2.40*	1.66 – 3.47
CCP+cMBP-	85	119	71.4	2.27*	1.44 – 3.60			53	108	49.1	1.66*	1.06 – 2.58
CCP-	93	186	50.0	1	(ref)			65	179	36.3	1	(ref)
CCP+cCCP3+	227	275	82.5	4.73*	3.10 – 7.23			138	245	56.3	2.26*	1.52 – 3.36
CCP+cCCP3-	10	17	58.8	1.43	0.52 – 3.91			9	17	52.9	1.97	0.73 – 5.36
CCP-	77	159	48.4	1	(ref)			55	153	35.9	1	(ref)
CCP+MCV+	224	273	82.1	4.87*	3.14 – 7.55			139	244	57.0	2.36*	1.56 – 3.58
CCP+MCV-	12	18	66.7	2.13	0.76 – 5.96			7	17	41.2	1.25	0.45 – 3.46

Table 3. Odds ratio for developing antibodies against citrullinated epitopes in the presence of HLA SE alleles or TE. (continued)

	SE-positive		N total	%	OR	95% CI	Smoking N	N Total	%	OR	95% CI
	N										
CCP-	94	175	53.7	1	(ref)		68	172	39.5	1	(ref)
CCP+RF+	210	260	80.8	3.62*	2.36 – 5.55		138	234	59.0	2.20*	1.47 – 3.29
CCP+RF-	32	38	84.2	4.60*	1.83 – 11.55		15	36	41.7	1.09	0.53 – 2.27
CCP-	27	45	60.0	1	(ref)		17	45	37.8	1	(ref)
CCP+ANF+	155	189	82.0	3.04*	1.51 – 6.14		97	172	56.4	2.13*	1.09 – 4.18
CCP+ANF-	81	102	79.4	2.57*	1.20 – 5.53		54	94	57.4	2.22*	1.07-4.61
CCP-	153	293	52.2	1	(ref)		117	300	39.0	1	(ref)
CCP+4-8 reactivities #	167	197	84.8	5.09*	3.24 – 8.00		100	172	58.1	2.17*	1.48 – 3.18
CCP+0-3 reactivities #	58	81	71.6	2.31*	1.35 – 3.94		40	77	51.9	1.69*	1.02 – 2.80

OR=odds ratio, CI=confidence interval, SE-positive=presence of one or two shared epitope alleles. ACPA-negative individuals were used as reference group. #Number of different citrullinated epitopes recognized per individual, including cVim1-16, cVim59-74, cFib α , cFib β , cEno5-20, cMBP, MCV and CCP3. * Statistically significant effect at 0.05 level of significance.

Table 4. Odds ratio for developing antibodies against citrullinated epitopes in the presence of HLA SE alleles or TE within the ACPA-positive stratum.

	SE-positive N	N total	%	OR	95% CI	Smoking N	N Total	%	OR	95% CI
cVim1-16+	33	41	80.5	0.97	0.43 – 2.23	23	37	62.2	1.39	0.69 – 2.83
cVim1-16-	233	288	80.9			145	268	54.1		
cVim59-74+	148	164	90.2	3.70*	2.00 – 6.84	86	150	57.3	1.20	0.76 – 1.88
cVim59-74-	120	168	71.4			83	157	52.9		
cFib α+	67	87	77.0	0.73	0.40 – 1.33	39	78	50.0	0.76	0.46 – 1.28
cFib α-	201	245	82.0			130	229	56.8		
cFib β+	184	227	81.1	1.14	0.63 – 2.07	115	205	56.1	1.12	0.68 – 1.84
cFib β-	75	95	78.9			49	92	53.3		
cEno+	98	110	89.1	2.50*	1.27 – 4.91	64	100	64.0	1.73*	1.06 – 2.82
cEno-	170	222	76.6			105	207	50.7		
cMBP+	183	213	85.9	2.44*	1.40 – 4.25	116	199	58.3	1.45	0.91 – 2.32
cMBP-	85	119	71.4			53	108	49.1		
CCP3+	227	275	82.5	3.31*	1.20 – 9.13	138	245	56.3	1.15	0.43 – 3.07
CCP3-	10	17	58.8			9	17	52.9		
MCV+	224	273	82.1	2.29	0.82 – 6.39	139	244	57.0	1.89	0.70 – 5.13
MCV-	12	18	66.7			7	17	41.2		
RF+	210	260	80.8	0.79	0.31 – 1.99	138	234	59.0	2.01	0.99 – 4.10
RF-	32	38	84.2			15	36	41.7		
ANF+	155	189	82.0	1.18	0.64 – 2.17	97	172	56.4	0.96	0.58 – 1.59
ANF-	81	102	79.4			54	94	57.4		
0-3 reactivities #	58	81	71.6	2.21*	1.19 – 4.10	40	77	51.9	1.29	0.75 – 2.21
4-8 reactivities #	167	197	84.8			100	172	58.1		

OR=odds ratio, CI=confidence interval, SE-positive=presence of one or two shared epitope alleles. # Number of different citrullinated epitopes recognized per individual, including cVim1-16, cVim59-74, cFibα, cFibβ, cEno5-20, cMBP, MCV and CCP3. * Statistically significant effect at 0.05 level of significance.

recognition of certain epitopes rather than the known association between TE and ACPA status, we analyzed the association within the ACPA-positive stratum. Only an association remained between TE and cEno-recognition ($OR = 1.7$ (95% CI 1.1-2.8)), while the other citrullinated epitopes were no longer significantly associated with TE (Table 4). This may indicate that TE might play a modest role in broadening of the immune response against some, but not all, citrullinated antigens.

Effect of risk factor interaction on ACPA fine specificity

Next we wished to investigate the effect of biological interaction between HLA SE alleles and smoking with regard to ACPA fine specificity. The recognition of most fine specificity peptides indicated biological interaction between those two risk factors when using the ACPA-negative patients as reference (Table 5). Significant interaction was present in the recognition of cVim59-74, cFib β and cMBP. The interaction effects were most prominent in the subset of people recognizing cEno ($OR=16$ (95% CI 6.1-42.7), $RERI=9.8$ (95% CI -0.5-20.1)) and cVim59-74 molecules ($OR=14$ (95% CI 6.3-31.5) $RERI=7.8$ (95% CI 0.5-15.1)) (Figure 1A). Even stronger effects were observed when individuals carrying two HLA SE alleles were analyzed for recognition of cVim59-74, yielding an OR of 32.0 (95% CI 10.9-94.1), $RERI=19.7$ (95% CI -10.3-49.7)) as depicted in Figure 2.

Intriguingly, interaction effects were also noted in the subset of ACPA-positive subjects that did not react with for example, cVim1-16. This observation indicated that the interactions detected are mainly explained by the known effects between HLA SE alleles and TE on ACPA status. To control for this possibility we next performed similar analyses after stratifying for ACPA status, in the ACPA-positive subjects only. We reasoned that if the interaction would only affect specific reactivities the interaction should be detectable in the total ACPA-positive subset as well. Remarkably no significant effects were observed anymore after stratification for ACPA status (Table 6). For example HLA SE alleles and TE no longer showed a significant interaction on the recognition of cVim59-74 with a combined effect OR of 4.0 (95% CI 1.7-9.6) ($RERI=0.3$ (95% CI -0.5-0.7)), which is comparable to the association found for HLA SE alleles and cVim59-74 recognition only (i.e.: $OR = 3.7$ (95% CI 2.0-6.8)). Similar effects were observed for the other fine specificity reactivities (Figure 1B).

In agreement with the results presented above, the AP due to interaction was comparable among all the citrullinated reactivities and ACPA (Figure 3). As the AP was calculated before stratification for ACPA, these results indicate that the interaction effects between TE and HLA SE alleles did not affect the fine specificity, but rather the ACPA status. Only cVim1-16 displayed a slightly higher AP, but this might be a spurious finding due to the small number of RA patients recognizing cVim1-16 (6%). Analyzing the interaction between TE and only HLA DRB1*04 SE alleles resulted in similar findings with strong interactive effects disappearing after stratification for ACPA status (data not shown).

Table 5. Risk of autoantibody-positive disease conferred by the presence of HLA SE alleles, TE and their interaction.

	OR SE	OR smoking	OR SE+smoking	RERI	AP	S
CCP2+	2.74 (1.67 – 4.48)	1.14 (0.61 – 2.11)	5.99 (3.61 – 9.95)	3.11 (0.87 – 5.36)	0.52 (0.29 – 0.75)	2.66 (1.30 – 5.45)*
cVim1-16+	1.07 (0.35 – 3.33)	0.26 (0.03 – 2.18)	5.40 (2.05 – 14.19)	5.07 (0.66 – 9.48)	0.94 (0.70 – 1.18)	-6.55 (N/A)
cVim1-16-	3.03 (1.80 – 5.08)	1.26 (0.66 – 2.40)	6.10 (3.57 – 10.44)	2.82 (0.45 – 5.20)	0.46 (0.21 – 0.72)	2.24 (1.14 – 4.39)*
cVim59-74+	6.10 (2.74 – 13.60)	1.18 (0.39 – 3.60)	14.10 (6.31 – 31.48)	7.81 (0.51 – 15.12)	0.55 (0.32 – 0.78)	2.48 (1.34 – 4.57)*
cVim59-74-	1.67 (0.95 – 2.93)	1.09 (0.55 – 2.18)	3.49 (1.97 – 6.18)	1.73 (0.19 – 3.28)	0.50 (0.17 – 0.82)	3.29 (0.69 – 15.61)
cFib α+	2.49 (1.14 – 5.45)	1.24 (0.46 – 3.34)	4.54 (2.06 – 9.99)	1.82 (-0.68 – 4.31)	0.40 (-0.03 – 0.83)	2.05 (0.65 – 6.47)
cFib α-	2.80 (1.62 – 4.86)	1.03 (0.50 – 2.12)	6.59 (3.77 – 11.51)	3.75 (1.08 – 6.43)	0.57 (0.35 – 0.79)	3.05 (1.37 – 6.78)*
cFib β+	2.97 (1.67 – 5.28)	1.31 (0.64 – 2.68)	6.52 (3.63 – 11.70)	3.24 (0.40 – 6.08)	0.50 (0.21 – 0.79)	2.42 (1.06 – 5.52)*
cFib β-	2.08 (1.01 – 4.27)	0.71 (0.25 – 1.97)	4.65 (2.28 – 9.47)	2.86 (0.44 – 5.29)	0.62 (0.31 – 0.92)	4.66 (0.65 – 33.35)
cEno+	5.40 (2.00 – 14.62)	1.89 (0.55 – 6.52)	16.10 (6.07 – 42.74)	9.81 (-0.53 – 20.14)	0.61 (0.38 – 0.84)	2.85 (1.40 – 5.83)
cEno-	2.28 (1.35 – 3.86)	0.98 (0.50 – 1.94)	4.24 (2.47 – 7.28)	1.98 (0.26 – 3.70)	0.47 (0.18 – 0.76)	2.57 (0.96 – 6.89)
MBP+	3.63 (1.94 – 6.79)	1.18 (0.52 – 2.70)	9.15 (4.88 – 17.17)	5.34 (1.30 – 9.38)	0.59 (0.37 – 0.80)	2.90 (1.45 – 5.79)*
MBP-	1.90 (1.00 – 3.59)	1.05 (0.47 – 2.36)	3.18 (1.65 – 6.10)	1.23 (-0.40 – 2.85)	0.39 (-0.04 – 0.82)	2.30 (0.53 – 9.92)
MCV+	3.40 (1.84 – 6.29)	1.32 (0.62 – 2.79)	10.77 (5.48 – 21.18)	7.05 (1.12 – 12.99)	0.65 (0.44 – 0.87)	3.59 (1.63 – 7.91)*
MCV-	1.47 (0.39 – 5.54)	0.76 (0.13 – 4.40)	2.67 (0.65 – 10.93)	1.44 (-1.67 – 4.55)	0.54 (-0.31 – 1.39)	7.38 (0.0 – >100)
CCP3+	3.20 (1.78 – 5.76)	1.16 (0.55 – 2.44)	9.15 (4.88 – 17.17)	5.80 (1.24 – 10.35)	0.63 (0.42 – 0.84)	3.46 (1.58 – 7.62)*
CCP3-	1.58 (0.36 – 6.93)	2.06 (0.43 – 9.74)	2.48 (0.52 – 11.85)	-0.15 (-4.02 – 3.73)	-0.06 (-1.65 – 1.53)	0.91 (0.08 – 10.35)
RF+	2.34 (1.28 – 4.30)	1.16 (0.56 – 2.42)	6.23 (3.33 – 11.66)	3.73 (0.80 – 6.66)	0.60 (0.36 – 0.84)	3.48 (1.28 – 9.48)*
RF-	3.41 (1.07 – 10.87)	0.33 (0.04 – 3.10)	4.63 (1.40 – 15.33)	1.89 (-1.72 – 5.50)	0.41 (-0.20 – 1.01)	2.08 (0.40 – 10.75)
ANF+	2.13 (0.82 – 5.56)	1.09 (0.34 – 3.54)	5.49 (1.92 – 15.65)	3.26 (-1.19 – 7.70)	0.59 (0.18 – 1.01)	3.66 (0.57 – 23.40)
ANF-	1.32 (0.47 – 3.73)	0.83 (0.23 – 3.03)	4.26 (1.42 – 12.82)	3.10 (-0.60 – 6.80)	0.73 (0.34 – 1.11)	20.79 (0 – >100)
4-8 reactivities #	4.10 (2.09 – 8.03)	1.55 (0.67 – 3.59)	9.69 (4.93 – 19.05)	5.05 (0.67 – 9.43)	0.52 (0.28 – 0.77)	2.39 (1.25 – 4.54)*
0-3 reactivities #	1.51 (0.73 – 3.14)	0.88 (0.35 – 2.25)	3.14 (1.53 – 6.45)	1.74 (0.00 – 3.48)	0.55 (0.15 – 0.96)	5.37 (0.15 – 192.71)

The interaction was considered statistically significant when the confidence intervals of the RERI and AP excluded 0 and the Synergy index excluded 1. N/A= Not applicable. Anti-CCP2-negative individuals were used as reference category.

#Number of different citrullinated epitopes recognized per individual, including cVim1-16, cVim59-74, cFibα, cFibβ, cEno5-20, cMBP, MCV and CCP3.

* Statistically significant effect at 0.05 level of significance.

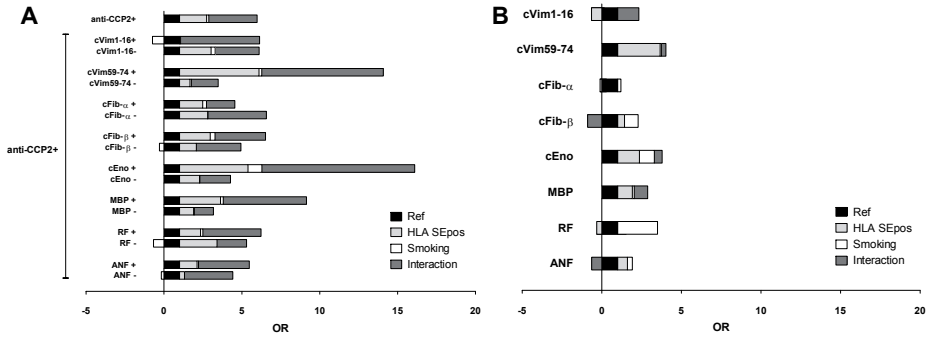


Figure 1: Risk of autoantibody-positive disease conferred by HLA SE positivity, TE and their interaction. Bars are colored black from OR 0 to 1, light grey to indicate the risk conferred by the presence (1 or 2 alleles) of HLA SE alleles, white for the risk conferred by TE and dark grey for the interaction. Analyses were performed using the ACPA-negative individuals as reference group (1A) and the same analysis was performed within the ACPA-positive stratum (1B).

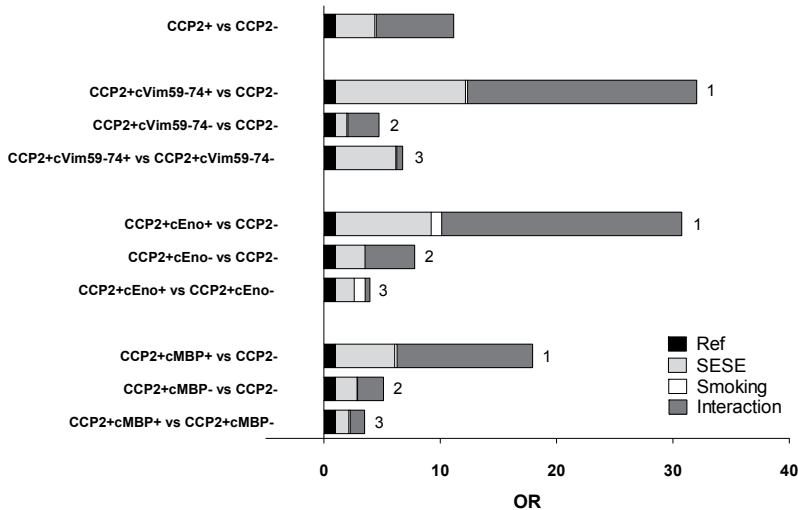


Figure 2: Risk of autoantibody-positive disease conferred by HLA SE homozygosity (SESE), TE and their interaction.

Bars are colored black from OR 0 to 1, light grey to indicate the risk conferred by the presence of two HLA SE alleles, white for the risk conferred by TE and dark grey for the interaction. Analyses were performed using the ACPA-negative individuals as reference group (bars 1 and 2) and the same analysis was performed within the ACPA-positive stratum (bar 3).

Moreover, the interaction effects between HLA SE status and TE observed in the unstratified analysis for recognition of more than 3 citrullinated epitopes (OR 9.7 (95% CI 4.9-19.1), RERI=5.1 (95% CI 0.7-9.4)) also became smaller and non significant after stratification for ACPA (OR 3.1 (95% CI 1.3-7.3), RERI=-0.4 (95% CI -3.0-2.2)). Similarly, the subset of patients carrying two HLA SE alleles recognizing for example cVim59-74 did

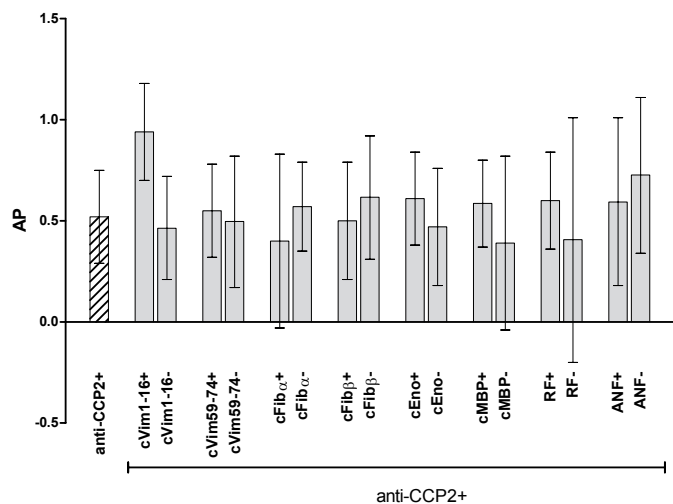


Figure 3: Attributable proportion due to interaction between HLA SE alleles and TE.
Attributable proportion due to interaction was calculated by dividing the relative excess risk due to interaction by the total risk of autoantibody positive disease in patients with 1 or 2 HLA SE alleles and TE (AP=RER1/R11) for the different fine specificity reactivities. All the reactivities show a similar attributable proportion compared to anti-CCP2 positive individuals. ACPA-negative individuals were used as reference group.

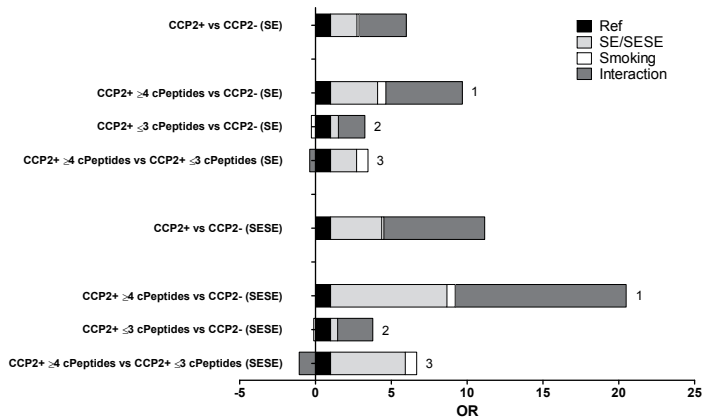


Figure 4: Risk of autoantibody-positive disease with recognition of a limited or extended number of citrullinated epitopes conferred by HLA SE-alleles, TE and their interaction.
Bars are colored black from OR 0 to 1, light grey to indicate the risk conferred by the presence of (1 or 2) HLA SE alleles or HLA SE homozygosity (SESE), white for the risk conferred by TE and dark grey for the interaction. Analyses were performed using the ACPA-negative individuals as reference group (bars 1 and 2) and the same analysis was performed within the ACPA-positive stratum (bar 3). Recognising 0-3 citrullinated epitopes was considered limited recognition and recognising 4-8 citrullinated peptides as extended recognition. Eight epitopes were included in this analysis (cVim1-16, cVim59-74, cFibα, cFibβ, cEno5-20, cMBP, CCP3 and MCV).

Table 6. Risk of autoantibody-positive disease conferred by the presence of HLA SE alleles, TE and their interaction within the ACPA-positive stratum.

	OR SE	OR TE	OR SE+TE	RERI	AP	S
cVim1-16	0.36 (0.11 – 1.14)	0.20 (0.02 – 1.81)	0.89 (0.33 – 2.39)	1.33 (0.63 – 2.02)	1.50 (0.34 – 2.66)	0.08 (0.00 – >100)
cVim59-74	3.67 (1.51 – 8.88)	1.08 (0.32 – 3.66)	4.04 (1.71 – 9.55)	0.29 (-2.02 – 2.59)	0.07 (-0.50 – 0.65)	1.10 (0.47 – 2.57)
cFib α	0.89 (0.38 – 2.10)	1.20 (0.39 – 3.69)	0.69 (0.30 – 1.59)	-0.40 (- 1.92 – 1.13)	-0.58 (-2.59 – 1.43)	-3.56 (N/A)
cFib β	1.43 (0.63 – 3.24)	1.86 (0.59 – 5.89)	1.40 (0.64 – 3.07)	-0.88 (- 3.18 – 1.42)	-0.63 (-2.14 – 0.88)	0.31 (0.04 – 2.34)
cEno	2.37 (0.84 – 6.72)	1.93 (0.51 – 7.27)	3.80 (1.38 – 10.41)	0.50 (- 2.00 – 2.99)	0.13 (-0.54 – 0.80)	1.22 (0.40 – 3.73)
MBP	1.92 (0.87 – 4.21)	1.13 (0.40 – 3.20)	2.88 (1.34 – 6.22)	0.84 (- 0.76 – 2.45)	0.30 (-0.25 – 0.84)	1.81 (0.37 – 8.83)
MCV	2.32 (0.60 – 8.93)	1.74 (0.29 – 10.51)	4.04 (1.01 – 16.18)	0.98 (- 3.84 – 5.79)	0.24 (-0.83 – 1.32)	1.48 (0.19 – 11.65)
CCP3	2.03 (0.45 – 9.10)	0.56 (0.11 – 2.84)	3.69 (0.78 – 17.54)	2.10 (-2.25 – 6.45)	0.57 (-0.18 – 1.32)	4.57 (0.03 – >100)
RF	0.69 (0.21 – 2.24)	3.50 (0.36 – 33.82)	1.35 (0.41 – 4.45)	-1.84 (-9.53 – 5.84)	-1.37 (-6.81 – 4.08)	0.16 (0.00 – 12.89)
ANF	1.61 (0.67 – 3.87)	1.31 (0.42 – 4.13)	1.29 (0.56 – 2.96)	-0.64 (-2.52 – 1.25)	-0.49 (-1.81 – 0.82)	0.31 (0.03 – 3.41)
0-3 vs 4-8 reactivities #	2.72 (1.11 – 6.65)	1.75 (0.55 – 5.58)	3.09 (1.31 – 7.30)	-0.38 (-2.99 – 2.24)	-0.12 (-0.97 – 0.72)	0.85 (0.29 – 2.43)

The interaction was considered statistically significant when the confidence intervals of the RERI and AP excluded 0 and the Synergy index excluded 1. N/A= Not applicable. # Number of different citrullinated epitopes recognized per individual, including cVim1-16, cVim59-74, cFibα, cFibβ, cEno5-20, cMBP, MCV and CCP3.

not show signs of interaction anymore after stratification (Figure 4). Together these data indicate that an interaction between HLA SE alleles and TE contributes to ACPA status, but is not likely to contribute to the recognition of specific citrullinated antigens.

DISCUSSION

Our results show that the apparent interaction between HLA SE alleles and TE for ACPA fine specificity disappears after stratification for ACPA status, indicating that this interaction does not greatly affect the fine specificity of the ACPA-response.

Recently it has been suggested that a gene-environment interaction is present between HLA SE alleles and TE in shaping an autoimmune response specifically directed against a peptide derived from enolase ²³. Upon further experimentation similar results were found with regard to the recognition of other citrullinated epitopes that were derived from vimentin and fibrinogen ²⁴. Nonetheless, these findings could be of relevance as interactions between genotype, smoking and autoimmunity to certain citrullinated antigens could further expand our understanding of RA pathogenesis.

However, in these cases no stratification for ACPA status was performed, allowing the possibility that the interaction effects were not explained through their influence on the formation of auto-immune reactions against specific citrullinated antigens, but rather on the formation of ACPA. In this manuscript we specifically analyzed this possibility. Careful analyses of these original data published by us and Mahdi et al ^{23,24}, suggested that no such interaction effects were present as, for example, the AP was similar for ACPA-positive disease in the presence but importantly also in the absence of a specific ACPA reactivity. Therefore, we reasoned that if such interaction effects would only shape the response to certain specific, but not to other citrullinated epitopes, these effects should still be observed in the ACPA-positive stratum as well. We now show that this is not the case as the OR's observed in the interaction analysis were similar to the OR's observed for the presence of HLA SE alleles only after stratification for ACPA status. Together, these data thus indicate that the gene-environment interaction between TE and HLA SE alleles does not seem to influence the reactivity of the ACPA response. Rather, the presence of the HLA SE alleles seems to be the main factor in shaping the antigen recognition of the ACPA response as the association between HLA SE alleles and certain fine specificities like cVim59-74 and cEno remained, even after stratification for ACPA status, as has been reported before ¹⁷. Thus, these data indicate that ACPA fine specificity recognition is mainly dependent on HLA SE status, and that TE and the interaction between HLA SE alleles and TE have a relatively minor contribution. These data also suggest that smoking promotes non-specific citrullination rather than citrullination of specific antigens.

The findings presented in this manuscript add to the discussion on the role of TE and possibly other environmental triggers such as infection with certain pathogens, in the pathogenesis of RA. For example, it has been postulated that the fact that immunity to some citrullinated antigens associates strongly with both TE and SE alleles argues in favor of the hypothesis that smoking may induce citrullination of certain proteins in the lungs and that immunity to these post-translationally modified proteins may be determined by certain HLA alleles ³⁸. This hypothesis is intriguing and warrants further investigation, also because it would incriminate certain citrullinated proteins and/or epitopes in the break of tolerance against citrullinated antigens. However, our data now indicate that the presumed interaction effects described by us and by Mahdi et al ^{23,24} should not be taken as an argument to support this hypothesis, but rather that the main driver of ACPA fine specificity is found in the HLA SE alleles. In contrast, the interaction effects between HLA SE alleles and smoking are more likely to be confined to the predisposition to ACPA-positive disease.

A potential limitation of our study is that the results are not corrected for multiple testing, allowing the possibility that some associations are false-positive. However, the (large) OR's found are in line with previously published data ^{17,23}. Likewise we cannot formally exclude the presence of interaction between HLA SE alleles and TE for the peptides analyzed, due to insufficient power. The observed RERI for e.g. cVim59-74 (i.e.: 0.29 (95% CI -2.02 – 2.59) is statistically non-significant ³⁹. Clearly a large number of patients would be required to obtain significant RERI values. The confidence interval surrounding this RERI further indicates that it is unlikely that a RERI above 2.6 is or will be found in larger studies. Thus even if such an interaction would exist, it is modest compared to the previous analyses and does not have a very strong influence on the constitution of the fine specificity repertoire. In contrast, our data indicate that the HLA SE status is mainly driving the observations. Although our data indicate that no clear link exists between environmental and genetic risk factors in the development of autoimmunity against specific citrullinated antigens, other potential citrullinated autoantigens will be awaiting further investigation and clearly we cannot exclude that such an interaction is involved in the development of antibodies against other citrullinated antigens. This would especially be the case when the analyses are focused on 'heavy smoking' as cumulative smoking exposure might affect such a presumed specific interaction. We did not perform such an analysis because of insufficient power.

All together our findings indicate that gene-environment interactions play no major role in shaping the reactivity of the ACPA response by broadening the ACPA recognition profile, but rather that the HLA SE alleles do have a significant contribution to the ACPA recognition profile.

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Chapter 12

Value of anti-modified citrullinated vimentin and third-generation anti-cyclic citrullinated peptide compared with second-generation anti-cyclic citrullinated peptide and rheumatoid factor in predicting disease outcome in undifferentiated arthritis and rheumatoid arthritis

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ABSTRACT

Objective Autoantibodies such as rheumatoid factor (RF) and the second generation anti-cyclic citrullinated peptide autoantibodies (anti-CCP2) are frequently measured in clinical practice because of their association with disease outcome in undifferentiated arthritis (UA) and rheumatoid arthritis (RA). Recently two new tests were developed: anti-CCP3 and anti-modified citrullinated vimentin autoantibodies (anti-MCV). To facilitate the decision of which autoantibody to test in daily practice, this study evaluates aforementioned autoantibodies and combinations of them for predicting three outcome measures: progression from UA to RA, the rate of joint destruction and achieving sustained DMARD-free remission in RA.

Methods 625 UA patients were studied for the progression to RA after 1 year. 687 RA patients were studied for achieving sustained DMARD-free remission and the rate of joint destruction during a median follow-up of 5 years. Positive predictive values (PPVs) for RA development and the associations with the disease course in RA were compared for single tests (anti-CCP2, anti-CCP3, anti-MCV, RF) and for combinations.

Results Using a single test in UA patients revealed that anti-CCP2 tended to have the highest PPV for RA development (67.1%), but the 95% confidence intervals of the other tests overlapped. Using a single test in RA, all tests showed comparable associations with the rate of joint destruction and achievement of remission. In ACPA-positive and ACPA-negative RA, RF-presence did not associate with more joint destruction. For all outcome measures, combining two autoantibody tests did not increase the predictive accuracy compared to performing one test.

Conclusion For clinical practice, a single autoantibody test is sufficient for risk estimation in UA and RA.

INTRODUCTION

Rheumatoid arthritis (RA) is considered to have an autoimmune origin because of the presence of self-reactive autoantibodies. In addition to rheumatoid factor (RF), which is to date the only serologic measure included in the 1987 American College of Rheumatology criteria for RA, several other autoantibodies have been described in recent years.¹ The discovery of anti-citrullinated protein autoantibodies (ACPA) has led to the development of various new tests for autoantibodies in RA. The first generation anti-cyclic citrullinated-peptide (anti-CCP) test, directed against a synthetic citrullinated peptide, revealed a higher specificity than RF (91-96% vs. 74-91%).²⁻⁶ Subsequently, a commercially available second generation anti-CCP test (anti-CCP2) was developed, showing an even better specificity (90-97%).³⁻⁸ RF and anti-CCP2 autoantibodies can also be present in the preclinical phase and are associated with future RA development.^{9,10} Consequently, tests for anti-CCP2 and RF are nowadays widespread used as diagnostic tools in clinical practice.

Recently two other serological tests emerged, anti-CCP3 and anti-MCV. The anti-CCP3 test has been reported to have sensitivities and specificities comparable to anti-CCP2 (69-83% and 93-95% respectively).^{8,11} The second novel autoantibody test targets modified citrullinated vimentin (MCV). This test has its origin in the older anti-Sa autoantibody test that has been shown to target citrullinated vimentin.¹² Compared to anti-CCP2, studies reported somewhat lower specificities and higher sensitivities for anti-MCV (79-92% and 70-84%).^{3-5,13}

The aforementioned data were obtained by case-control studies comparing RA patients with non-RA patients or healthy individuals and the resulting test characteristics quantify the proportion of patients that are identified as positive by the test (sensitivity) or the proportion of healthy individuals that are identified as negative (specificity). As such, these measures, as well as the likelihood ratio of a test, provide information on the quality of the test. In clinical practice, the value of determining ACPA or RF relates to their ability to predict the disease course. The chance for an individual patient to have a certain disease course is expressed by the positive predictive value (PPV) and negative predictive value (NPV). A clinical state in which knowledge on the presence of RF and ACPA can be particularly helpful is undifferentiated arthritis (UA). In this subgroup of early arthritis patients no diagnosis can be established according to existing classification criteria and the presence of RF or anti-CCP2 indicates an increased risk for RA development.^{14,15} Thus far the PPV and NPV for the risk to develop RA in UA have not been studied for anti-CCP3 and anti-MCV and the four autoantibodies have not been subjected to a head-to-head comparison. Furthermore, the additive value of testing several combinations of autoantibodies for the prediction of RA development in individual UA patients has not been addressed. Therefore, the first aim of this study is to compare anti-CCP2, anti-CCP3,

anti-MCV and RF in the prediction of the RA development in patients with UA and to explore whether testing combinations of autoantibodies increases the predictive accuracy.

RF and anti-CCP2 are not only important predictors for RA development, but are also some of the most potent predictors for the outcome of RA, as measured by the rate of radiological joint destruction.¹⁶⁻¹⁸ Thus far, only one study compared radiological progression for anti-MCV and anti-CCP2 in 273 RA patients and provided suggestive evidence that anti-MCV is a better predictor than anti-CCP2.¹⁹ The effect of testing combinations of all four autoantibodies however was not studied. Thus, the second aim of the present study is to compare anti-CCP2, anti-CCP3, anti-MCV and RF in the prediction of the rate of joint destruction and to explore whether combinations of autoantibodies can increase the predictive ability, making use of a longitudinal cohort of 687 RA patients with a median follow-up of 5 years.

A second disease outcome of RA is the achievement of remission which with the introduction of new aggressive treatment modalities has increasingly become an attainable goal. We chose a strict definition and defined sustained disease-modifying antirheumatic drugs (DMARD)-free remission as the persistent absence of synovitis for at least 1 year after cessation of DMARD-therapy.²⁰ Since the predictive value of the four autoantibodies in relation to remission is scarcely explored, the present study compares the four tests for their ability to predict sustained DMARD-free remission in RA patients treated with conventional DMARDs.

In summary, to support the choice on which autoantibody to test in daily practice, this study uses a large longitudinal cohort to evaluate the value of determining anti-CCP2, anti-CCP3, anti-MCV and RF for predicting three outcome measures: progression from UA to RA, the rate of joint destruction and the chance of achieving sustained DMARD-free remission in RA. In addition, the predictive value of combining several tests is investigated.

PATIENTS AND METHODS

Patients

All patients included in this study are selected from the Leiden Early Arthritis Clinic (EAC) cohort that was started in 1993.²¹ Patients were referred by general practitioners when arthritis was suspected. Inclusion took place when arthritis was confirmed at physical examination and symptom duration was less than 2 years. Written informed consent was obtained from all participants. The study was approved by the local Medical Ethical Committee.

At inclusion, patients were asked about their joint symptoms and subjected to a physical examination. Blood samples were taken for routine diagnostic laboratory screening

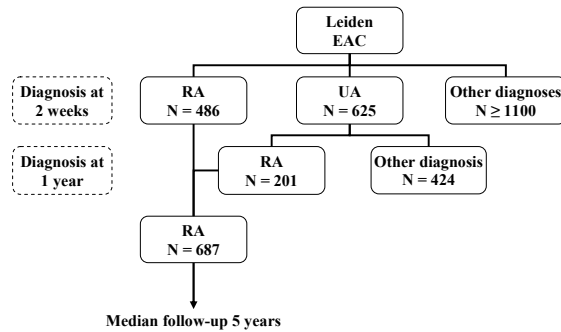


Figure 1: Flowchart of the study.

Of the 687 RA patients, 579 had radiographic data available and for 635 patients information on sustained DMARD-free remission was available. Of all UA patients RF, anti-CCP2, anti-CCP3 and anti-MCV were determined in 623, 624, 597 and 597 patients respectively. Of all RA patients with radiographs available RF, anti-CCP2, anti-CCP3 and anti-MCV were determined in 572, 565, 544 and 544 patients respectively. Of all RA patients with data on sustained DMARD-free remission, RF, anti-CCP2, anti-CCP3 and anti-MCV were determined in 615, 603, 579 and 579 patients respectively

(including IgM-RF) and stored to determine other autoantibodies at a later time. Follow-up visits were performed on a yearly basis and included radiographs of hands and feet.

Since the start of the EAC treatment strategies for RA have changed; four different strategies were applied depending on the inclusion period. Patients included between 1993 and 1995 were treated initially with analgesics and subsequently with chloroquine or salazopyrin if they had persistent active disease (delayed treatment).²² From 1996 to 1998 RA patients were promptly treated with either chloroquine or salazopyrin (early treatment).^{21,22} From 1998 to 2002 patients were promptly treated with either salazopyrin or methotrexate (early treatment) and patients included in 2002 or later were promptly treated with either salazopyrin or methotrexate combined with treatment adjustments based on the disease activity (early and disease activity based treatment). Treatment of UA patients was not protocolized.

UA was defined as not fulfilling one of the existing classification criteria for rheumatologic diagnoses two weeks after the first presentation.²³ Thus other rheumatic diseases like Sjögren's syndrom, psoriatic arthritis, spondylarthropathies, etc. that were established at baseline were excluded (Figure 1). 625 patients with UA, consecutively included between 1993 and 2006, were studied. After 1 year of follow-up, 201 patients (32.2%) had progressed to RA, fulfilling the 1987 ACR criteria for RA. In addition, 687 patients with RA included between 1993 and 2006 were studied for their disease outcome; 486 patients fulfilled the ACR criteria for RA already at inclusion and 201 patients were initially diagnosed with UA and developed RA within the first year of follow-up (Figure 1).

Autoantibodies

IgM–RF was determined by enzyme-linked immunosorbent assay (ELISA). Anti-CCP2 autoantibodies (total IgG) were measured by ELISA (Immunoscan RA Mark 2; Euro-Diagnostica, Arnhem, The Netherlands). The cutoff level for anti-CCP2 autoantibody-positivity was set at 25 arbitrary units, according to the manufacturer's instructions. Anti-CCP3 autoantibodies (IgA and IgG subforms) and anti-MCV autoantibodies were measured with ELISA as well (Quanta lite CCP 3.1 IgG/IgA, INOVA Diagnostics Inc., San Diego, CA, USA and Orgentec Diagnostika GmbH, Mainz, Germany respectively). According to the manuals, the cutoff level for both tests was 20 arbitrary units. The numbers of patients in which RF, anti-CCP2, anti-CCP3 and anti-MCV measurements were performed varied slightly (available data for RF, anti-CCP2, anti-CCP3 and anti-MCV were respectively 665, 653, 629 and 629 out of 687 RA patients and 623, 624, 597 and 597 out of 625 UA patients). This is due to the difference in timing of performing the tests. RF was determined routinely at inclusion whereas the ACPA were determined using stored serum samples. The recently introduced anti-CCP3 and anti-MCV assays were performed on all available sera in 2008, whereas the anti-CCP2 assay was performed earlier.

Radiographs

Radiographs of hands and feet were taken on consecutive years starting at baseline and were scored according to the Sharp-van der Heijde method.²⁴ From all RA patients, 579 had data on both autoantibodies and radiographs. To encompass a reliable sample size during follow-up, radiographic data were restricted to a maximum of 7 years of follow-up. The number of available radiographs varied per time-point and declined from 552 at baseline to 478 after 1 year, 426, 358, 299, 270, 207 and 156 after 2 until 7 years of follow-up respectively. Due to the study design (an inception cohort) not all patients had a similar duration of follow-up (median 5 years). All radiographs were scored by one experienced scorer (ML) who was blinded with respect to the patient's autoantibody status, treatment and other clinical data. Scoring was performed with known time order, which is more sensitive to change compared to scoring with unknown time sequence.²⁵ From the total number of scored radiographs, 499 radiographs were rescored by the same reader, consisting of 149 baseline radiographs and 350 radiographs during follow-up belonging to 60 randomly selected RA patients. The intraobserver intraclass correlation coefficients were 0.91 for all scored radiographs, 0.84 for baseline radiographs and 0.97 for the radiographic progression rate.

Sustained DMARD-free remission in RA

Remission was defined in its most stringent form as the persistent absence of synovitis for at least one year after cessation of DMARD therapy and the identification of remission by the patient's rheumatologist.²⁶ The remission status could be reliably ascertained in 635

RA patients. Most patients who achieved remission were followed-up longer than the minimum requirement of 1 year; the median time of observation after discontinuation of DMARDs in the absence of swollen joints was 2.5 years. Patients who had a recurrence of their arthritis after discharge, could easily return to the Leiden University Medical Center, the only referral center for Rheumatology in a health care region of approximately 400.000 inhabitants. The frequency of relapse was recorded and patients with relapse were included in the non-remission group (n=6).

Statistical analysis

The PPV (proportion of UA patients with a positive test that progressed to RA) and the NPV (proportion of UA patients that did not develop RA) were determined.

As radiographic data are not normally distributed, non-parametric Mann-Whitney tests were used to compare the Sharp-van der Heijde scores at individual time points for patients with and without autoantibodies or for autoantibody combinations. In addition, to take advantage of the prospective character of the data consisting of repeated measurements, and to avoid multiple testing by performing statistical tests for each time point, a linear mixed model with an autoregressive correlation structure with heterogeneous variances was used. This model estimates the linear progression rate in radiological joint destruction using normalized, log-transformed Sharp-van der Heijde scores, taking missing observations into account. This means that it compares the progression rates for the different patient groups. In the mixed model analyses, corrections were applied for age, gender and inclusion period/treatment strategy. Correction for treatment strategy was performed by including the inclusion period in the linear mixed model. This was done because treatment modalities improved over time and an influence of the treatment strategy (reflected by the inclusion period) on the progression of radiographic joint damage was observed previously as well as in the present study (data not shown). In addition, the available follow-up duration differed between patients and therefore the number of radiographs per time point decreased during follow-up. As the patients with the longest follow-up were included in the earliest inclusion period and thus have been treated with the least aggressive treatment strategy, correction for inclusion period was performed. In order to prevent overfitting of the data no corrections were applied for other variables. Analysis of sustained DMARD-free remission was performed by Cox regression analysis, to take into account the differences in follow-up times among patients. For patients that achieved remission the dependent variable is the "time-to-event", indicating the time until reaching remission. For non-remission patients the time to last follow-up was used, with a maximum of 10 years. SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) was used. P-values <0.05 were considered significant. All reported p-values are two-sided.

RESULTS

Progression from UA to RA

From the total EAC, consisting of >2,000 patients with early arthritis, UA patients (n=625) were selected and studied for progression to RA after 1 year of follow-up. At inclusion, the mean age was 50.9 ($\pm$ 17.0) years, 371 (59.1%) patients were female and the self-reported symptom duration was 5.5 ($\pm$ 8.5) months. Anti-CCP2, anti-CCP3, anti-MCV and RF were present in 149 (23.9%), 172 (28.7%), 199 (31.7%) and 155 (24.8%) patients respectively. The presence of autoantibodies overlapped; UA patients who tested positive for anti-CCP2 were also frequently positive for other autoantibodies (Figure 2).

The PPV was compared for the four autoantibodies (Table 1). Anti-CCP2 had the highest PPV, 67.1%, compared to 64.0%, 56.3% and 61.7% for anti-CCP3, anti-MCV and RF respectively. The NPVs of all four tests were comparable (~80%). Thus, in case one auto-

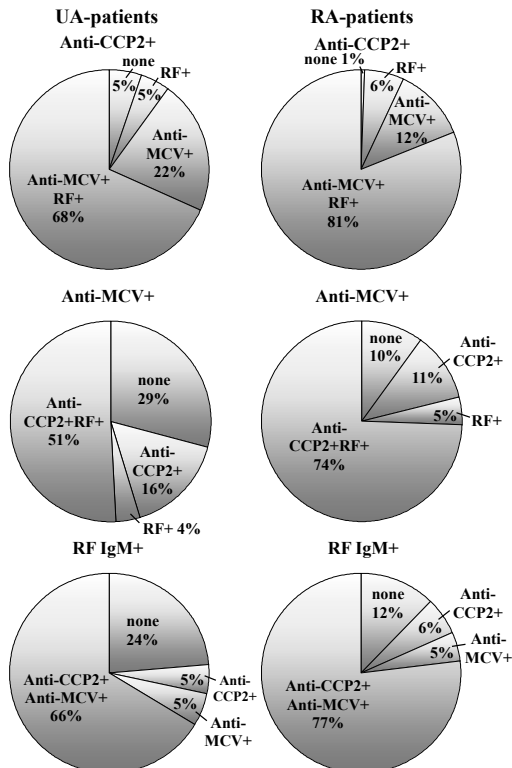


Figure 2: Prevalence of other autoantibodies in case of a positive test result for anti-CCP2, anti-MCV or RF for both UA and RA.

The prevalence of other autoantibodies given one positive autoantibody test is depicted. This figure was derived using 596 UA and 540 RA patients for whom data on all autoantibodies was present. None: no other autoantibodies present.

Table 1. Positive predictive value, negative predictive value, sensitivity, specificity and likelihood ratios of anti-CCP2, anti-CCP3, anti-MCV and RF for predicting progression from UA to RA.

Autoantibody Test	PPV		NPV		Sensitivity		Specificity		Likelihood Ratio	
	% (95%CI)	True pos./all pos.tests	% (95%CI)	True neg./all neg. tests	% (95%CI)	True pos./all RA	% (95%CI)	True neg./all non-RA	LR+	LR-
Anti-CCP2	67.1 (59.6-74.7)	100/149	79.0 (75.3-82.6)	375/475	50.0 (43.1-56.9)	100/200	88.4 (85.4-91.5)	375/424	4.33	0.57
Anti-CCP3	64.0 (56.8-73.1)	110/172	80.0 (76.2-83.8)	340/425	56.4 (49.5-63.4)	110/195	84.6 (81.1-88.1)	340/402	3.66	0.52
Anti-MCV	56.3 (49.4-63.2)	112/199	79.2 (75.2-83.1)	315/398	57.4 (50.5-64.4)	112/195	78.4 (74.3-82.4)	315/402	2.66	0.54
RF	61.7 (54.0-69.4)	95/154	77.8 (74.1-81.6)	365/469	47.7 (40.8-54.7)	95/199	86.1 (82.8-89.4)	365/424	3.43	0.61

PPV: positive predictive value; NPV: negative predictive value; 95%CI: 95% Confidence Interval; pos.: positive; neg.: negative; LR: likelihood ratio

antibody test is performed, a positive anti-CCP2 test tends to correlate with the highest risk of RA development, but overlapping 95%CI's hamper a definite differentiation.

Next it was determined whether performing two autoantibody tests results in a better estimation of the risk for RA than performing one test. Since anti-CCP2 and anti-CCP3 are related tests and anti-CCP2 positive patients were in 90% also positive for anti-CCP3, the possible combinations of anti-MCV, anti-CCP2 and RF were first assessed. The proportions of patients that developed RA in case of two positive test results (PPV) were: anti-CCP2+/anti-MCV+ 69.9% (95%CI 62.1-77.7), anti-CCP2+/RF+ 74.1% (95%CI 65.8-82.3) and anti-MCV+/RF+ 70.6% (95%CI 62.1-79.2). Additional analyses using anti-CCP3 instead of anti-CCP2 yielded comparable results (data not shown). Altogether, these data show that when two tests are performed none of these combinations is clearly superior to the other. Furthermore, no additive value of performing two instead of one autoantibody test could be observed.

When performing a single autoantibody test, no information about the presence or absence of the other autoantibodies is obtained. However, the eventual coexisting presence of other unmeasured autoantibodies can affect the risk for RA. To determine the risk for RA as conferred to by the individual autoantibodies or by the number of autoantibodies, the PPVs for progression to RA were determined in the group of 587 UA patients for whom information on all three autoantibodies was available. The difference with above mentioned data is that the presence of two autoantibodies now indicates that the third is absent, whereas in above mentioned data only two tests were performed and the third autoantibody test could be positive as well as negative. The PPV for RA development in patients without autoantibodies was 17.9% (95%CI 13.9-22.0). In the presence of one autoantibody the PPV was 27.5% (95%CI 17.9-35.0) and increased significantly to 61.2%

Table 2. Positive predictive values of the number of positive antibodies for progression from UA to RA.

Number of Positive Antibodies	PPV % (95% CI)	True pos./ all pos. tests	Antibody Combinations	PPV % (95% CI)	True pos./ all pos. tests
0	17.9 (13.9-22.0)	61/340	-	-	-
1	27.5 (18.8-36.1)	28/102	Anti-CCP2+/anti-MCV-/RF-	12.5 (-10.4-35.4)	1/8
			Anti-CCP2-/anti-MCV+/RF-	29.3 (17.6-41.0)	17/58
			Anti-CCP2-/anti-MCV-/RF+	27.8 (13.2-42.4)	10/36
2	61.2 (47.6-74.9)	30/49	Anti-CCP2+/anti-MCV+/RF-	58.1 (40.7-75.4)	18/31
			Anti-CCP2+/anti-MCV-/RF+	83.3 (53.5-113.2)	5/6
			Anti-CCP2-/anti-MCV+/RF+	58.3 (30.4-86.2)	7/12
3	71.9 (62.9-80.9)	69/96	-	-	-

These data are based on the patients with data available for all three antibody-tests (anti-CCP2, anti-MCV and RF, n=587). UA: undifferentiated arthritis; PPV: positive predictive value; 95%CI: 95 % Confidence Interval; pos.: positive.

(95%CI 47,6-74,9) in the presence of two autoantibodies (Table 2). The PPV was the highest in the presence of 3 autoantibodies (71.9%, 95%CI 62,9-80.9).

In conclusion, when performing one autoantibody test in clinical practice, none of the four tests is clearly superior. Although the presence of 2 autoantibodies significantly increased the risk for RA compared to the presence of 1 autoantibody, for clinical use performing two tests does not significantly increase the predictive performance compared to performing one test. This finding is likely explained by the presence of other, non-measured autoantibodies that affect the risk for RA.

Joint destruction in RA

Baseline characteristics of the 579 studied RA patients were: mean age 56.2 ($\pm$ 15.5) years, 405 patients (69.9%) were female, anti-CCP2, anti-CCP3, anti-MCV and RF were present in 313 (55.4%), 322 (59.2%), 331 (60.8%) and 334 (58.4%) patients respectively. Anti-CCP2 positive patients also tested positive for anti-MCV and RF in 93% and 87% of cases respectively (Figure 2).

First, the association between autoantibody-positivity and rate of joint destruction was assessed when performing only one autoantibody test. For all autoantibody tests (anti-CCP2, anti-CCP3, anti-MCV and RF) a positive test result was associated with a higher Sharp-van der Heijde score at all time points except baseline (M-W, $p < 0.001$) and a higher rate of joint destruction over a period of 7 years (mixed model, $p < 0.001$) compared to a negative test result. Figure 3A shows that there is no difference among the four tests with regard to predictive ability for joint destruction.

Next, it was investigated whether the addition of a second autoantibody test increased the predictive value for the rate of joint destruction. As depicted in Figure 3B no dif-

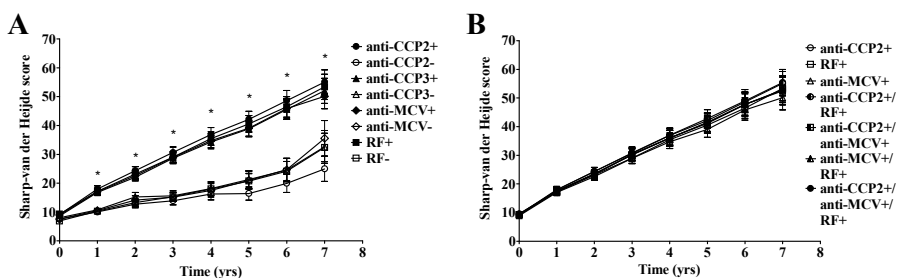


Figure 3: Mean Sharp-van der Heijde scores ($\pm$ SEM) for the different autoantibody tests (A) and for combinations of positive tests (B).

The number of patients in Figure 3A for anti-CCP2-positive/negative, anti-CCP3-positive/negative, anti-MCV-positive/negative and RF-positive/negative was respectively 313/252, 322/222, 331/213 and 334/238.

*Mann-Whitney, $p < 0.001$, for comparison of positive with negative test. For all four tests the p-value was < 0.001 using mixed models analysis.

In Figure 3B, for anti-CCP2+, RF+, anti-MCV+, anti-CCP2+/RF+, anti-CCP2+/anti-MCV+, anti-MCV+/RF+ and anti-CCP2+/anti-MCV+/RF+ the number of patients was 313, 334, 331, 271, 283, 259 and 244 respectively.

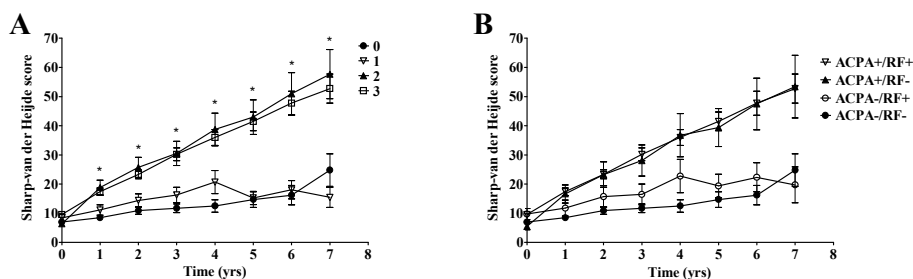


Figure 4: Mean Sharp-van der Heijde scores ($\pm$ SEM) for the number of positive autoantibody tests (A) and for the effect of RF in the presence or absence of ACPA (B).

Figure 4A: depicted are the mean Sharp-van der Heijde scores for the number of positive autoantibody tests (with the other tests negative) using data for RF, anti-CCP2 and anti-MCV (total $n=540$). The number of patients positive for 0, 1, 2 or 3 autoantibodies was 152, 74, 70 and 244 respectively. Comparing 3 with 0, 3 with 1, 2 with 1 and 2 with 0 autoantibodies revealed a $p<0.05$ at all time points except baseline (M-W) and a $p<0.001$ for the 7 year period (mixed model).

Figure 4B: depicted are the mean Sharp-van der Heijde scores for the following groups ACPA-RF-, ACPA-RF+, ACPA+RF- and ACPA+RF+. The patients numbers per group were 152, 39, 36 and 244. $P=0.864$ for comparison of ACPA-RF- with ACPA-RF+ and $p=0.702$ for comparison ACPA+RF- and ACPA+RF+ (both mixed model)

ferences were seen between testing positive for anti-CCP2, anti-MCV or RF alone and combinations of these autoantibodies. These results indicate that for clinical use, a positive test for one of these autoantibodies predicts a severe disease course, and a second or third autoantibody test does not increase the predictive accuracy. Testing for anti-CCP3 instead of anti-CCP2 gave comparable results (data not shown).

To identify the contribution of the individual autoantibodies to the association with the rate of joint destruction, the effect of the number of positive autoantibodies (with the known absence of the other autoantibodies) was investigated using data on RF, anti-CCP2 and anti-MCV. The presence of either 2 or 3 autoantibodies was associated with a higher rate of joint destruction compared to 0 or 1 autoantibody (Figure 4A) (mixed model, $p<0.001$ for both 2 and 3 compared to 0 as well as 1 autoantibody). No significant difference was observed between the presence of 2 or 3 autoantibodies or between 0 and 1 autoantibody. It should be noted that the group with 1 autoantibody consisted almost exclusively of patients that were anti-MCV+ ($n=33$) or RF+ ($n=39$); only two patients were positive for anti-CCP2 and negative for anti-MCV and RF.

To more specifically investigate the role of RF in relation to ACPA (anti-CCP2, anti-CCP3 or anti-MCV) and the rate of joint destruction, the additional effect of RF in the presence or absence of ACPA was determined (Figure 4B). This revealed that in the presence of ACPA, but also in the absence of ACPA, RF did not significantly contribute to the rate of joint destruction.

Sustained DMARD-free remission in RA

From a total of 635 RA patients, 78 patients achieved sustained DMARD-free remission after a median follow-up of 39.5 months. These 78 patients had a mean age of 59.4 (± 15.7) years and 57 patients (73.1%) were female. Anti-CCP2 autoantibodies were present in 11.8% and anti-CCP3, anti-MCV autoantibodies and RF in 21.9%, 28.8% and 25.0%.

The four autoantibody tests were compared for their association with the achievement of remission (Figure 5A). The Hazard Ratio (HR) of each of the four tests for not achieving sustained DMARD-free remission was 11.6 (95%CI 5.8-23.4) for anti-CCP2, 6.0 (95%CI 3.4-10.4) for anti-CCP3, 4.9 (95%CI 3.0-8.2) for anti-MCV and 4.7 (95%CI 2.8-8.0) for RF.

Subsequently, the additive value of performing two autoantibody tests compared to one test was investigated. The HRs were 15.6 (95%CI 6.7-36.4), 14.0 (95%CI 6.4-31.0) and 11.5 (95%CI 5.4-24.5) for combinations of anti-CCP2 and RF, anti-CCP2 and anti-MCV and anti-MCV and RF respectively. These data indicate that to predict the chance of remission, performing two tests has no additional value compared to anti-CCP2 alone.

To investigate whether the number of present autoantibodies affected the chance of achieving sustained DMARD-free remission, the HRs were determined for the presence of 1, 2 or 3 positive autoantibodies (with the other autoantibodies known to be absent) using data on anti-CCP2, anti-MCV and RF (Figure 5B). The HRs for not achieving sustained DMARD-free remission were as follows, 3.7 (95%CI 1.1-12.3), 15.5 (95%CI 5.9-41.2) and 17.1 (95%CI 6.8-43.3) for the presence of, respectively, 1, 2, and 3 autoantibodies compared to no autoantibodies. Thus, however the 95%CIs were overlapping, the results suggestively indicate that the more autoantibodies are present, the lower the chance is to achieve sustained DMARD-free mission.

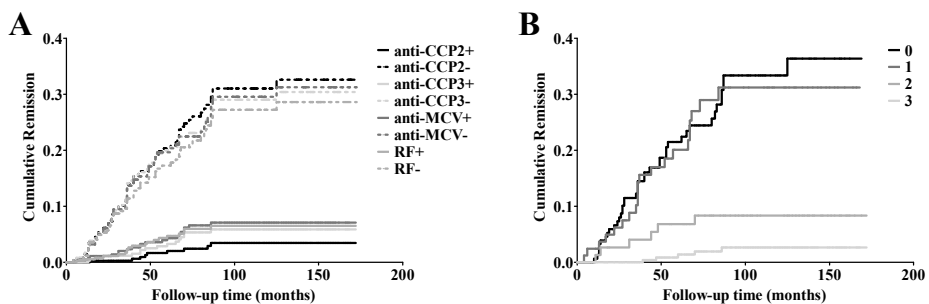


Figure 5: Effect of the presence of a positive autoantibody test (A) and the number of autoantibodies (B) on sustained DMARD-free remission.

DISCUSSION

Among the autoantibodies tested in RA, only RF and ACPA are considered clinically useful. In clinical practice a physician or patient is interested in the chance for the individual to progress to RA or not, given a positive or negative test result respectively. These risks are reflected by the PPV and NPV. In addition, in early arthritis the predictive value of these autoantibody tests is valued the most in patients in whom at presentation no definite diagnosis can be established (UA), as only one-third of these patients progress to RA after one year.¹⁴ Also in RA, the autoantibody tests form one of the most potent predictors of the severity of the future disease course. As such, information on the results of the autoantibody tests can influence treatment decision in individual patients with UA and RA.²³ Nevertheless, it is thus far unknown which test, or which combination of tests, is most powerful in predicting the progression from UA to RA and the disease progression in RA and therefore the present study was undertaken.

To evaluate the prediction from UA to RA using a single test, the PPVs of the four tests were compared. This revealed that a positive anti-CCP2 test tended to have the highest predictive value (PPV 67% compared to 62% and 56% for RF and anti-MCV), but due to overlapping 95% CIs a definite differentiation could not be made. In addition, performing a second test did show a tendency for a higher chance on RA development in case both tests were positive (highest risk for RA in anti-CCP2+, RF+ patients, PPV 74%), but also here the 95% CIs overlapped. To formally conclude whether the PPVs of 67% and 74% are statistically significantly different or not, >1800 UA-patients would be required (using a p-value of 5% and a power of 90%). Based on the present data it can be concluded that addition of a second test does not result in an increased predictive accuracy. Notably, UA patients who tested positive for anti-CCP2 were also frequently positive for other autoantibodies (Figure 2). Thus the finding of comparable prognostic performances of one or two tests is likely due to co-existing presence of autoantibodies that are unmeasured with a single test but that do affect the risk-estimation for RA.

The development of RA was assessed after 1 year of follow-up. This time-point was chosen in order to have a similar duration of follow-up for all studied patients. However, this may have introduced misclassification as patients with UA may have progressed to RA after more than 1 year of follow-up. With all available follow-ups, 25 patients (4.4%) progressed to RA later than 1 year, indicating that the current PPVs may be marginally underestimated. Since this misclassification is present in the total group of UA patients this does not hamper a comparison of tests.

Two measures were studied for the severity of the disease course in RA: achieving sustained DMARD-free remission and the level of radiological joint destruction during a median follow-up of 5 years. Although Figure 3A and 5A may lead to the impression that anti-CCP2 positive patients have a higher rate of joint destruction and achieve sustained

DMARD-free remission less frequently than patients positive for the other autoantibodies, these differences were not statistically significant. Similar to the data on RA development, performing a second test appeared not to result in a more accurate prediction for the disease outcome in RA. This observation, which is in contrast to an earlier report,²⁷ can be explained by the presence of non-measured autoantibodies that are associated with a progressive course of RA.

To obtain a more detailed comprehension of the current results, the contribution of the individual autoantibodies to disease progression (RA development, joint destruction and achievement of remission) was investigated. This showed that the presence of two autoantibodies indicated a significantly increased risk compared to the presence of one autoantibody. However, the group with one autoantibody present consisted mostly of anti-MCV or RF-positive patients. Patients that were positive only for anti-CCP2 were very rare. Therefore it cannot be excluded that the increased risk in the presence of two compared to one autoantibody is due to the effect of anti-CCP2 rather than to the effect of an additional autoantibody. Nevertheless, in general it was observed that a higher number of autoantibodies present resulted in a higher risk for RA or for progressive disease. This is in line with recent published data showing that a broader autoantibody response is associated with disease progression.²⁸

As the association of RF with the presence of RA is primarily explained by its association with ACPA,²⁹ we investigated whether we could observe a similar effect for the progression of joint destruction in RA. Intriguingly, the rate of joint destruction both in the ACPA-positive and ACPA-negative groups was not affected by the presence or absence of RF. This finding further supports the notion that RF does not, by itself, contribute to disease progression.

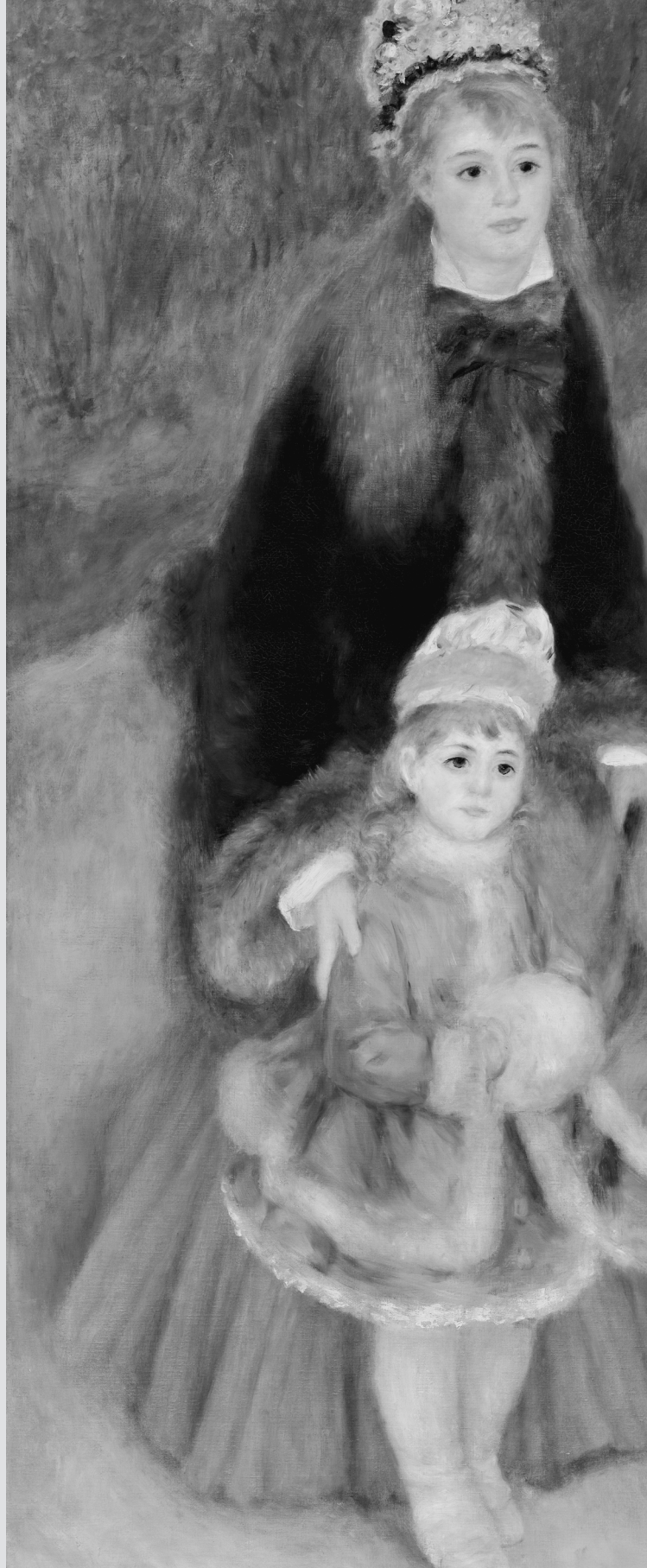
In order to get an impression of the effect of anti-MCV itself on the rate of joint destruction, RA patients that were positive only for anti-MCV ($n=33$) were studied. This revealed that the rate of joint destruction was comparable to that of the patients with no autoantibodies (data not shown), indicating that anti-MCV alone does not strongly affect the level of joint damage in RA.

In conclusion, our results indicate that for risk estimation of the disease course in clinical practice, performing a single autoantibody test is sufficient, both in UA and RA.

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Part III

Remission



Chapter 13

Prevalence of and predictive factors for sustained disease-modifying antirheumatic drug-free remission in rheumatoid arthritis

Results from two large early arthritis cohorts

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ABSTRACT

Objective Remission has become an attainable goal of rheumatoid arthritis (RA) treatment, especially since the advent of biological anti-rheumatic therapy. Because little is known about patients who achieve remission with conventional treatment, we used two large independent inception cohorts to study the prevalence and predictive factors for disease modifying anti-rheumatic drug (DMARD)-free sustained remission after treatment with conventional therapy.

Methods Remission was assessed in patients from the Leiden Early Arthritis Clinic (EAC, n=454) and the British Early RA study (ERAS, n=895) who fulfilled the 1987 ACR criteria for RA and were treated with conventional therapy. Sustained DMARD-free remission was defined as fulfilling the following criteria for at least one year: 1) no current DMARD-use, 2) no swollen joints and 3) classification as remission by the patient's rheumatologist. Predictive factors were identified by Cox regression analysis.

Results Sustained DMARD-free remission was achieved by 68/454 (15%) of patients in the EAC and 84/895 (9.4%) in the ERAS. Five factors were associated with sustained DMARD-free remission in both cohorts: acute onset, short symptom duration before inclusion, non-smoking, absence of IgM rheumatoid factor and of HLA shared epitope alleles. In the ERAS, low disease activity at baseline was also predictive of remission. Multivariate analyses revealed symptom duration and the absence of autoantibodies (anti-CCP2/RF) as independent predictors.

Conclusion Sustained DMARD-free remission in RA patients treated with conventional therapy is not uncommon. Symptom duration at presentation and the absence of autoantibodies are associated with sustained DMARD-free remission.

INTRODUCTION

Patients with rheumatoid arthritis (RA) vary considerably in terms of their disease course and outcome. The spectrum of possible disease outcomes extends from debilitating, destructive joint disease on one side, to remission, the most favorable outcome, on the other side ¹. Since the introduction of biological anti-rheumatic treatment, remission is increasingly being reported as a primary disease outcome of new therapeutic trials ². In addition to the potent suppression of disease activity which can be achieved with these novel treatment agents, the recent insight that early initiation of anti-rheumatic therapy leads to better long-term outcomes ^{3,4}, has led to a shift in the current goals of RA treatment towards aiming for remission.

When studying remission in RA, there are several aspects of remission which need to be taken into account and which are inconsistently defined and used in the literature. The first inconsistency involves the duration of the remission. Clinical trials frequently use a disease activity score (DAS)-based definition of remission which does not require a specified follow-up period, but can be fulfilled at one single point in time.

The second factor which needs to be taken into account is the use of anti-rheumatic treatment. Remission can be interpreted as either a state of minimal disease activity while using anti-rheumatic treatment, or as persistent resolution of the disease after discontinuation of therapy ^{5,6}. The DAS-based remission criteria do not require remission to be drug-free, because they were intended to monitor treatment response in clinical trials ^{7,8}. Remission rates as reported in clinical trials therefore often reflect the number of patients which achieve minimal disease activity while using novel therapeutic agents. Data on how often remission persists after discontinuation of treatment are scarce ⁹, though this may better reflect disease resolution.

A third aspect of remission studies is the type of treatment which was administered. Despite the fact that remission rates after treatment with new therapeutic agents are now often reported, there are few data on remission after treatment with conventional therapy such as non-steroidal anti-inflammatory drugs (NSAIDs) and non-biological disease modifying antirheumatic drug (DMARD) therapy. Such studies would however, allow a better interpretation of the remission rates reported by clinical trials of novel agents.

In this study, we investigated the prevalence and prognostic factors for sustained DMARD-free remission in two large, independent inception cohorts of RA patients treated with conventional therapy. In order to investigate remission as a definitive disease outcome, stringent criteria were used to define remission: the sustained absence of synovitis for at least one year after the discontinuation of therapy with DMARDs. This definition is an approximation of a definitive cure of the disease, and as such, is close to the meaning of remission as it is used for other diseases such as malignancies.

Investigating remission as a definitive disease outcome, resembling cure, is very important from a pathophysiological point of view. Knowledge of which clinical or immunological patient characteristics are associated with remission could fuel new hypotheses about the biological pathways involved in disease persistence and resolution, and would increase our understanding of the disease course of RA.

METHODS

Patient population

The study population consisted of two cohorts: the Leiden Early Arthritis Clinic (EAC) and the British Early RA Study (ERAS).

The Leiden EAC is an inception cohort of patients with recent-onset arthritis (less than 2 years of complaints) that was initiated at the department of rheumatology of the Leiden University Medical Center in 1993 ¹⁰. The present study included patients who fulfilled the 1987 revised ACR criteria for RA at baseline (n=369) or within the first year of follow-up (n=85), and who presented to the EAC between 1993 and 2002. The treatment strategy differed according to the inclusion period. Patients included between 1993 and 1995 were initially treated with analgesics and subsequently with chloroquine or sulfasalazine if they had persistent active disease. Between 1996 and 1998, patients who were included were promptly treated with chloroquine or sulfasalazine, while after 1998 the initial treatment strategy consisted of either methotrexate or sulfasalazine. Patients included in the EAC after January 2003 were not part of the current study because, due to their limited duration of follow-up, they only had a short period of time to achieve sustained DMARD-free remission, and therefore had a high risk of misclassification. These patients who presented after January 2003 did not differ markedly from the patients who were included earlier with regard to the rate and the predictive factors of sustained DMARD-free remission (data not shown), but they were not part of the present study because of the high risk of misclassification as mentioned above. Follow-up visits with standard clinical assessments were performed two weeks after the first presentation and yearly thereafter. Demographic characteristics, a 44 swollen joint count (SJC) and the modified Stanford Health Assessment Questionnaire (HAQ) were recorded at each visit. Laboratory evaluation consisted of erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) measurements, determination of IgM rheumatoid factor (RF) and anti-cyclic citrullinated peptide 2 antibodies (anti-CCP2). Furthermore, genotyping of the HLA-DRB1 region was performed to determine the number of RA-associated Shared Epitope (SE) alleles ¹¹. Radiographs of hand and feet were scored according to the Sharp-van der Heijde scoring method (SHS) ¹².

To determine if the prevalence and predictive factors which were identified in the Leiden EAC cohort could be replicated, a second inception cohort was used: the ERAS. This cohort consists of RA patients who were recruited from 9 rheumatology departments in the UK from 1986 until 1996. During this period, consecutive patients who presented with RA according to the ACR criteria were included in the ERAS if their symptoms of RA had lasted less than 2 years and no second-line antirheumatic medication had been used. ERAS patients were treated according to the rheumatologist's preference, which generally entailed a short course of analgesics, followed by sequential monotherapy or combination therapy for more severe RA with methotrexate, sulfasalazine and hydroxychloroquine as favored drugs.

Baseline and yearly assessments comprised of the Ritchie articular index, a 44 SJC, the HAQ and measurements of ESR and IgM RF. Disease activity scores (DAS) were calculated according to the original formula ¹³. In addition, HLA-DRB1 genotyping was performed. Radiographs of hand and feet were scored according to the Larsen scoring method ¹⁴. In contrast to the EAC, measurements of CRP and anti-CCP were not performed in the ERAS.

Both in the ERAS and the EAC, a number of patients failed to complete follow-up: $n=136$ (23%) in the EAC and $n=384$ (30%) in the ERAS. The most common reasons in both cohorts were death, moving and withdrawal of consent. Because the definitive disease outcome (remission versus non-remission) could not be accurately determined for the deceased patients, the present report shows the results of the analyses without these patients. However, when the deceased patients were included in the non-remission group, the identified predictive factors did not change (data not shown). In total, 454 EAC patients and 895 ERAS patients were included in the present study.

Definition of remission

Sustained DMARD-free remission was defined according to the following three criteria: 1) no current use of DMARDs, 2) no swollen joints and 3) classification as DMARD-free remission by the patient's rheumatologist. Corticosteroids were considered to be equivalent to DMARDs for the present study, while NSAIDs did not qualify as DMARDs. Patients had to fulfill all three criteria in order to be diagnosed with remission. To ensure that remission was not temporary, but rather sustained and long-lasting, the absence of swollen joints had to have been observed by a rheumatologist for at least one year after discontinuation of DMARD-therapy. In both EAC and ERAS cohorts, all patients were seen approximately every four months by their rheumatologists, even when they had very low disease activity. In addition, standardized follow-up visits to collect data for the EAC and ERAS were performed annually. In the EAC, all patients with remission were discharged from the outpatient clinic after at least one year of observation after discontinuation of DMARDs in the absence of joint swelling. Most patients in the EAC who achieved remission were followed-up longer than the minimum requirement of one year; the median

time of observation after discontinuation of DMARDs in the absence of swollen joints was 2.5 years. Patients who had a recurrence of their arthritis after discharge, could easily return to the Leiden University Medical Center, the only referral center for Rheumatology in a health care region of approximately 400.000 inhabitants. The frequency of relapse was recorded and patients with relapse ($n=6$) were included in the non-remission group. In the ERAS, the majority of patients who had achieved remission continued to undergo their yearly assessments, which provided an opportunity to assess if there was continued absence of joint swelling.

Statistical analysis

Summary statistics were generated to investigate the prevalence of sustained DMARD-free remission and the baseline characteristics of remission and non-remission patients. Patients included in the ERAS had a longer average period of follow-up than patients in the EAC, due to the fact that the ERAS was initiated in 1986, while the EAC was started in 1993. To avoid skewing of the results due to the difference in follow-up time, the present analysis used data from the first 10 years of follow-up for all patients.

Baseline variables were assessed for their ability to predict remission by univariate and multivariate regression analysis. To take into account the difference in follow-up times among patients, analyses were performed by Cox regression analysis, after verification that the proportional hazards assumption was satisfied. In Cox regression models the dependent variable is the "time-to-event", which consisted of the time to remission for the remission patients, and the time to last follow-up (with a maximum of 10 years) for the non-remission patients. The time of remission was defined in the EAC as the date at which DMARDs were discontinued due to remission, and in the ERAS as the date of the first annual study visit when patients were in DMARD-free remission. The analysis was also performed with a later date defined as the time of remission (date described above plus one year), which led to similar results. This indicates that the predictive factors were stable regardless of the exact date used to define remission.

In order to investigate the predictive ability of baseline characteristics in univariate analysis, each variable was included as a covariate in a separate non-conditional Cox regression analysis. The results of the univariate analyses were subjected to correction for multiple testing by the Holm method ¹⁵. Subsequently, multivariate Cox regression analysis was performed to identify significant independent predictors for achieving remission. As possible explanatory variables, all baseline variables with a p-value below 0.10 in univariate analysis were included in the model. A two-step modeling approach was performed which in the first step identified independent predictive variables by a backward step selection procedure that removed variables with a p-value greater than 0.10. To verify that the identified predictive variables were indeed independent predictors

for the entire cohort, they were then entered as covariates into a second multivariate Cox regression analysis (enter model).

If there is a highly significant correlation between two variables they cannot be entered into the same multivariate model, as this results in over-correction and annulment of the effect. In both cohorts, symptom duration and radiographic damage (as measured by SHS in the EAC and Larsen score in the ERAS) were highly correlated. Symptom duration was included in the multivariate models because a longer symptom duration most likely caused a higher radiological score and not vice versa. In the ERAS, the Ritchie articular index, SJC and DAS were highly correlated due to the fact that the DAS is a component measure consisting of amongst others, the Ritchie articular index and the SJC, and therefore the DAS was omitted from the multivariate model.

In the EAC, a number of cases ($n < 50$) had missing values for baseline continuous variables, which would have resulted in their exclusion from the multivariate model. For these patients with missing values, multiple imputation analysis was performed using STATA with the *ice* and *mim* packages (StataCorp. 2007. Stata statistical software: Release 10. College Station, TX, USA). Multiple imputation was applied to the final Cox regression model identified through the preliminary variable selection step (predictive variables: symptom duration, CRP and anti-CCP). The full database was used to generate 25 imputations, each from 10 cycles and incorporating both survival and censoring information within the multiple imputation prediction equations. In the ERAS, the number of cases with missing variables was very limited and for that reason no imputation was performed.

All analyses were performed using the Statistical Package for the Social Sciences (SPSS) 14.0, except for the multiple imputation. P-values below 0.05 were considered to be statistically significant, except for the results of univariate analysis for which subsequent correction for multiple testing was performed.

RESULTS

Prevalence of sustained DMARD-free remission

The baseline characteristics of the RA patients in the EAC and ERAS are shown in Table 1. On average, ERAS patients had a longer disease duration and a higher number of swollen joints at baseline, and were more often IgM RF and HLA SE allele positive than EAC patients.

Sustained DMARD-free remission was achieved by $68/454 = 15.0\%$ of patients in the EAC and $84/895 = 9.4\%$ of patients in the ERAS. The median time to remission was 43 months in both cohorts (interquartile range (IQR) EAC: 24-67, ERAS: 18-60) (Figure 1A). A subgroup analysis was performed to investigate if the prevalence of remission in the EAC would have been different if only the 369 patients who presented with RA at baseline

Table 1. Baseline characteristics of patients in both cohorts.

	EAC n=454	ERAS n=895
Period of inclusion	1993-2003	1986-1996
Age in years, mean (SD)	56 (16)	52 (13)
Female gender	69%	69%
Symptom duration in months, mean (SD)	6.4 (9)	8.3 (6)
44 swollen joint count, median (IQR)	8 (4-14)	14 (7-25)
IgM Rheumatoid factor positive	58%	63%
HLA SE positive	66%	72%
Sharp van der Heijde score, mean (SD)	8.0 (11)	NA
Larsen score, mean (SD)	NA	3.7 (9)

NA: not available

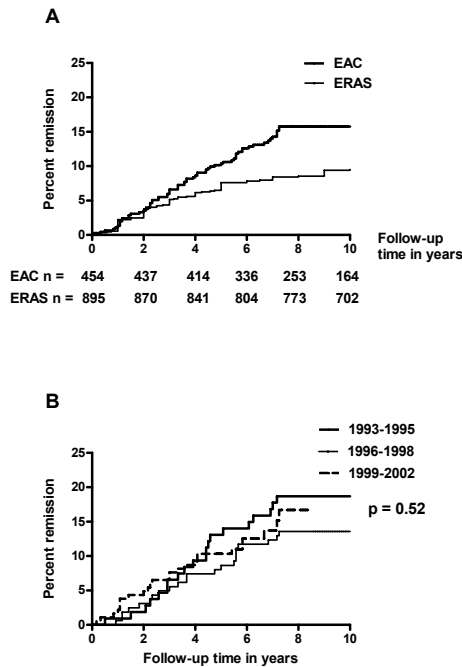


Figure 1: Kaplan-Meijer curves of the percentage of patients with sustained DMARD-free remission.

Figure: 1A: The y-axis depicts the percentage of patients who achieved remission. The x-axis reflects the follow-up time in years. Indicated below the x-axis is the number of patients available for analysis at each timepoint.

Figure 1B: Kaplan-Meijer curves of the percentage of patients in the EAC with sustained DMARD-free remission, stratified for period of inclusion. Differences between the different strata are non-significant: p-value log rank test across all strata: 0.52. The line representing the patients included from 1999 until 2002 terminates at 8 years of follow-up due to the fact that this was the maximum follow-up for patients in this stratum (data were collected in 2007).

had been included (leaving out the 85 patients who developed RA within the first year of follow-up). This revealed a similar prevalence of sustained DMARD-free remission as in the entire group: 14.9% (55/369).

The prevalence of remission steadily increased in the first part of the follow-up period until approximately seven years after inclusion, after which it remained relatively stable. To investigate if patients who were included at a later point in time and who had therefore been treated with a different treatment strategy had a higher chance of achieving remission, or achieved remission faster, analyses in both cohorts were stratified for inclusion period. This revealed that the rate of achieving remission did not differ between patients who were included in different inclusion periods (log rank test: p-value: 0.52) as shown for the EAC in Figure 1B. The same was true for patients who were included in different time periods in the ERAS (data not shown).

Univariate analysis

In order to investigate which baseline patient characteristics can predict sustained DMARD-free remission, univariate Cox regression analysis was performed. The following variables were associated with sustained DMARD-free remission in the EAC: symptom duration at baseline, smoking, CRP, RF IgM, anti-CCP2 antibodies, radiographic damage as measured by SHS, and HLA SE alleles (Table 2). A subgroup analysis of the 369 patients who presented with RA at baseline (without the 85 patients who developed RA within the first year of follow-up) again led to the same results. Of these identified factors, symptom duration at baseline, acute start of complaints, the presence of RF IgM, HLA SE alleles, and radiographic damage could be replicated in the ERAS (Figure 2).

In addition, univariate analysis in the ERAS revealed several other predictive variables (44 SJC, Ritchie articular index, HAQ and DAS4, Table 3). A sub-analysis in the ERAS on

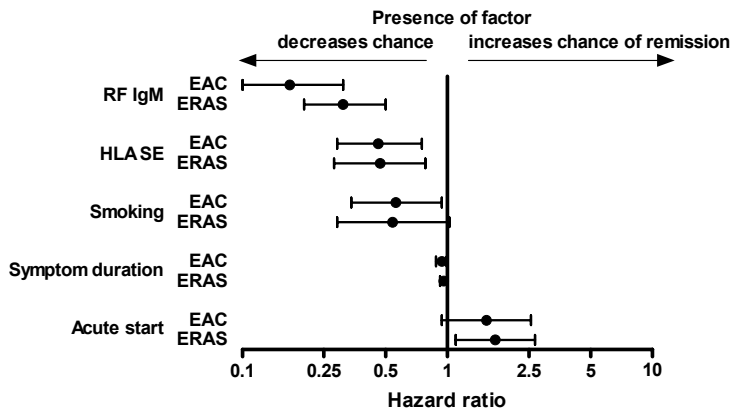


Figure 2: Predictors for sustained DMARD-free remission identified in both cohorts.
Depicted are the hazard ratio's with 95% confidence intervals.

Table 2. EAC univariate analysis: baseline characteristics of patients who did and did not achieve sustained DMARD-free remission.

Patient characteristic †	Remission n=68	No remission n=386	Hazard Ratio ‡	95% CI #	P *
Age in yrs, mean (SD)	59 (17)	56 (16)	1.02	0.99-1.03	0.067
Female gender, n (%)	50 (74%)	262 (68%)	1.28	0.74-2.19	0.38
Symptom duration at baseline in months, median (IQR)	3 (1-6)	5 (2-9)	0.94	0.88-0.99	0.021
mean (SD)	4.6 (5)	6.7 (9)			
Smoking, n (%)	22 (33%)	175 (47%)	0.56	0.34-0.94	0.028
Family history positive for RA, n (%)	12 (19%)	111 (31%)	0.55	0.30-1.04	0.064
Absence of comorbidities, n (%)	43 (64%)	233 (63%)	0.98	0.59-1.61	0.92
Acute start of complaints, n (%)	43 (64%)	192 (53%)	1.55	0.94-2.56	0.084
Start of complaints in small joints, n (%)	38 (57%)	175 (47%)	1.48	0.91-2.40	0.11
Symmetrical start of complaints, n (%)	46 (72%)	239 (67%)	1.24	0.72-2.14	0.44
44 Swollen joint count, median (IQR) §	8.0 (4-12.5)	8.0 (4-14)	1.00	0.96-1.04	0.82
mean (SD)	9.6 (7)	9.8 (7)			
HAQ, median (IQR)	1.1 (0.6-1.6)	1.0 (0.5-1.5)	1.06	0.74-1.52	0.76
mean (SD)	1.1 (0.7)	1.1 (0.7)			
ESR in mm/h, mean (SD)	38 (25)	44 (28)	0.99	0.98-1.00	0.14
CRP in mg/l, median (IQR)	17 (8-31)	20 (9-45)	0.99	0.98-1.00	0.039
mean (SD)	24 (24)	35 (40)			
RF IgM positive, n (%)	14 (21%)	247 (64%)	0.17	0.10-0.31	<0.001*
Anti-CCP2 positive, n (%)	8 (13%)	236 (66%)	0.09	0.04-0.18	<0.001*
SHS, median (IQR) ¶	3 (1-8)	5 (2-11)	0.95	0.90-0.99	0.026
mean (SD)	5 (6)	9 (12)			
HLA SE positive, n (%)	33 (50%)	247 (69%)	0.46	0.29-0.75	0.002*

† For variables which were normally distributed, mean and standard deviation (SD) are reported. For non-normally distributed variables, median and interquartile range (IQR) are reported and also the mean and standard deviation since Cox regression uses parametric analysis. For dichotomous variables, the number and the percentage of patients are listed, relative to the total number of patients for whom information about the characteristic under investigation was available.

‡ The hazard ratio, which is the effect measure generated by Cox regression analysis, can be interpreted similar to an odds ratio, meaning that a higher hazard ratio signifies a higher chance of remission.

95% CI denotes the 95% confidence interval.

* p-values below 0.05 are printed in bold. Asterisks indicate p-values significant after correction for multiple testing by the Holm method.

§ Ritchie articular index and DAS were not reported in the EAC.

¶ SHS denotes the Sharp van der Heijde score for radiographic damage.

Table 3. ERAS univariate analysis: baseline characteristics of patients who did and did not achieve sustained DMARD-free remission.

Patient characteristic	Remission n=84	No remission n=811	Hazard Ratio	95% CI	P *
Age in yrs, mean (SD)	51 (16)	52 (13)	1.00	0.98-1.01	0.58
Female gender, n (%)	53 (63%)	560 (69%)	0.78	0.50-1.22	0.28
Symptom duration at baseline in months, median (IQR)	6.0 (3-9)	7.0 (4-12)	0.96	0.92-1.00	0.038
mean (SD)	7.0 (5)	8.5 (6)			
Smoking, n (%)	13 (28%)	268 (42%)	0.54	0.29-1.02	0.059
Family history positive for RA, n (%)	20 (24%)	211 (26%)	0.87	0.53-1.44	0.59
BMI, mean (SD)	25 (4)	26 (5)	0.98	0.93-1.04	0.54
Acute start of complaints, n (%)	51 (61%)	383 (48%)	1.71	1.10-2.67	0.017*
Start of complaints in small joints, n (%)	25 (30%)	204 (25%)	1.27	0.80-2.04	0.31
Symmetrical start of complaints, n (%)	63 (81%)	615 (78%)	1.18	0.67-2.07	0.56
44 Swollen joint count, median (IQR) §	9.5 (4-21)	15 (7-26)	0.97	0.95-0.99	0.005*
mean (SD)	13 (12)	17 (13)			
Ritchie articular index, median (IQR)	4.0 (2-12)	10 (5-17)	0.91	0.88-0.95	<0.001*
mean (SD)	6.5 (6)	13 (11)			
HAQ, median (IQR)	0.6 (0.1-1.1)	1.0 (0.5-1.6)	0.51	0.36-0.71	<0.001*
mean (SD)	0.8 (0.7)	1.1 (0.7)			
ESR in mm/h, mean (SD) †	36 (30)	40 (27)	0.99	0.99-1.0	0.21
RF IgM positive, n (%)	29 (36%)	520 (66%)	0.31	0.20-0.50	<0.001*
DAS4, mean (SD)	3.3 (1)	4.3 (2)	0.65	0.55-0.76	<0.001*
Larsen score, median (IQR)	0.0 (0-2)	0.0 (0-4)	0.94	0.88-1.00	0.050
mean (SD)	1.7 (3)	3.9 (9)			
HLA SE positive, n (%)	34 (56%)	440 (74%)	0.47	0.28-0.78	0.003*

* p-values below 0.05 are printed in bold. Asterisks indicate p-values significant after correction for multiple testing by the Holm method.

† CRP and anti-CCP2 were not measured in the ERAS.

the effect of current or past smoking as compared to never smoking as the reference group, revealed that current smoking was associated with the lowest chance of achieving remission (hazard ratio (HR) with 95% confidence interval (CI): 0.27 (0.08-0.87)), followed by past smoking (HR (95%CI): 0.78 (0.39-1.58)). In both EAC and ERAS, there was not a significant gene dose effect of the HLA SE alleles.

To correct for multiple testing, the Holm method was applied which revealed that in both cohorts, autoantibody status, presence of the HLA SE alleles and, in the ERAS, the Ritchie articular index, 44 SJC, DAS and HAQ at baseline were significant predictors of remission.

Multivariate analysis

To investigate which of the patient characteristics were independent predictors for remission, multivariate Cox regression analysis was performed by a two-step modeling approach. Table 4 shows the results of the multivariate analysis for both cohorts. Three variables were found to be independent predictors of sustained DMARD-free remission in the EAC, two of which were significant ($p < 0.05$): baseline CRP and anti-CCP status. In the ERAS, these two characteristics were not available but autoantibody status in the form of IgM RF was the most significant predictive variable. Symptom duration showed a trend towards significance in the EAC ($p = 0.07$), while significantly associated with remission in the ERAS ($p = 0.029$).

Table 4. Multivariate analysis: independent predictors for achieving sustained DMARD-free remission.

Cohort	Patient characteristic	Hazard Ratio	95% CI	P
EAC	Symptom duration in months	0.95	0.90-1.00	0.07
	CRP in mg/l	0.99	0.98-1.0	0.040
	Anti-CCP2	0.09	0.04-0.20	<0.001
ERAS	Symptom duration in months	0.94	0.89-0.99	0.029
	Acute start of complaints	2.03	1.15-3.59	0.015
	Ritchie articular index	0.92	0.88-0.97	0.001
	HAQ	0.66	0.44-0.99	0.044
	RF IgM	0.28	0.16-0.49	<0.001
	HLA SE	0.44	0.26-0.73	0.002

The fact that CRP and anti-CCP status were not available in the ERAS, made it difficult to assess to what extent the results of the ERAS replicated the findings in the EAC. The multivariate analysis of the EAC was therefore also performed after exclusion of these variables. This analysis revealed the following independent predictive variables (HR, 95%CI): symptom duration (0.96, 0.91-1.01), IgM RF (0.19, 0.11-0.35) and HLA SE alleles (0.59, 0.36-0.96). These three factors were also identified as independent predictors in the ERAS cohort, which therefore provides a complete replication of the data of the EAC.

Radiological progression

Although the present study aimed to find baseline characteristics predictive of achieving sustained DMARD-free remission, the rate of joint destruction during follow-up was also compared in the remission versus the non-remission group in the EAC. The remission patients had a lower level of joint damage with a median SHS (with IQR) after 1, 3 and 5 years of follow-up of 4 (1-9), 6 (1-14) and 6 (3-15), compared to the median SHS of the non-remission patients of 11 (5-24), 20 (9-39) and 26 (12-50).

Remission defined according to the ACR criteria

To determine whether the results would have been different if remission had been defined as specified by the ACR criteria, this definition was also applied to the EAC. Remission according to the ACR criteria was achieved by 97/454 (21.4%) of patients, including all of the 68 patients who achieved sustained, DMARD-free remission. The additional 29 patients were still using DMARDs at the time when they fulfilled the ACR remission criteria. Investigation of which baseline factors were predictive of achieving remission as defined by the ACR criteria revealed almost completely the same predictive factors (data not shown).

DISCUSSION

The present study investigated the prevalence and predictive factors for sustained DMARD-free remission in RA patients treated with conventional therapy. The long-term follow-up data from the Dutch EAC and the British ERAS cohorts revealed that sustained DMARD-free remission occurs in 9 to 15% of RA patients. Furthermore, sustained DMARD-free remission can be predicted by several clinical variables at first presentation which are routinely assessed in outpatient clinic. Most predictive characteristics which were identified in the EAC could be replicated in the ERAS. In this study, sustained DMARD-free was defined in its most stringent form which closely resembles a cure of the disease. The present findings therefore provide important new insights with regards to disease resolution and the factors underlying this process.

Notably, the frequency of sustained DMARD-free remission was roughly comparable in the ERAS and the EAC cohort, being 9.4 and 15% respectively. The finding that the prevalence was somewhat lower in the ERAS may be due to the fact that patients in the ERAS had a longer symptom duration at presentation, higher 44 SJC and were more often IgM RF positive and HLA SE positive than patients included in the EAC. These factors were found to be independently associated with a smaller chance of achieving remission in both cohorts, and are known to be associated with a severe disease course. This indicates that the patients included in the ERAS were less prone to achieve sustained DMARD-free remission than patients in the EAC.

Previous studies on remission in patients treated with conventional therapy have also reported remission rates between 10 and 20%^{1, 16-19}. However, remission was not defined as a sustained disease outcome in these previous investigations, but rather as an episodic phenomenon. In the present study, the long-term follow-up data from the EAC and ERAS cohorts enabled us to investigate remission as a definite disease outcome, which most closely resembles a cure of the disease.

The possibility that treatment differences between the EAC and the ERAS may have affected the prevalence of remission cannot be entirely excluded. However, in the EAC, stratification for inclusion period (reflecting different treatment strategies) showed comparable remission rates for the three inclusion periods. This indicates that changes in treatment strategy during the course of the study did not have a major effect on the prevalence of sustained DMARD-free remission. Furthermore, in order to exclude the possibility that patients who achieved remission had been treated more stringently, a detailed investigation of medication use was performed in both cohorts. This revealed that the patients who achieved remission had not been treated more stringently, but rather tended to have been treated less aggressively, e.g. with NSAIDs only (in approximately 25% of patients) or with less potent DMARDs (data not shown). In both cohorts, patients who had been treated with DMARDs had a significantly lower chance of ever achieving remission (EAC: HR (with 95% CI) =0.12 (0.07-0.21), ERAS: HR=0.08 (0.05-0.12)). This suggests that the rheumatologists deemed the remission patients to have less severe disease which did not need to be treated with DMARDs. This is in line with the other results of the univariate analysis, which reveal that remission patients had less radiological damage (in the EAC and ERAS) and lower swollen joint counts, Ritchie articular index and HAQ (in the ERAS) at baseline. The effect of ever using DMARDs was not included in the multivariate models because the aim of the analysis was to identify baseline characteristics of the patients which were predictive of remission.

One may consider the observed prevalence of remission (9-15%) to be surprisingly high, leading to the question whether these patients “really” had RA. However, all patients fulfilled the 1987 ACR criteria for RA; a set of criteria that is not perfect, but is nonetheless the most frequently applied. In addition, remission was achieved after a median of 3.5 years, indicating that patients had a chronic polyarthritis for this lengthy period. Finally, the majority of remission patients had radiological joint destruction (although less severe than in the non-remission group); a hallmark which is considered characteristic for RA.

The current study identified several predictive factors for sustained DMARD-free remission. Autoantibody status, HLA SE alleles, smoking, an acute onset of complaints and the symptom duration before inclusion, were found to be predictive in both cohorts. The findings on the association between the absence of autoantibodies and remission are in line with prior reports that have documented a strong association between autoantibody status (IgM RF and anti-CCP2) and the rate of joint destruction in RA²⁰⁻²². The current observations illustrate that these autoantibodies are also important persistency factors.

The majority of predictive factors identified in the EAC could be replicated in the ERAS, particularly when the same baseline characteristics were included in the multivariate models. The ERAS then provided a perfect replication of the results of the EAC. There were also some differences between the two cohorts with regard to the predictive factors which were identified. In the ERAS, disease activity at baseline, as reflected by the Ritchie

articular index, 44 SJC, DAS and HAQ, was inversely associated with achieving remission, while in the EAC, the 44 SJC and HAQ were not associated with remission. Part of this difference may be attributable to the on average higher disease activity in the ERAS at baseline (as apparent from the higher 44 SJC) and accompanying larger range of values, which lends more power to the ERAS to find an association between disease activity at baseline and definite disease outcome.

Interestingly, several factors which were recently described to predict remission after treatment with anti-TNF therapy such as age, gender and comorbidity could not be replicated in the present study^{23,24}. This could very well be the result of the different definitions of remission which were used. In these reports, remission was defined as a DAS below 1.6 or DAS28 below 2.6 during treatment, and it is therefore conceivable that the factors which were identified predict treatment response, rather than long-term disease resolution²⁵. The difference in predictive factors emphasizes that low disease activity during treatment is different from remission which persists after discontinuation of anti-rheumatic therapy.

In conclusion, 9-15% of RA patients treated with conventional therapy achieved sustained DMARD-free remission. Several factors which can easily be determined in clinical practice such as symptom duration before presentation and the absence of IgM RF and anti-CCP antibodies are consistently associated with sustained DMARD-free remission. In light of our findings that RA can resolve in approximately one-tenth of patients, additional studies are warranted to further elucidate the underlying mechanisms.

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Chapter 14

**Sustained drug-free remission
in rheumatoid arthritis after
DAS-driven or non-DAS-
driven therapy: a comparison
of two cohort studies**

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ABSTRACT

Objectives To compare the prevalence of and predictors for sustained drug-free remission in two cohorts of patients with recent-onset rheumatoid arthritis (RA) treated with disease activity score (DAS)-driven therapy or non-DAS-driven therapy.

Methods Sustained drug-free remission was assessed after 5 years of follow-up in 508 patients treated with DAS-driven therapy ($\text{DAS} \leq 2.4$) in a randomized treatment cohort, and in 424 patients who received non-DAS-driven therapy in a prospective inception cohort. The design of the DAS-driven cohort required systematic joint assessments with DAS-driven restart of therapy. Predictors for remission were identified by univariable and multivariable logistic regression in each cohort separately and in a combined multivariate logistic regression analysis corrected for propensity scores, including a sensitivity analysis on patients receiving initial monotherapy.

Results Patients in the DAS-driven cohort had more active disease at baseline, but the prevalence of sustained drug-free remission was similar after DAS-driven (9.8%) and non-DAS-driven therapy (10.6%). Among patients with antibodies to citrullinated proteins (ACPA), drug-free remission was more frequently achieved after DAS-driven than after non-DAS-driven therapy (5.4% versus 2.1%, OR 2.68, 95%CI 0.97-7.43). Absence of ACPA and short symptom duration were independent predictors for sustained drug-free remission in both cohorts. Initial treatment choice and inclusion period were not predictive. The sensitivity analysis yielded comparable results.

Conclusions Retrospectively comparing a DAS-driven to a non-DAS-driven therapy cohort, the occurrence and predictors of sustained drug-free remission were similar. The DAS-driven cohort had a more unfavorable prognosis. DAS-driven therapy may improve the chance of sustained drug-free remission in ACPA-positive patients with recent-onset RA.

INTRODUCTION

In rheumatoid arthritis (RA), an early start of treatment, use of combination therapy with corticosteroids or tumor necrosis factor (TNF)-blockers, and a strategy of tight control have significantly improved the clinical and radiographic outcomes of patients.¹⁻⁶ Continued tight control aimed at a predefined goal of minimal disease activity has resulted in suppressed inflammation, improved functional status and high remission percentages, irrespective of the initial choice of therapy.^{7, 8} Recently an international task force has reviewed and summarized all the evidence on tight control of RA and has formulated recommendations to enhance the implementation of 'treating to target' in clinical practice.^{9, 10}

According to these recommendations, the primary target of RA treatment should be a state of clinical remission, defined as the absence of signs and symptoms of inflammatory disease activity, assessed by a validated (composite) measure such as the disease activity score (DAS).^{10, 11} Ideally, clinical remission is maintained even after discontinuation of treatment, resulting in what could be called 'cure'. In the BeSt study, which combined an early start of treatment with DAS-driven therapy adjustments, the attainment of clinical remission ($\text{DAS44} < 1.6$) led to tapering and ultimately discontinuation of disease modifying anti-rheumatic drugs (DMARDs) according to the prespecified treatment protocol. After five years, 10-19% of the patients in the four strategy groups were in drug-free remission with a median duration of 23 months.⁷

Does DAS-driven therapy result in more drug-free remission than routine non-DAS-driven treatment? It is known that a proportion of patients treated according to routine care, can discontinue their anti-rheumatic medication.¹²⁻¹⁴ DAS-driven therapy might have a benefit by increasing the proportion of patients who achieve and maintain sustained drug-free remission, by inducing remission earlier in the disease course, in patients with more severe disease, or all of these alternatives.

To investigate this, we set out to retrospectively compare the prevalence and predictors of sustained drug-free remission in two cohorts of recent-onset RA patients, originating from the same region, and treated with either DAS-driven or non-DAS-driven therapy. Because anti-citrullinated protein antibodies (ACPA) are known to be an important predictive factor for disease outcome in RA, and current pathophysiological concepts consider ACPA-positive and ACPA-negative RA as two distinct disease entities^{14, 15}, all outcomes were investigated separately in ACPA-positive and ACPA-negative patients.

MATERIALS AND METHODS

Patients

Two cohorts of patients were compared:

1. the BeSt study (DAS-driven therapy cohort): 508 patients with recent-onset RA according to the 1987 American College of Rheumatology (ACR) criteria ¹⁶, a symptom duration < 2 years and active disease at baseline ($\geq 6/66$ swollen joints, $\geq 6/68$ tender joints, and either an erythrocyte sedimentation rate (ESR) ≥ 28 mm/hr or a global health score ≥ 20 mm on a visual analogue scale (VAS) of 0-100 mm), who were randomized to four different DAS-driven treatment strategies. The inclusion period was 2000-2002. ¹⁷
2. the Leiden Early Arthritis Clinic (EAC) (non-DAS-driven therapy cohort): 424 patients with recent-onset RA according to the 1987 ACR criteria from an observational inception cohort of consecutive arthritis patients with a symptom duration < 2 years. The inclusion period was 1993-2002. ¹⁸ In contrast to a previous report on the EAC ¹⁴, 30 patients who also participated in the BeSt study were excluded.

The protocols of both cohorts were approved by the local ethics committees and the LUMC medical ethics committee, and all participants provided written informed consent.

Treatment

In the BeSt study, patients were randomized to one of four treatment groups: initial methotrexate monotherapy subsequently replaced by or extended with other DMARDs (group 1 and 2), initial combination therapy including conventional DMARDs and prednisone (group 3) or initial combination therapy including infliximab (group 4). Every three months, the DAS44 was calculated and treatment was adjusted following a strict protocol, aiming at a $\text{DAS44} \leq 2.4$. If the DAS44 was ≤ 2.4 for at least 6 consecutive months, medication was tapered to monotherapy in maintenance dose. Subsequently, from the third year of the study, if the DAS44 was < 1.6 for at least an additional 6 consecutive months, the last DMARD was tapered and discontinued. If the DAS44 increased to > 1.6 , the last DMARD was immediately restarted. Details of the treatment protocol can be found elsewhere. ^{17, 19}

Patients in the EAC were treated according to the clinical judgment of their rheumatologist, via non-DAS-based efficacy evaluations. ¹⁸ The treatment strategy differed according to the inclusion period. Patients included from 1993 to 1996 were initially treated with analgesics and subsequently with chloroquine. Between 1996 and 1998, patients were initially treated with chloroquine or sulfasalazine, while after 1998 the initial treatment strategy consisted of either sulfasalazine or methotrexate. ¹⁴ The initial treatment of the patients described in the current manuscript consisted of non-steroidal anti-inflammatory drugs (NSAIDs) in 27% or DMARDs (with or without NSAIDs) in 73% of patients. Of all

patients, 37% started with DMARD-combination therapy, and 15% initially (also) used corticosteroids. Tapering and discontinuation of the medication was not protocolized.

Definition of remission

In the DAS-driven cohort, sustained drug-free remission was defined as a continued DAS44 < 1.6 after discontinuation of DMARDs. In 85% of the patients in drug-free remission the swollen joint count (SJC) was zero, as based on systematically performed 66/68 joint counts at the three-monthly follow-up visits. In the non-DAS-driven cohort patients, the DAS was not routinely performed, nor was it calculated post-hoc or imputed. A full joint count was registered yearly. In the non-DAS-driven cohort, drug-free remission was defined according to the rheumatologist and required the absence of swollen joints after cessation of therapy.

In both cohorts, drug-free remission had to be present for at least one year, and had to persist until the end of follow-up, which for the patients in the DAS-driven cohort was the 5-year follow-up time point. In the non-DAS-driven cohort, patients thus defined as being in drug-free remission were discharged from the outpatient clinic after on average 2.5 years of drug-free remission. After that there was no systematic follow-up, but if patients had a recurrence of their arthritis after discharge, they were instructed to return to the Leiden University Medical Center. During the period under investigation, one patient was re-referred with a relapse and was therefore included in the non-remission group.

Seventy-two patients (14%) in the DAS-driven cohort dropped out or were lost to follow up, and 31 patients (7.3%) in the non-DAS-driven cohort were lost to follow-up before the 5-year endpoint due to withdrawal, death or moving. An intention to treat analysis with last observation carried forward approach was used.

Statistical analysis

The prevalence of sustained drug-free remission was assessed in both treatment cohorts (DAS-driven and non-DAS-driven). Since ACPA has been previously reported as a predictive factor for drug-free remission, results were stratified for ACPA and odds ratios (OR) with 95% confidence intervals (CI) for DAS-driven versus non-DAS-driven therapy were calculated.

Baseline variables were assessed for their ability to predict the outcome of sustained drug-free remission by univariable logistic regression analysis in both cohorts separately. Predictors which had previously been reported and with a p -value < 0.10 in univariable analysis were taken along in a multivariable analysis with a backward selection procedure (removal criterion: $p > 0.10$), yielding independent predictors for sustained drug-free remission in both cohorts separately.

Next, we investigated whether DAS-driven treatment was a predictor for sustained drug-free remission by performing a multivariable logistic regression analysis on a combined

dataset of both cohorts. The treatment strategy (DAS-driven versus non-DAS-driven) was indicated by a variable (“therapy”) in this combined dataset. To take into account that baseline patient characteristics differed between the two cohorts, baseline dissimilarities were corrected for by propensity scores, constructed from the variables age, gender, symptom duration, swollen joint count, ESR, rheumatoid factor (RF) and ACPA and all significant two-way interactions.²⁰ Via interaction terms we analyzed whether the effect of DAS-driven therapy on drug-free remission was different for ACPA-positive or ACPA-negative patients.

Subsequently, sensitivity analyses were performed to assess whether the early use of combination therapy in the DAS-driven cohort or the earlier inclusion period (associated with treatment choice) in the non-DAS-driven cohort had influenced the analyses. The sensitivity analysis was performed on the patients who received initial DMARD monotherapy, being the patients from arm 1 and 2 in the DAS-driven cohort, and patients included after 1998 in the non-DAS-driven cohort.

Table 1. Baseline characteristics of patients in the DAS-driven therapy cohort (BeSt) and the non-DAS-driven therapy cohort (EAC).

Baseline characteristics	DAS-driven therapy (n=508)	Non-DAS-driven therapy (n=424)	p-value
Age, mean $\pm$ SD years	54 (13.7)	56 (16.1)	0.06
Female gender, no. (%)	346 (68%)	288 (68%)	0.90
Symptom duration, median (IQR) weeks	23 (14-53)	20 (11-39)	<0.001
BMI, mean (SD)	25.6 (4.0)	25.5 (3.6)	0.62
DAS, mean (SD)	4.4 (0.86)	NA	-
HAQ, mean (SD)	1.4 (0.67)	1.1 (0.72)	<0.001
SJC, median (IQR)	14 (10-19)	8 (5-12)	<0.001
CRP, median (IQR) mg/L	21 (9-55)	19 (9-44)	0.41
ESR, median (IQR) mm/hr	37 (19-56)	37 (21-59)	0.24
RF-positive, no. (%)	329 (65%)	237 (56%)	0.008
ACPA-positive, no. (%)	294 (62%)	238 (56%)	0.10
SE, no. (%)	281 (67%)	270 (67%)	0.98

For dichotomous variables, the number and the percentage of patients are listed, relative to the total number of patients for whom information about the characteristic under investigation was available. P-values are listed for the comparison between the two cohorts, using t-test for normally distributed variables, Mann-Whitney for non-Gaussian distributions and Chi-square for dichotomous variables. NA=not applicable.

RESULTS

Cohort characteristics and prevalence of sustained drug-free remission

At baseline, due to the inclusion criteria of the BeSt study, patients in the DAS-driven therapy cohort had a longer symptom duration, more active disease characterized by worse function measured by the health assessment questionnaire (HAQ) and a higher SJC, and were more often RF- or ACPA-positive than patients in the non-DAS-driven cohort (Table 1). After 5 years of follow-up, the prevalence of sustained drug-free remission was $50/508 = 9.8\%$ in the DAS-driven therapy cohort and $45/424 = 10.6\%$ in the non-DAS-driven cohort (Table 2). All four treatment arms of the DAS-driven study were included in the present analysis and there were no significant differences between the treatment arms. Stratified for ACPA, the prevalence of sustained drug-free remission was approximately 20% in ACPA-negative RA patients, irrespective of the therapy cohort. The prevalence of drug-free remission was markedly lower in ACPA-positive patients (2-5%). ACPA-positive patients treated with DAS-driven therapy had a higher chance of achieving drug-free remission compared with non-DAS-driven therapy (OR 2.68, 95%CI 0.97-7.43, $p=0.058$).

Table 2. Prevalence of sustained drug-free remission.

		Drug-free remission	No drug-free remission	OR (95% CI)
Total	DAS-driven therapy	50 (9.8%)	458 (90.2%)	0.92 (0.60-1.41)
	non-DAS-driven therapy	45 (10.6%)	378 (89.4%)	
ACPA-positive	DAS-driven therapy	16 (5.4%)	278 (94.6%)	2.68 (0.97-7.43)
	non-DAS-driven therapy	5 (2.1%)	233 (97.9%)	
ACPA-negative	DAS-driven therapy	34 (18.6%)	149 (81.4%)	0.83 (0.50-1.38)
	non-DAS-driven therapy	40 (21.6%)	145 (78.4%)	

Prevalence of sustained drug-free remission in the DAS-driven and the non-DAS-driven therapy cohort, in the total group and stratified for ACPA-positive and ACPA-negative patients. Odds ratios (OR) with 95% confidence intervals (CI) represent the chance for drug-free remission with DAS-driven versus non-DAS-driven therapy.

In the DAS-driven cohort, the discontinuation of antirheumatic therapy was prohibited by treatment protocol before two-and-a-half years of follow-up. The mean time (with standard deviation: SD) to drug-free remission was 3.1 years (SD: 0.5 years) in the DAS-driven cohort and 2.6 years (SD: 1.3 years) in the non-DAS-driven therapy cohort.

Predictive factors

In the DAS-driven cohort, male gender, lower DAS and HAQ at baseline and the absence of RF, ACPA and shared epitope (SE) alleles were significantly univariately associated with sustained drug-free remission (Table 3). The allocated initial treatment or treatment arm (mono- versus combination therapy), was not a predictive factor (data not shown). The

Table 3. Characteristics of patients who did or did not achieve sustained drug-free remission.

Potential predictors for remission	DAS-driven therapy			Non-DAS-driven therapy		
	Remission (n=50)	No remission (n=458)	OR (95%CI)	Remission (n=45)	No remission (n=379)	OR (95%CI)
Age, mean $\pm$ SD years	55 (16)	54 (14)	1.01 (0.98;1.03)	60 (17)	56 (16)	1.02 (0.998;1.04)
Male gender, no. (%)	23 (46%)	139 (30%)	2.27 (1.26;4.09)*	16 (36%)	120 (32%)	1.19 (0.62;2.28)
Symptom duration, median (IQR) weeks	19 (11-34)	24 (14-55)	0.99 (0.98;1.00)	13 (6-28)	21 (11-41)	0.98 (0.96;0.99)*
Smoking, no. (%)	14 (28%)	163 (36%)	0.69 (0.36;1.33)	14 (31%)	170 (49%)	0.48 (0.25;0.93)*
BMI, mean $\pm$ SD	25 (3)	26 (4)	0.96 (0.88;1.04)	25 (3)	26 (4)	0.95 (0.83;1.08)
DAS, mean $\pm$ SD	4.19 (0.88)	4.45 (0.86)	0.70 (0.49;1.00)*	NA	NA	NA
HAQ, mean $\pm$ SD	1.22 (0.66)	1.42 (0.67)	0.63 (0.40;0.98)*	1.17 (0.73)	1.04 (0.72)	1.26 (0.78;2.03)
SJC, median (IQR)	15 (10-18)	13 (10-19)	1.01 (0.97;1.06)	8 (4-11)	8 (5-12)	0.99 (0.94;1.04)
Total SHS, median (IQR)	3 (1.0-6.4)	4 (1.5-9.0)	0.98 (0.94;1.02)	3 (1.0-11.0)	6 (2.0-12.0)	0.97 (0.93;1.01)
Erosive $\geq$ 1, no. (%)	31 (65%)	326 (72%)	0.70 (0.37;1.31)	31 (72%)	300 (83%)	0.52 (0.99;1.01)
CRP, median (IQR) mg/L	16 (6-36)	22 (9-56)	1.00 (0.99;1.01)	15 (8-32)	20 (9-44)	1.00 (0.99;1.01)
ESR, median (IQR) mm/hr	29 (15-44)	37 (20-57)	0.99 (0.98;1.00)	34 (17-54)	37 (22-60)	1.00 (0.99;1.01)
RF-positive, no. (%)	22 (44%)	307 (67%)	0.39 (0.21;0.70)*	11 (24%)	226 (60%)	0.22 (0.11;0.44)*
ACPA-positive, no. (%)	16 (32%)	281 (65%)	0.25 (0.14;0.47)*	5 (11%)	233 (62%)	0.08 (0.03;0.20)*
SE, no. (%)	23 (50%)	258 (69%)	0.46 (0.25;0.85)*	20 (44%)	250 (69%)	0.35 (0.19;0.66)*

Characteristics of patients who did or did not achieve sustained drug-free remission after 5 years in the DAS-driven therapy cohort (BeSt) and non-DAS-driven therapy cohort (EAC). Odds ratios (OR) and 95% confidence intervals (CI) are presented from the univariable logistic regression analyses.

* = statistically significant, NA=not available, BMI=body mass index, DAS=disease activity score, HAQ=health assessment questionnaire, SJC=swollen joint count, SHS=Sharp/van der Heijde score, CRP=C-reactive protein, ESR=erythrocyte sedimentation rate, RF=rheumatoid factor, ACPA=anti-citrullinated protein antibodies, SE=shared epitope.

multivariate model revealed male gender, short symptom duration, the absence of ACPA and lower baseline DAS as independent predictors for sustained drug-free remission in the DAS-driven cohort (Table 4 I). If the DAS was replaced with the HAQ, HAQ was also an independent predictor with borderline significance (OR 0.61, 95% CI: 0.37-1.006, $p=0.053$). RF was a significant independent predictor if put in the model instead of ACPA (OR 0.33, 95% CI: 0.18-0.60, $p<0.001$), but was not significant if included in addition to ACPA.

Also in the non-DAS-driven cohort, absence of RF, ACPA and SE were significant univariate predictors (Table 3). In addition, shorter symptom duration and non-smoking were

Table 4. Independent predictors for sustained drug-free remission in the DAS-driven therapy cohort (I) and the non-DAS-driven therapy cohort (II).

Predictor	OR (95% CI)	p-value
I DAS-driven cohort		
Male gender	2.39 (1.26-4.53)	0.008
Symptom duration (weeks)	0.99 (0.98-1.00)	0.099
ACPA-positivity	0.20 (0.10-0.39)	<0.001
DAS	0.63 (0.43-0.94)	0.022
II Non-DAS-driven cohort		
Symptom duration (weeks)	0.99 (0.97-1.00)	0.108
ACPA-positivity	0.09 (0.04-0.24)	<0.001

identified as predictors. The inclusion period as surrogate variable for the initial treatment, was not predictive for sustained drug-free remission (data not shown). The multivariable model revealed a shorter symptom duration and the absence of ACPA as independent predictors for sustained drug-free remission in the non-DAS-driven cohort (Table 4 II).

Table 5. Independent predictors of sustained drug-free remission from the combined multivariable logistic regression analysis.

Predictor	OR (95% CI)	p-value
Model I		
Male gender	1.89 (1.18 - 3.01)	0.01
Symptom duration (weeks)	0.99 (0.98 - 1.00)	0.06
ACPA-positivity	0.18 (0.10 - 0.30)	<0.01
DAS-driven vs. non-DAS-driven therapy	1.42 (0.84 - 2.40)	0.19
Model II After adding interaction ACPA*therapy		
DAS-driven therapy:		
In ACPA-positive patients	2.95 (1.03 - 8.44)	0.04
In ACPA-negative patients	1.12 (0.62 - 2.03)	0.72

The analysis was corrected for baseline cohort differences via propensity scores. Model I represents the results without interaction terms. Model II shows the effect of DAS-driven therapy separately in ACPA-positive and negative patients.

Again, RF was only a significant independent predictor if put in the model instead of ACPA (OR 0.25, 95% CI: 0.12-0.51, $p < 0.001$).

Looking at the effect of ACPA-positivity, the odds ratio (OR) for sustained drug-free remission was two times higher with DAS-driven therapy (OR 0.20) than with non-DAS-driven therapy (OR 0.09), suggesting that the negative effect of having ACPA on achieving sustained drug-free remission may be counterbalanced to some extent by a benefit of DAS-driven therapy.

The multivariable logistic regression analysis with the two datasets combined included age, gender, symptom duration, smoking, ESR, C-reactive protein (CRP), RF, ACPA and treatment cohort as explanatory variables and was corrected for propensity scores to take into account the differences in baseline characteristics between the two cohorts. The model revealed male gender, short symptom duration, and the absence of ACPA as independent predictors for sustained drug-free remission (Table 5, I). DAS-driven therapy was not associated with a significantly higher chance for sustained drug-free remission in this multivariable model. However, a significant interaction was present between treatment cohort and ACPA (OR 3.2, 95% CI: 1.04-10.1, $p = 0.043$). We therefore investigated the effect of treatment cohort separately for ACPA-positive and ACPA-negative patients. As shown in Table 5 II, DAS-driven therapy was associated with a three times higher chance for sustained drug-free remission in ACPA-positive patients, but not in ACPA-negative patients, confirming our univariable findings.

Sensitivity analysis

The analyses in the total cohorts did not reveal treatment allocation in the DAS-driven cohort nor the inclusion period in the non-DAS-driven cohort as factors associated with achieving drug-free remission. Nevertheless, to investigate if the differences in initial treatment and the inclusion period within and between the cohorts influenced our results, we performed a sensitivity analysis on the patients who received comparable initial DMARD monotherapy in a similar time period. The prevalence of sustained-drug free remission was 25/247 (10.1%) in the patients treated with initial methotrexate monotherapy in the DAS-driven cohort and 19/199 (9.5%) in the patients included after 1998 in the non-DAS-driven cohort, treated with either initial methotrexate or sulfasalazine monotherapy. In the DAS-driven cohort, independent multivariate predictors for drug-free remission remained male gender (OR 2.1, 95% CI: 0.84-5.35, $p = 0.112$), absence of ACPA (OR 0.23, 95% CI: 0.09-0.59, $p = 0.002$) and short symptom duration (OR 0.98, 95% CI: 0.96-0.99, $p = 0.038$). In the non-DAS-driven cohort, only absence of ACPA remained a significant independent predictor (OR 0.09, 95% CI: 0.02-0.41, $p = 0.002$).

DISCUSSION

Although it is clear that tight-control strategies such as DAS-driven therapy result in better clinical and radiographic outcomes, and recommendations for 'treating RA to target' have recently been formulated, it has not yet been investigated whether DAS-driven therapy also results in more drug-free remission than non-DAS-driven routine treatment evaluations.^{9, 10} In this study, we found that in two cohorts of patients with recent-onset RA, treated with either DAS-driven or non-DAS-driven therapy, the occurrence of sustained drug-free remission was fairly similar, as were predictors of sustained drug-free remission: a short symptom duration and the absence of ACPA.

Since there were significant differences between the two cohorts at baseline, the comparison is not straightforward. A randomized controlled trial would be the ideal setting to investigate the effect of DAS-driven versus non-DAS-driven therapy on achieving sustained drug-free remission. However, as DAS-driven therapy leads to better functional and radiological outcomes than non-DAS-driven therapy^{2, 6, 19} such a design may be considered unethical. In addition, large patient numbers per group would be required, since sustained drug-free remission does not occur often. Therefore, we resorted to a retrospective comparison of two existing large cohorts.

Patients in the DAS-driven cohort, which started as the BeSt trial, had more severe disease and therefore poorer prognosis, based on the inclusion criteria of the trial¹⁷, whereas the non-DAS-driven cohort also included less severe patients¹⁸. In addition, the definition of sustained drug-free remission and frequency of follow-up differed between both cohorts, although it does not appear very likely that this will have had a major impact on the results. In the DAS-driven cohort, remission required a sustained DAS < 1.6 at three-monthly intervals and as soon as the DAS rose above 1.6, medication was restarted. In 15% of patients in remission based on a DAS < 1.6, residual joint swelling (based on a 66/68 joint count) was still present. In the non-DAS driven cohort, remission was based on the rheumatologist's opinion, including absence of swollen joints at yearly evaluations. Restart of therapy, duration or frequency of follow-up were not protocolized.

It is difficult to speculate what the results might have been like if the cohorts had been more similar. Considering that the DAS-driven cohort had more disease activity at baseline and perhaps a more stringent definition of remission, one could argue that DAS-driven therapy leads to more sustained drug-free remission than is reflected by the current study. On the other hand, the earlier inclusion period and relative lack of combination therapy might have posed a disadvantage for the patients in the non-DAS-driven therapy cohort. The inclusion period may have represented different practices in timing and choice of prescribing anti-rheumatic drugs.²¹ Part of the patients in the non-DAS-driven cohort who were included at an earlier point in time, were not promptly treated with DMARDs, and a delay of DMARD therapy is associated with worse disease outcome.^{1, 5} Initial combination

therapy, used in a subpopulation of the DAS-driven cohort, is associated with early and better suppression of disease activity, but was seldom prescribed in the non-DAS-driven cohort. It is remarkable in this respect that neither the specific treatment arm in the DAS-driven cohort (the BeSt study), nor the inclusion period and corresponding treatment strategy in the non-DAS-driven cohort (the EAC), were associated with sustained drug-free remission, confirming previous reports.^{14, 19} This suggests that the effect of the differences in inclusion period and associated medication use on our results may have been limited.

It is difficult to separate the effect of using more (novel) DMARDs from the effect of DAS-driven strategy, as a tight control strategy with frequent DAS-measurements inherently leads to rapid changes in medication. However, in our sensitivity analysis we partly disentangled these effects by excluding all the patients with initial combination therapy, as well as the patients included before 1999, which did not result in a change in the observed remission rates.

With, or despite, the differences between the two cohorts, the rate of sustained drug-free remission did not differ between the DAS-driven and non-DAS-driven cohort. ACPA-negative patients achieved sustained drug-free remission more easily than ACPA-positive patients, whether DAS-driven or not, but ACPA-positive patients, despite having a worse prognosis in general²², had a slightly higher chance for achieving drug-free remission in the DAS-driven cohort than in the non-DAS-driven cohort. The more advanced analysis methods (employing propensity scores and interaction terms) yielded results compatible with the straightforward comparison of the prevalence of sustained drug-free remission: more remission in ACPA-positive patients after treatment with DAS-driven therapy (borderline significance). This argues in favor of the robustness of the analysis. We tentatively propose that this may indicate that with DAS-driven therapy sustained drug-free remission can be achieved more readily, even in patients with more unfavorable prognostic characteristics.

The absolute number of patients with sustained drug-free remission in both our cohorts is still low and an important question is if and how we can attain more sustained drug-free remission in the future. Is sustained drug-free remission intrinsically possible only for certain patients, or should we be able to induce it in all patients? The similar rates of sustained drug-free remission in both cohorts as well as the higher sustained drug-free remission rate among ACPA-negative patients support the former, but the fact that with DAS-driven treatment drug-free remission was also attainable in more poor prognosis ACPA-positive patients support the latter. In addition, the finding of short symptom duration as a predictor of sustained drug-free remission may indicate a 'window of opportunity'. The studied DAS-driven cohort aimed at a $\text{DAS44} \leq 2.4$, a cut-off which allows for some residual inflammation. Thus, actively aiming at even lower levels of disease activity, as well as starting treatment earlier and including the use of drug combinations and biologicals might result in more patients achieving early and sustained drug-free remission in patients with RA.

In summary, in a comparison of a DAS-driven and a non-DAS-driven therapy cohort, the occurrence and predictors of sustained drug-free remission appeared similar, but the DAS-driven cohort had more patients with an unfavorable prognosis. The fact that the presence of ACPA at presentation is associated with a significantly lower chance of achieving remission, calls attention once more to the severe disease course associated with ACPA-positive RA. The results of this study suggest that ACPA-positive patients may especially benefit from tight disease control strategies. Future trials will hopefully shed more light on the optimal treatment strategy for this group of patients with their inherently poor prognosis.

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Chapter 15

Summary and discussion

The results of the studies presented in this dissertation will be summarized and discussed in three parts, corresponding to the structure of the thesis.

PART 1: RISK FACTORS FOR AND HERITABILITY OF RHEUMATOID ARTHRITIS

The effect of HLA DRB1 alleles on the risk of rheumatoid arthritis

Rheumatoid arthritis (RA) has a complex, multifaceted etiology, of which many elements remain unknown. It is clear that both genetic and environmental risk factors play a role in disease development. The field of risk factor investigation in RA has witnessed a paradigm shift due to the description of anti-citrullinated protein antibodies (ACPA) several years ago ¹. ACPA-positive and ACPA-negative disease differ not only in their clinical disease phenotype, with ACPA-positive patients having more severe and persistent disease, but also with regard to their association with different genetic and environmental risk factors^{2,3}. The discovery that the strongest genetic risk factor for RA: the HLA shared epitope (SE) alleles specifically predispose to ACPA-positive disease has proven to be crucial for the current concepts about the pathophysiological processes underlying the disease ^{4,5}.

In addition to the HLA-DRB1 alleles which contribute to RA susceptibility, other HLA-DRB1 alleles confer protection against disease ⁶⁻⁸. These protective HLA-DRB1 alleles have been categorized according to several different classifications usually based on shared amino acid sequences among the protective alleles, analogous to the shared epitope classification of susceptibility alleles. For one of these classifications, the so-called DERAA alleles, based on the shared sequence DERAA at position 70-74 of the HLA-DR beta 1 chain ⁹, it was shown that their effect was truly protective and not merely the results of the absence of predisposing shared epitope alleles ¹⁰. Due to limitations of sample size, it was unclear whether the protective effect of the various alleles differed among ACPA-positive or ACPA-negative RA. In order to obtain a better understanding of which HLA DRB1 alleles were associated with a consistent protective effect among different populations, and to investigate whether these protective effects were specific for ACPA-positive or ACPA-negative RA, we performed a meta-analysis as described in **chapter 2**.

Unexpectedly, the results revealed that none of the previous classifications of protective HLA alleles was completely accurate. Protection was limited to the ACPA-positive subset of RA and only conveyed by HLA-DRB1*13 alleles, or more specifically by DRB1*1301. The protective effects of the DERAA- and D70-alleles could be explained by DRB1*13, which is a part of both of these classifications. Therefore the presence of a DERAA- or D70-sequence in itself is not sufficient to confer protection. It should be noted that although our study included over 2700 patients and 3000 controls from large data-sets from four different European populations, smaller protective effects which may be present for other alleles than DRB1*1301 cannot be excluded.

These findings raise the question what unique feature of the DRB1*1301 alleles may be responsible for this protective effect. Intriguingly, the peptide sequence of DRB1*1301 does not harbor a particular stretch of amino acids which is truly exclusive to DRB1*1301. On

the contrary, there is quite extensive homology between the DRB1*1301 and DRB1*1102 alleles, despite the fact that no protective effect could be detected for the DRB1*1102 alleles. Therefore, it is not apparent at the first glance that DRB1*1301 would be capable of presenting a unique peptide, although this cannot be ruled out solely on the basis of its sequence.

It is noteworthy nonetheless that the difference between DRB1*1301 and the shared epitope alleles in the region of the shared epitope defining sequence: position 70 to 74 is limited to the two amino acids at position 70 and 71. While the shared epitope alleles as well as DRB1*09, which is also associated with predisposition to ACPA-positive RA, all harbor a positively charged or neutral amino acid in these two positions, DRB1*1301 carries two negatively charged amino acids in this region which constitutes binding pocket 4 of the HLA-DRB1 molecule. Despite the fact that this is also the case for all other alleles containing the previously described DERAA-sequence, it may nevertheless contribute to the protective effect exerted by DRB1*1301, in conjunction with an as yet unknown unique feature of the DRB1*1301 molecule or genomic region.

If one were to assume that the protective effect associated with DRB1*1301 were due to antigen-presenting properties of the DRB1*1301 molecule, this would lead to one of the following two hypotheses. Hypothesis 1: DRB1*1301 would have to be a very poor presenter of a pathogenic peptide, presentation of which by all other HLA-DRB1 molecules, and especially by the shared epitope (SE) alleles, could lead to the stimulation of pathogenic T cells. Despite the fact that there is some evidence that SE alleles can present citrullinated antigens ^{11, 12}, one may wonder whether it is plausible that the properties of all other DRB1 molecules would be similar in this respect. It is also difficult to envisage how DRB1*1301 would confer protection in a heterozygous setting, if there were another allele present which could present pathogenic peptides. In this case, the dose of antigen presentation would have to be a decisive element in order for there to be a protective effect based on the presence of only one DRB1*1301 allele.

Another possibility is hypothesis 2: DRB1*1301 would have to be a potent presenter of a tolerizing peptide. This peptide would have to be practically constitutively present and lead to tolerance against the antigens responsible for inciting the autoimmune response in RA. In this setting however, one would expect that the presence of a DRB1*13 allele would have a dominant protective effect, taking into account that tolerance is considered to be an all-or-nothing process. In a heterozygous setting, the effect of the other allele would cease to matter, which cannot be reconciled with data showing that patients heterozygous for DRB1*1301 and SE still have an increased risk of developing ACPA-positive RA.

Despite these objections, the advantage of assuming that antigen presentation lies at the basis of the protective effect of DRB1*1301, is that it is readily compatible with the interesting North-South gradient which was observed in the strength of the associations of several HLA-DRB1 alleles. This suggests that environmental factors influence the effect of

HLA-DRB1*1301 which is in agreement with the fact that the amount and types of antigen available for presentation may vary in different environments. Although this is an attractive hypothesis, there are also other conceivable explanations for the geographical differences we observed. For example, differences in genetic background might be responsible for the variation in the effect of DRB1*13 due to intergenic interactions.

To clarify whether the protective effect associated with DRB1*1301 is due to its antigen presenting properties, it would be worthwhile to further characterize its peptide binding characteristics, especially with regard to citrullinated peptides. It may also be interesting to investigate the characteristics of the ACPA response (as described in part 2 of the thesis) in the rare DRB1*1301-positive individuals who do develop ACPA-positive RA. If the fine specificity of their ACPA response were to differ from DRB1*1301-negative patients, this would argue in favor of a role of DRB1*1301 in the presentation of / induction of tolerance to citrullinated peptides.

With regard to HLA-DRB1*1301 it is remarkable that this haplotype / these alleles have been described to be associated with protection against several other diseases as well, such as narcolepsy¹³, chronic hepatitis B¹⁴ and hepatitis C¹⁵. This may suggest the presence of a more general immunoregulatory factor within the HLA-DRB1*1301 haplotype. On the other hand, the fact that HLA-DRB1*1301 alleles have also been reported to be associated with an enhanced risk of other diseases such as primary sclerosing cholangitis¹⁶ and type 1 autoimmune hepatitis¹⁷ argues against this hypothesis.

In addition to the possibility that the protective effect may be due to the HLA-DRB1*1301 molecule itself as elaborated above, it also appears highly probable that the causative gene variant is instead located within the extended HLA-DRB1*1301 haplotype. Several studies have described that genetic variants and single nucleotide polymorphisms (SNPs) within the HLA region can contribute to the genetic risk of ACPA-positive RA, independently of the HLA-DRB1 alleles^{18,19}. In this area of research, denser mapping of the HLA region and ultimately, functional studies of the involved genetic variants will be required to fully elucidate the role of HLA-DRB1*1301 or its associated haplotype in rheumatoid arthritis.

The heritability of rheumatoid arthritis

To date, the majority of genetic risk factors, including the HLA shared epitope alleles which confer the highest risk of RA of all known genetic risk factors, have been found to be associated with ACPA-positive RA. These findings led to the hypothesis that the influence of genetic factors may be larger in ACPA-positive compared to ACPA-negative RA.

The study presented in **chapter 3** therefore investigated the heritability and the contribution of the HLA SE alleles to the genetic variance of both disease subsets using data from a large RA twin cohort from the United Kingdom. Surprisingly, we discovered that the heritability of ACPA-positive and ACPA-negative RA was similar and amounted to approxi-

mately 66% in both cases. As was to be expected, the HLA SE alleles hardly contributed to ACPA-negative RA, but did explain 18% of the genetic variance of ACPA-positive RA.

As a result of recent genome-wide association studies (GWAS), a large number of new single nucleotide polymorphisms (SNPs) have been described to be associated with RA ^{20, 21}. In a continuation of our first heritability study, we investigated how much of the genetic variance could be explained by these new genetic risk factors. In light of the results of chapter 2 and the strength of the protective effect associated with DRB1*1301, we also wished to take into account to what extent DRB1*1301 contributed to the genetic variance of RA. The results of these studies are presented in **chapter 4**, and unexpectedly, showed that the contribution of both the protective HLA DRB1*1301 alleles and the separate SNPs to the different subsets of RA was very small. The most likely explanation for the limited consequences of the HLA DRB1*1301 alleles with regard to the explained genetic variance, is the modest prevalence of these alleles. In case of the SNPs, the contribution to the genetic variance is most likely constrained by the low effect sizes with which they are associated. These numbers illustrate that largest part of the substantial influence of genetic risk factors in RA remains unexplained, and the disease therefore still suffers from “missing heritability”, as do many other illnesses ²². Various explanations for this missing heritability have been put forward.

Perhaps many other common variants, tagged by SNPs, remain to be discovered, although it appears likely that they will be associated with increasingly smaller odds ratio's. Thus, the number of SNPs which would be required to explain a reasonable part of the genetic variance would seem to be enormous. On the other hand, the true causal genetic variants, which are tagged by the SNPs, may be associated with significantly larger effect sizes than the SNPs themselves. Thereby, the current figures which are based on the SNP effect sizes may prove to be under-estimates of the true contributions of the definitive genetic risk factors.

Another prevalent hypothesis is that so-called rare variants with a low minor allele frequency could play a major role ^{23, 24}. If these rare variants are associated with substantial odds ratio's and are inherited in a Mendelian fashion, a small number of families could explain a considerable part of the genetic variance of RA ²⁵. In the most extreme form, such rare variants could be associated with a very high penetrance and a severe disease phenotype, as has been described for mutations in the gene for the interleukin 10-receptor in inflammatory bowel disease ²⁶. If milder gene variants in the same gene subsequently prove to be present in patients with a normal disease phenotype, rare variants in this manner can also provide very valuable information about pathophysiology of the disease in general.

Structural genomic variation, including inversions, translocations and copy number variants (CNV) may also account for a part of the missing heritability. However, the impact of such variation has been called into question by recent data indicating that the SNP

genotyping arrays used for the GWAS have captured most of the variability due to this trait ²⁷. Interaction between different risk factors may also contribute to the heritability, both in the sense of gene-gene (intergenic) interaction and gene-environment interaction. With regard to intergenic interaction, conflicting results have been reported with a study in RA describing no such interaction between the known genetic risk factors and a number of recently identified SNPs ²⁸. On the other hand, in a study investigating which part of the genetic variance in individual height can be explained by genetic risk factors, intergenic interaction was found to potentially have a very large impact ²⁹. Finally, it may also be worthwhile to reassess the current estimates of heritability, despite the fact that the numbers which are currently reported, including those we found, are very much in line with previous estimates.

In conclusion, there are many possible explanations for the current enigma of missing heritability. Besides the quest for the solution to this problem which will undoubtedly involve the identification of more genetic variants, it is important to be aware of the fact that translation of genetic findings to the resulting pathophysiological processes and thus to clinically applicable knowledge, is currently lagging behind. It will be equally important to conquer the challenges in this area in order to be able to use the data for the benefit of RA patients.

The effect of alcohol consumption on the risk of rheumatoid arthritis

After the preceding studies of genetic risk factors in RA, we also investigated the effect of one of the environmental risk factors which has been described in association with RA: alcohol consumption. In the past, a possible protective effect of alcohol has been reported in several cohorts with relatively limited sample size ³⁰⁻³², and more recently in two Scandinavian cohorts ³³. As described in **chapter 5**, we investigated the association of alcohol not only with rheumatoid arthritis, but also with other kinds of inflammatory arthritis (reactive arthritis, spondylarthritis, psoriatic arthritis) and osteoarthritis. While alcohol was indeed associated with a significantly lower risk of RA, this protective effect was also present with regard to the other forms of arthritis. In conjunction with the observation that there was a negative correlation between the degree of systemic inflammation, as measured by the erythrocyte sedimentation rate (ESR), and alcohol consumption, these results suggest that alcohol intake may be inversely associated with systemic inflammatory activity in general, rather than with RA in particular. Slightly contradictory to these findings was our observation that the protective effect of alcohol consumption was significantly larger in patients with ACPA-positive RA as compared to patients with other forms of arthritis. This effect persisted after correction for the ESR, indicating that the reduction of alcohol consumption in this subgroup was not solely due to the association with an increased level of systemic inflammation.

The inverse correlation between alcohol consumption and systemic inflammation can be interpreted in two ways. One hypothesis is that alcohol may suppress both the innate and adaptive immune system leading to reduced pro-inflammatory cytokine production and in this manner possibly to a decrease or complete absence of joint inflammation where it otherwise would have occurred. This theory is supported by immunological studies demonstrating reduced dendritic cell and monocyte responses in the presence of alcohol^{34, 35}, and by experiments in mice showing that alcohol may prevent the development of destructive arthritis³⁶. Recent results from the prospective Nurses' Health Study³⁷ showing a U-shaped association between alcohol consumption and markers of inflammation prior to the onset of RA, also suggest that alcohol may have an anti-inflammatory effect.

With regard to our data, it appears that if alcohol were to suppress inflammation, one might have expected a dose-response relationship with heavy drinking conferring a stronger protective effect than light drinking. The fact that we did not observe a dose-response relationship is therefore not in line with a dose-dependent suppressive effect of alcohol on inflammation. Furthermore, the general immunosuppressive effect of alcohol would presumably persist after disease development, yet we did not observe an increase in remission, nor a decrease in radiographic joint destruction in RA patients in association with alcohol consumption. However, other cohorts have reported an association between alcohol consumption and decreased disease severity^{38, 39}, indicating that a beneficial effect of alcohol may also be present after disease onset.

The second possible explanation is that patients with arthritis and a high degree of systemic inflammatory activity may drink less alcohol, or report drinking less alcohol, due to their illness. The fact that alcohol has also been reported to be inversely associated with the risk of other inflammatory diseases^{40, 41}, could argue in favor of this hypothesis. Furthermore, a recent case-control study in systemic lupus erythematosus (SLE)⁴² where in contrast to our investigation, information on alcohol consumption prior to the moment of diagnosis was also available, described that there was no association between alcohol consumption before SLE diagnosis and the risk of SLE. However, once patients had been diagnosed, they reported drinking significantly less alcohol than controls. These results suggest that part of the protective association between alcohol consumption and the development of arthritis which we and others have described, may be due to recall or interviewer bias.

An observation that cannot be explained by these hypotheses which assume a general immunosuppressive effect of alcohol or recall or interviewer bias, is the particularly strong negative association between alcohol and ACPA-positive RA. It is remarkable that a previous Scandinavian study³³ also described this effect, which raises the question whether alcohol may specifically interfere with the pathophysiological processes underlying ACPA-positive RA. This question was addressed in the Scandinavian study by interaction analyses which showed that the effect of alcohol was more pronounced among HLA SE-

positive patients and smokers. This suggests that alcohol may affect the same pathophysiological pathway in which the HLA SE alleles and smoking are involved.

In summary, it remains difficult at present to draw firm conclusions about the effect of alcohol consumption on the risk of developing rheumatoid arthritis. On the basis of our results, it appears that an important issue to address is whether the protective association may be due to recall bias. This could be achieved by case-control studies using a more detailed questionnaire with questions about alcohol ingestion before and after the start of symptoms. Alternatively, prospective studies which could record alcohol use prior to the onset of disease, might offer even more reliable data in this regard. In addition, investigations concerning patients with other chronic inflammatory diseases could elucidate whether the possible protective effect of alcohol is specific for arthritic conditions or due to a more general anti-inflammatory effect. In light of the large interest of the media for this topic, a careful communication strategy of the results of further research to a larger audience will be key, especially with regard to the large public health risks of excessive alcohol consumption.

PART 2: CHARACTERISTICS OF ACPA

Glycosylation

As described above, the discovery of ACPA has led to a major change in the hypotheses about the pathophysiological basis of RA. The fact that the prevalence and titer of ACPA increase before disease onset⁴³⁻⁴⁵ could be compatible with a direct pathogenic role for these antibodies. Findings from an animal model describing the induction of arthritis by citrullinated fibrinogen⁴⁶ also support this idea. Because the (patho)physiological effect of an antibody response is critically dependent on several specific antibody characteristics, we decided to examine various features of the ACPA response in part two of this thesis.

Chapter 6 focuses on carbohydrate structures known as glycans which are linked to the Fc part of antibodies. They are crucial for the structural stability of the antibody and influence its effector functions such as complement activation, antibody-dependent cellular cytotoxicity and binding to cellular Fc-receptors. IgG antibodies in the sera of RA patients are known to contain mostly G0 glycans, meaning that these glycan chains lack galactose. To investigate whether the glycan chains carried by ACPA differ from the glycans carried by other antibodies in the sera of RA patients, we developed a technique to analyze Fc-linked glycans in an antigen-specific manner⁴⁷.

A comparison of serum ACPA IgG1 to total serum IgG1 revealed that ACPA were associated with a characteristic glycan profile lacking sialic acid residues. ACPA from synovial fluid (SF) of RA patients were highly agalactosylated and due to the attachment of sialic acid to galactose, thereby also lacked sialic acid. Differences in glycosylation were also

detected within RA patients depending on the presence of rheumatoid factor (RF). ACPA from RF-positive patients contained significantly more G0 (agalactosylated) glycoforms than ACPA from RF-negative patients.

These results are readily compatible with the functions currently attributed to the various glycoforms. The presence of sialic acid residues is thought to mediate anti-inflammatory activity of human IgG⁴⁸, therefore a lack of sialic acid as was detected in association with serum ACPA may increase its pathogenic potential. With regard to the agalactosylated glycoforms, it has been previously described that the relative amount of these glycoforms in the sera of RA patients correlates with disease activity and decreases during treatment⁴⁹⁻⁵¹. The observation that in comparison to ACPA from serum, ACPA from synovial fluid (SF) exhibit a significant reduction in galactose residues therefore suggests that SF ACPA may either be closer to, or more intimately involved in inflammation. It is unlikely that the inflammatory environment in synovial fluid leads to enzymatic modification of glycan residues, since this would affect all antibodies in the SF, while in our experiments the glycan profile of total IgG isolated from SF did not differ from the profile of total IgG isolated from serum. Together, these results suggest that the glycosylation profile of ACPA could facilitate a pathogenic role of these antibodies.

The detected difference in ACPA glycosylation between RF-positive and RF-negative patients is intriguing. One possible explanation may be that agalactosylated antibodies may lead to the production of RF, because a lack of galactose residues could lead to a conformational change in IgG Fc structure revealing new epitopes to which RF could bind. On the other hand, if one were to assume that the predominance of G0 glycoforms is secondary to inflammation in rheumatoid arthritis, RF-positive individuals may have more severe inflammation which could lead to selective agalactosylation of the most recently formed antibodies: ACPA. There are however, several findings arguing against the hypothesis that the changes in antibody glycosylation in RA are secondary to inflammation, including a recent report describing aberrant IgG glycosylation years before the onset of arthritis⁵². It is also noteworthy that the RF-positive and RF-negative patients we investigated did not differ significantly with regard to measures of inflammation such as erythrocyte sedimentation rate and C-reactive protein.

Isotypes and fine specificity

In **chapter 7** we investigated the association between disease course and another characteristic of the ACPA response: the antibody isotypes. The different human antibody isotypes are known to vary in their ability to recruit effector mechanisms, and are thereby key determinants of antibody function. The results described in this chapter can best be understood in the light of the findings regarding another antibody characteristic: the fine specificity. This designates the exact antigen recognition properties of an antibody. The development of ACPA fine specificity was investigated before disease onset and during

disease progression as described in **chapter 8**. Furthermore, the clinical effects of individual differences in ACPA fine specificity repertoire were examined in more detail in **chapter 9**. Specifically, we analyzed whether certain ACPA fine specificities were associated with the extent of radiographic joint damage. Together with a previously published article by Verpoort et al ⁵³, these manuscripts provide an overview of the development of ACPA fine specificity and isotype use during disease onset and of the predictive value of these ACPA characteristics for the future disease course.

In the Leiden Early Arthritis Clinic (EAC), patients who presented with undifferentiated arthritis (UA) and whose disease did or did not progress to RA, were used to investigate the association of ACPA isotypes and fine specificity with disease outcome. At the time of disease onset, ACPA-positive UA patients who later developed RA recognized significantly more peptides and had a broader isotype use, than ACPA-positive UA patients whose disease did not progress. Thus, despite the same clinical phenotype of UA, the immunological differences with regard to the ACPA response foretold the future progression of the disease.

Remarkably, after disease onset neither the fine specificity repertoire nor the isotype use expanded, despite a progressively worsening of the disease in UA patients who developed RA. These findings were corroborated by investigating patients with longstanding RA, in whom no differences could be detected between the fine specificity and isotype profiles at baseline and after 7 years of follow-up. Despite the fact that we cannot exclude that the use of a larger number of citrullinated peptides might have led to different results, these findings suggested that the evolution of these ACPA characteristics predominantly occurs before disease onset. To further investigate the immunological development of the ACPA response prior to disease manifestation, we made use of a unique serum collection from blood donors who had donated blood before and shortly after the onset of RA. In line with the above hypothesis, the number of recognized citrullinated peptides was found to increase in the period preceding disease onset. Although this cohort has not been used to study ACPA isotypes before disease manifestation, it seems very probable that most of the isotype switching also occurs at that early point in time, in light of the differences in isotype use which are already present at presentation among UA patients.

A graphic depiction of the currently most probable timeline according to which ACPA characteristics develop, is shown in Figure 1 in relation to the various clinical stages of RA development. An increase prior to disease onset has been reported for both ACPA-levels ⁴⁴ and for ACPA fine specificity as presented in this thesis. With regard to ACPA isotype use, the development prior to the stage of undifferentiated arthritis is unknown, but seems likely to follow a similar pattern. Analogous to the reduction in ACPA levels which has been described previously ^{54, 55}, we found a slight decrease in ACPA fine specificity recognition and isotype use during the early disease course. During longer-term follow-up the fine specificity and isotype repertoire remained relatively stable.

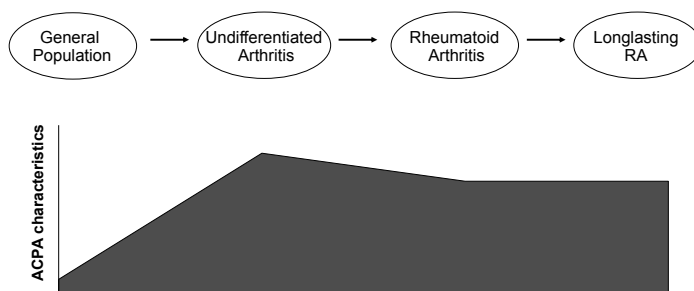


Figure 1. Development of ACPA characteristics (such as levels and epitope recognition) over time, in relationship to the clinical disease stages of RA.

In order to examine the clinical consequences of inter-individual differences in fine specificity repertoire and isotype distribution, we analyzed the association between radiographic joint damage and these ACPA characteristics. This revealed a difference between the two antibody properties since a larger number of isotypes was indicative of a higher risk of radiographic damage (chapter 7), but no such association was found for an expanded ACPA fine specificity repertoire (chapter 9).

On the whole, one of the most important conclusions of these findings is that the immunological development of the ACPA response takes place before disease onset. The accumulation of autoantibody reactivities before disease manifestation has also been reported in studies of other human diseases, such as SLE ⁵⁶ and pemphigus ⁵⁷, as well as in animal models such as the collagen-induced arthritis model (CIA) ⁵⁸. Our observations with regard to the development of the ACPA fine specificity repertoire have been confirmed by a recent study in ACPA-positive arthralgia patients ⁵⁹, demonstrating that differences in citrullinated antigen recognition already exist in the arthralgia phase before the development of arthritis. The lack of further broadening of antigen recognition and isotype repertoire after the start of the disease, could to some extent be due to the use of immunosuppressive medication which was prescribed to RA patients, and sometimes to extent to UA patients in the EAC. However, the fact that there was also no change in ACPA fine specificity in untreated arthralgia patients who subsequently developed RA ⁵⁹, suggests that this phenomenon may in fact reflect the natural course of the immune response.

An important implication of the fact that the ACPA response appears to mature so early, is that treatment which is initiated once symptoms have developed, may no longer be able to influence the characteristics of the ACPA response. Or, from a different point of view, one may argue that even if medication would affect the evolution of the ACPA response, it is hard to say if this would have any effect considering the advanced state of immunological maturation at presentation.

When patients first present with ACPA-positive UA, those among them who recognize many citrullinated peptides and use many isotypes are at increased risk to develop RA.

However, our results suggest that after the development of RA, a broader use of isotypes at baseline is associated with more radiographic joint destruction, while an expanded fine specificity repertoire at baseline is not. From an immunological perspective this could mean that at a later stage in the disease, the antibody effector mechanisms employed by the various isotypes are more decisive in determining the extent of tissue destruction than the exact peptide recognition properties of the antibody. On the other hand, there are several reasons to be cautious in the interpretation of these results.

First of all, the effect of ACPA fine specificity on radiographic joint destruction may have been underestimated due to the limited number of citrullinated epitopes under investigation. Although this issue was addressed in chapter 9 by using SE alleles as a surrogate marker, this likely only represents unmeasured ACPA fine specificities which develop under the influence of SE alleles. The impact of the ACPA isotype profile on future radiographic progression on the other hand may have been overestimated, and a direct comparison of the two studies (chapter 7 and 9) is hampered by the fact that different statistical methods were used due to recent advances in statistical techniques. It is also worth noting that both the total number of recognized peptides and the overall number of isotypes are associated with the total IgG anti-CCP2-level and it is challenging to determine whether these ACPA characteristics are predictive independently from this total serum level. The matching approach chosen in chapter 7 suggested that ACPA isotypes do supply additional information to the total IgG level, but these results need to be replicated in future studies.

Finally, it is interesting to re-examine the role of ACPA in the pathophysiology of RA based on the insights acquired from the studies of ACPA characteristics. Although the investigations described above were not designed to demonstrate a causal role for ACPA in the biological processes underlying RA, the results are compatible with the hypothesis that the exact nature and characteristics of the ACPA response may determine disease development and progression. The fact that ACPA can be detected years before the clinical diagnosis of RA and also in unaffected family members in North American Native populations with a high prevalence of RA ⁶⁰, suggests that not all ACPA are pathogenic. Prior to the onset of disease, the number of citrullinated epitopes which is recognized increases, the isotype usage expands, and perhaps ACPA acquire their specific glycosylation pattern associated with inflammation or inflammatory potential. It is possible that these changes result in antibodies which can now evoke or contribute to an inflammatory disease process. Although this hypothesis cannot be proven by the results described above, it is an attractive model for further research.

The influence of genetic and environmental risk factors on the ACPA fine specificity repertoire

The HLA SE alleles not only predispose to ACPA-positive RA, but are also involved in shaping the fine specificity of the ACPA response, as was shown in a previous report which demonstrated that these alleles predispose to the development of antibodies against citrullinated vimentin but not against citrullinated fibrinogen ⁶¹. On the basis of these results, we decided to investigate whether the combined effect of HLA SE alleles and smoking may also be associated with the recognition of distinct citrullinated proteins. In the predisposition to ACPA-positive RA, the combined effect of HLA SE alleles and smoking has been shown to exceed the sum of their single effects: a phenomenon known as biological interaction ^{62, 63}. Linking these epidemiological insights on risk factors for RA with biological studies of autoantibody features, **chapters 9, 10 and 11** provide a more in-depth analysis of the effect of these genetic and environmental risk factors and of their possible interaction with regard to the fine specificity repertoire of ACPA.

In chapter 9, the effect of the SE gene dose on ACPA fine specificity was analyzed. Reactivity to some citrullinated antigens was found in increased frequency in SE-positive patients, while no such effect was observed for other citrullinated antigens. Since patients with one or two SE alleles had a similar peptide recognition profile, it appears that one SE allele is enough to facilitate the development of certain ACPA fine specificities, and that a second SE allele does not enhance this effect. This would be compatible with a role for the HLA SE alleles in the presentation of citrullinated peptides, because presentation of an “arthritogenic” peptide by a limited number of HLA alleles would be enough to break tolerance and result in an autoimmune response. On the other hand, this does not explain the higher risk of RA which has been described in association with a double dose of SE in previous publications ^{64, 65}.

In addition to the most important genetic risk factor (the HLA SE alleles), chapter 10 and 11 investigated the influence of the most powerful environmental risk factor for ACPA-positive RA: smoking. Since a specific interaction between these two risk factors had been postulated in association with autoimmunity to citrullinated α -enolase peptide (CEP-1) ⁶⁶, we investigated whether the interaction was truly specific for this antigen or might also exist for other peptides. In the first analysis (chapter 10), it appeared that this gene-environment interaction was not specific for citrullinated α -enolase, but rather extended to other citrullinated antigens as well, particularly to citrullinated vimentin (cVim). As presented in chapter 11 however, the gene-environment interaction was no longer present when ACPA-positive cVim-positive patients were compared to ACPA-positive cVim-negative patients. The interaction between the SE alleles and smoking therefore does not affect the development of specific ACPA fine specificities, but rather predisposes to the development of ACPA in general. The difference described in chapter 10 between the risk factors associated with antibodies to citrullinated vimentin versus citrullinated fibrinogen

was not due to a gene-environment interaction, but solely to the effect of the SE alleles, which specifically predispose to the development of anti-cVim antibodies as has been described previously ⁶¹.

A popular scenario linking the genetic and environmental risk factors of RA consists of smoking inducing citrullination of certain proteins in the lungs, presentation of which by HLA SE alleles subsequently leads to a break in tolerance and the development of anti-citrullinated protein immunity. In this setting, association of immunity to certain citrullinated antigens with both HLA SE alleles and smoking would imply that the antigen in question may play a key role in the pathogenesis of RA and may lie at the basis of autoimmunity against citrullinated proteins. The contribution of chapter 11 to our understanding of the pathophysiology of RA is that it shows that with regard to the specific citrullinated proteins investigated thus far, an interaction effect between genetic and environmental risk factors cannot be found.

It should be noted that as in the other studies in this thesis concerning ACPA fine specificity, one cannot exclude that different results, in this case entailing a true gene-environment interaction between SE alleles and smoking, might be found for other citrullinated antigens. Even if this were the case however, the conclusion that the citrullinated antigen associated with these specific risk factors may be an antigen of etiologic importance warrants caution for several reasons. First of all, in vitro assays may not accurately mimic the way in which antigens are presented and recognized in vivo. Furthermore, the etiologic importance of a distinct antigen cannot be proven by risk factor association studies, although their results may be suggestive. The identification of a likely culprit antigen would require more extensive investigations of the in vivo immunologic processes.

The value of different ACPA tests in clinical practice

In the final chapter of part 2, we investigated the usefulness of the various commercial ACPA tests which are currently available for daily clinical practice. In addition to the anti-CCP2 test mentioned before, two new tests have been introduced: the anti-CCP3 test and an assay for anti-modified citrullinated vimentin antibodies (anti-MCV). **Chapter 12** describes the predictive value of these autoantibodies and of the combinations of the various tests for three outcome measures: progression from UA to RA, the rate of joint destruction and sustained DMARD-free remission in RA.

The results were similar for all three outcomes. None of the four autoantibody tests under investigation (anti-CCP2, anti-CCP3, anti-MCV and RF) had a significantly higher positive or negative predictive value than the other tests. Furthermore, there was no additive value in performing a second or third autoantibody test compared to performing a single assay, especially when the single assay was a CCP2- or CCP3-test. This last conclusion may seem contradictory to the finding that patients with two or three autoantibodies had worse outcomes than patients with no or with one autoantibody. Patients with two

or three autoantibodies had a higher chance of progression from UA to RA, a higher rate of joint destruction and a smaller chance of achieving sustained DMARD-free remission. However, it is important to keep in mind when interpreting these results that the single autoantibody present in the group of patients with only one autoantibody was almost always either anti-MCV or RF. It is therefore very likely that the increased risk of a poor outcome in the presence of two autoantibodies as compared to one autoantibody is mainly due to the predictive ability of the anti-CCP2-test. It is thus reasonable to conclude that if the anti-CCP2-test, or the comparable anti-CCP3-test, is used as the first autoantibody assay in arthritis patients, the additive value of an anti-MCV- or RF-assay is limited.

These findings are compatible with the results of chapter 9 which investigated the association of ACPA fine specificity with radiographic progression. Neither the commercially available assays using different citrullinated antigens, nor the ACPA fine specificity tests we developed in our laboratory, were predictive of a more severe disease course as measured by radiographic joint damage. As discussed above, these results do not necessarily mean that the ACPA fine specificity does not affect the disease course. It is possible that further insights into the recognition of citrullinated antigens *in vivo* will lead to the development of new tests with increased predictive value.

PART 3: REMISSION

The final part of this thesis consists of two observational studies examining the best possible clinical outcome of RA: sustained remission after discontinuation of disease modifying anti-rheumatic drugs (DMARDs). While RA was traditionally considered to be a chronic life-long disease, the recent improvements in therapeutic options have led to an increase in the number of patients that achieve remission ⁶⁷. Concurrently, this clinical outcome has become the focus of more research and discussion within the field of rheumatology ⁶⁸. The studies on remission included in this thesis were performed in order to answer several research questions.

Although remission is frequently reported as an outcome in trials of new anti-rheumatic drugs, there is a lack of recent data on the number of patients which attain remission with more conventional treatment, despite the fact that in daily practice most new RA patients are started on a conventional drug such as methotrexate ⁶⁹. Furthermore, the elucidation of which clinical characteristics at baseline are associated with remission can give clues about which therapeutic strategy is required to achieve remission in as many patients as possible. These data can also lead to an increase in our understanding of the pathophysiology of RA, and could lead to new hypotheses about the biological pathways involved in disease persistence and resolution.

In **chapter 13**, two large independent cohorts of patients treated with conventional therapy were therefore used to determine the prevalence and predictive factors for sustained DMARD-free remission. Long-term follow-up data from the Leiden EAC and the British Early RA study (ERAS) revealed that sustained DMARD-free remission occurs in 9 to 15% of RA patients. Several baseline patient characteristics were found to be independently associated with sustained DMARD-free remission in both cohorts: a short symptom duration before presentation and the absence of IgM rheumatoid factor or anti-CCP antibodies.

In order to compare these results to remission data from a cohort of patients treated with modern goal-steered therapy, we investigated the prevalence and predictive factors for sustained drug-free remission in the Leiden EAC and the BeSt (behandelstrategieën) trial, as described in **chapter 14**. The BeSt trial employed a tight control treatment strategy, meaning that the Disease Activity Score (DAS) were measured every 4 months, and treatment was adjusted following a strict protocol aimed at achieving a $\text{DAS44} \leq 2.4$. Patients participating in the BeSt study were randomized among four treatment groups. Since there was no significant difference between these groups with regard to the outcome of remission, all BeSt-patients were analyzed as part of the DAS-driven cohort in chapter 14. In comparison to patients treated with conventional treatment in the EAC (the non-DAS-driven therapy cohort), patients in the DAS-driven cohort had more active disease at baseline. Nonetheless, the prevalence of sustained drug-free remission was similar after DAS-driven and non-DAS-driven therapy (9.8% versus 10.6%). ACPA-positive patients more frequently achieved sustained drug-free remission after DAS-driven than after non-DAS-driven therapy (5.4% versus 2.1%, OR 2.68, 95% confidence interval 0.97-7.43). In both cohorts, absence of ACPA and short symptom duration were independent predictors for sustained drug-free remission.

In the interpretation of the findings of chapter 13 and 14, several points need to be taken into account. First of all, the definition of remission used for the EAC in these studies differs from the definitions employed in other reports⁷⁰⁻⁷². We aimed to investigate sustained DMARD-free remission as the clinical outcome most closely reflecting a cure of the disease. This required the absence of swollen joints as observed by a rheumatologist for at least one year after discontinuation of DMARD-therapy to ensure that remission was long-lasting. In contrast, clinical trials frequently use a DAS-based definition of remission which does not require a specified follow-up period but can be fulfilled at one single point in time.

One may argue that the definition of remission used in this thesis also had several drawbacks. One drawback could be that it was not based on a standardized clinical measure such as the DAS or the simplified disease activity index (SDAI)⁷³. However, considering that the diagnosis of synovitis is generally based on the presence of joint swelling, our definition requiring the absence of swollen joints could also be deemed a more appropriate measure than compound scores which include inflammatory markers such as CRP

or patient-reported outcomes such as visual analogue scales (VAS). Another possible weakness of the remission studies in chapter 13 and 14 could be that EAC patients with remission were discharged from the outpatient clinic after at least one year of observation. This precluded continued observation and indefinite affirmation of the prolonged state of remission. On the other hand, most remission patients in the EAC were followed-up longer than the minimum requirement of one year with a median time of observation of 2.5 years. Furthermore, it seems plausible that most patients who had a recurrence of their arthritis after discharge, were referred to the Leiden University Medical Center again, the only referral center for Rheumatology in a health care region of approximately 400.000 inhabitants. Indeed, six patients were found to have experienced a relapse and were included in the non-remission group.

Due to the increased interest in remission and the inconsistencies in the definitions of remission used to date as exemplified above, the American College of Rheumatology (ACR) and European League Against Rheumatism have recently issued a proposal for two possible definitions of remission in RA for clinical trials ⁷⁰, replacing the 1981 ACR-definition which was difficult to apply in clinical practice ⁷¹. These definitions will hopefully be embraced by the field and result in a more uniform approach in the research of this important clinical outcome.

In chapter 14, the baseline differences between the DAS-driven and the non-DAS-driven cohort posed analytical difficulties and rendered the comparison far from straightforward. While acknowledging the differences between the cohorts, several strategies were employed to ensure a valid comparison. Taking into account that the inclusion of the non-DAS-driven cohort (the EAC) had taken place at an earlier point in time, we performed a sensitivity analysis to specifically assess whether this earlier inclusion period had influenced the analyses. This sensitivity analysis showed no differences compared to the analysis of the entire cohorts. Furthermore, a multivariable analysis was performed in which the differences between the two cohorts were adjusted for by means of propensity scores. These adjustments in the statistical analysis do not diminish the fact that a randomized controlled trial would be the ideal setting to investigate the effect of DAS-driven versus non-DAS-driven therapy on achieving sustained drug-free remission. However, the repeated observation that DAS-driven therapy results in better functional and radiological outcomes than non-DAS-driven therapy ^{74, 75} raises the question whether such a randomized design would still be considered ethical.

The prevalence of sustained DMARD-free remission of 9-15% reported in chapters 13 and 14 may be considered surprisingly high, raising the questions whether these patients “really” had RA. It is therefore worthwhile to note that these patients not only fulfilled the 1987 ACR criteria for RA, but also had a median disease duration of 3.5 years before they achieved remission, meaning that they had RA disease activity for this lengthy period. Furthermore, most of the remission patients had radiological joint destruction albeit less

severe than in the non-remission group. Nonetheless, the patient characteristics which were identified as being associated with a higher chance of remission (short disease duration and absence of autoantibodies), do point to the fact that remission is generally achieved by patients who have a milder form of RA from the start. It is not unexpected that autoantibody-positive, and especially ACPA-positive patients, have the smallest chances of achieving remission, and it is promising that chapter 14 suggests that these patients may have the most to gain from tight control treatment strategies such as DAS-driven therapy.

Summarizing our findings with regard to remission, a substantial proportion of RA patients achieve sustained DMARD-free remission after treatment with conventional therapy. The presence of autoantibodies, and in particular ACPA, at presentation is associated with a significantly lower chance of achieving remission. Once more this calls attention to the severe disease course of ACPA-positive RA and the inherent differences between ACPA-positive and ACPA-negative disease. The comparison with the BeSt trial as a DAS-driven therapy cohort suggests that ACPA-positive patients may especially benefit from tight disease control strategies. Future trials will hopefully shed more light on the potential usefulness of taking ACPA-status into account in treatment decisions.

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Chapter 16

Nederlandse samenvatting

**Antistoffen tegen
gecitrullineerde eiwitten
in reumatoïde artritis –
van erfelijke aanleg tot
klinische ziekte-uitkomst**

REUMATOÏDE ARTRITIS EN ACPA

Reumatoïde artritis (RA) wordt gekenmerkt door gewrichtsontsteking (artritis) van met name de kleine hand- en voetgewrichten. Het is de meest voorkomende vorm van artritis met een prevalentie tussen 0.5 en 1% in westerse populaties. De aandoening komt ongeveer drie keer vaker bij vrouwen dan bij mannen voor en de prevalentie neemt toe met de leeftijd. Afgezien van de artritis die tot ernstige gewrichtsdestructie kan leiden, kunnen ook ontstekingsreacties in de inwendige organen optreden zoals in het hart, de longen en de nieren. Voor patiënten met reumatoïde artritis zijn de behandelmogelijkheden de laatste jaren sterk verbeterd. Door de opkomst van tumor necrosis factor (TNF)-blokkers en andere zogenoemde “biologicals” is het tegenwoordig mogelijk om bij een groot deel van de patiënten een zeer lage ziekte-activiteit te bereiken en zelfs te streven naar remissie. Onderzoek van de afgelopen jaren heeft laten zien dat reumatoïde artritis ingedeeld kan worden in twee verschillende vormen op basis van de aan- dan wel afwezigheid van antistoffen tegen gecitrullineerde eiwitten: anti-citrullinated protein antibodies (ACPA). De ACPA-positieve en ACPA-negatieve patiënten verschillen van elkaar met betrekking tot klinische ziekte-uitkomst, en met betrekking tot genetische en omgevingsfactoren die met een verhoogd risico op de ziekte gepaard gaan.

ACPA binden aan eiwitten/peptides die citrulline bevatten: een post-translationale modificatie van het natuurlijk voorkomende aminozuur arginine. Terwijl het voorkomen van arginine in menselijke eiwitten dus door de genen is vastgelegd, moet voor het ontstaan van citrulline deze arginine door een enzymatisch proces worden veranderd. Dit proces heet citrullinatie en wordt gekatalyseerd door enzymen die peptidyl arginine deiminasen worden genoemd. De precieze biologische functie van citrullinatie is nog onbekend. Gecitrullineerde eiwitten zijn aangetoond in meerdere ontstoken weefsels, zoals ontstoken gewrichten in verschillende vormen van artritis, in longen en andere organen.

Sinds de ontdekking van ACPA zijn inmiddels verschillende commerciële tests ontwikkeld die veelal gebruik maken van cyclische gecitrullineerde eiwitten (cyclic citrullinated proteins, CCP) als antigen om de aanwezigheid van ACPA aan te tonen. De diagnostische eigenschappen van deze bepalingen zijn meermaals onderzocht en vergeleken met het klassieke auto-antilichaam dat met RA geassocieerd is: IgM reumafactor (IgM-RF). Het belangrijkste verschil tussen beide tests is de toegenomen specificiteit van anti-CCP in vergelijking van IgM-RF (95 versus 85%). Voor beide auto-antilichamen is de sensitiviteit bij patiënten met vastgestelde RA 67%. ACPA blijken daarnaast van grote voorspellende waarde te zijn voor het ziektebeloop van artritis. Niet alleen zijn ACPA de beste voorspeller van het voortschrijden van ongedifferentieerde artritis (undifferentiated arthritis, UA) naar RA, maar ACPA-positieve RA patiënten hebben ook beduidend meer gewrichtsschade dan ACPA-negatieve patiënten. Uit studies die bloeddonoren hebben onderzocht die op een later tijdstip RA hebben ontwikkeld, is gebleken dat ACPA reeds voor uitbraak van de

ziekte aantoonbaar zijn. Evenals de observatie dat ACPA artritis in muizenmodellen doet verergeren, suggereert dit dat de immuunrespons tegen gecitrullineerde eiwitten mogelijk een rol speelt bij het ontstaan van RA. Dit heeft geleid tot nader onderzoek naar welke factoren geassocieerd zijn met het ontwikkelen van immuniteit tegen gecitrullineerde eiwitten.

RISICOFACTOREN EN PATHOFYSIOLOGISCH MODEL VOOR REUMATOÏDE ARTRITIS

Voor het ontstaan van RA zijn zowel genetische risicofactoren als omgevingsfactoren van belang. Van de genetische risicofactoren hebben de genen die coderen voor de humane leukocyten antigenen (HLA) het grootste effect. Bepaalde HLA DR-allelen die gekenmerkt worden door een vergelijkbare aminozuur-volgorde ter plaatse van positie 70-74 van het HLA-DRB1 molecuul (de zogenaamde “shared epitope” (SE) allelen) gaan gepaard met het grootste risico op RA. Na de ontdekking van ACPA is duidelijk geworden dat de SE allelen niet predisponeren voor RA in het algemeen, maar specifiek voor ACPA-positieve RA. Dit suggereert dat deze vorm van erfelijke belasting met name leidt tot het ontwikkelen van een immuunrespons gericht tegen gecitrullineerde eiwitten. Van vele andere genetische factoren is sindsdien ook beschreven dat zij bij voorkeur gepaard gaan met ACPA-positieve RA. Daarnaast is een minderheid van genetische risicofactoren mogelijk specifiek voor ACPA-negatieve RA. Met betrekking tot omgevingsfactoren is veelvuldig een associatie tussen roken en het ontstaan van RA beschreven. Na de opkomst van ACPA-bepalingen bleek dat roken eveneens alleen predisponeert voor de ontwikkeling van ACPA-positieve RA. Hierbij is opvallend dat het gezamenlijke effect van de SE allelen en roken groter is dan de som van de twee individuele effecten, passend bij een interactie tussen genetische en omgevings-risicofactoren.

Deze recente bevindingen hebben grote consequenties gehad voor ons begrip van de onderliggende biologische mechanismen die leiden tot het ontstaan van RA. In het huidige pathofysiologisch model van RA dat vooral van toepassing is op ACPA-positieve ziekte kunnen meerdere fases worden onderscheiden. In de eerste fase vóór het ontstaan van de ziekte leidt een erfelijke belasting samen met omgevingsfactoren tot het ontstaan van een adaptieve immuunrespons. Zo kunnen mogelijk de SE allelen in combinatie met roken aanleiding geven tot het ontstaan van immuniteit tegen gecitrullineerde eiwitten. In de tweede fase kan een tot nu toe onbekende prikkel vervolgens leiden tot het ontstaan van gewrichtsontsteking wat zich manifesteert als ongedifferentieerde artritis (UA). De geactiveerde T cellen, gericht tegen bijvoorbeeld gecitrullineerde eiwitten, kunnen nu naar het gewricht migreren en daar inflammatoire cytokines produceren. Daarnaast stimuleren de T cellen ook andere cellen in het gewricht, zoals osteoclasten, macrofagen en

fibroblasten, wat botresorptie en verdere productie van cytokines zoals TNF α , en matrix metalloproteinasen tot gevolg kan hebben. Ook de activatie van B cellen en de productie van auto-antilichamen zoals ACPA, kan leiden tot een verergering van het ontstekingsproces. Al deze factoren samen kunnen zorgen voor een voortschrijdend ziekteproces dat zich ontwikkelt tot RA en uiteindelijk gewrichtsdestructie veroorzaakt.

DEEL 1: ERFELIJKHEID EN ASSOCIATIE VAN GENETISCHE RISICOFACTOREN EN ALCOHOL MET ACPA-POSITIEVE EN ACPA-NEGATIEVE RA

Het onderzoek beschreven in **deel 1** van dit proefschrift houdt zich bezig met de erfelijkheid van RA, en met de precieze associatie van ACPA-positieve en ACPA-negatieve RA met verschillende genetische factoren (de HLA-DRB1 allelen) en de risicofactor alcohol.

Zoals eerder vermeld, is sinds vele jaren bekend dat bepaalde HLA-DRB1 allelen, de zogenaamde shared epitope (SE) allelen, de sterkste genetische risicofactor voor het ontwikkelen van reumatoïde artritis zijn. Zij zijn specifiek geassocieerd met een groter risico op ACPA-positieve RA. Van andere HLA allelen is beschreven dat zij gepaard zouden gaan met bescherming tegen RA, maar door enkele tegenstrijdige berichten in de literatuur was niet geheel duidelijk welke allelen precies beschermend zouden zijn. De beschermende allelen zijn in het verleden samengevat in verschillende classificaties, die analoog aan de SE classificatie, berustten op aminozuursequenties die bij alle beschermende allelen voorkwamen. Onduidelijk was eveneens of deze beschermende effecten mogelijk ook beperkt zijn tot uitsluitend ACPA-positieve of ACPA-negatieve patiënten. Zoals wordt beschreven in **hoofdstuk 2** hebben wij daarom een meta-analyse uitgevoerd met de individuele patiëntengegevens van vier grote Europese cohorten.

Hieruit bleek dat beschermende effecten alleen aantoonbaar zijn voor de HLA-DRB1*13 allelen, en in het bijzonder DRB1*1301. Deze allelen beschermen specifiek tegen het ontwikkelen van ACPA-positieve RA. Dit betekent ook dat de in het verleden geformuleerde classificaties van protectieve allelen allen tot op zekere hoogte onjuist zijn. Het beschermende effect dat werd toegeschreven aan deze groepen van allelen, kan geheel verklaard worden door het sterke effect van DRB1*1301. Hoe de bescherming door DRB1*1301 tegen ACPA-positieve RA precies tot stand komt, zal in toekomstige immunologische studies worden onderzocht. Opvallend was dat er geen significante associaties waren tussen HLA DRB1-allelen en ACPA-negatieve RA.

De meerderheid van de genetische risicofactoren die tot op heden zijn geïdentificeerd, is specifiek geassocieerd met ACPA-positieve RA. Dit suggereert dat de erfelijke belasting voor ACPA-positieve RA mogelijk groter is dan voor de ACPA-negatieve vorm. Dit was de aanleiding voor een studie waarin wij de erfelijkheid van zowel ACPA-positieve als ACPA-negatieve RA hebben onderzocht. De resultaten hiervan worden behandeld in **hoofdstuk**

3. Met behulp van data van een groot RA-tweelingencohort uit het Verenigd Koninkrijk vonden wij dat de erfelijkheid van ACPA-positieve en ACPA-negatieve RA vergelijkbaar is en ongeveer 66% bedraagt. Zoals verwacht droegen de HLA SE allelen zeer weinig bij aan de genetische variantie van ACPA-negatieve RA, maar verklaarden zij 18% van de genetische variantie van ACPA-positieve ziekte.

Als gevolg van de opkomst van genome-wide association studies (GWAS) bestaat een groot deel van de recentelijk beschreven genetische risicofactoren voor RA uit single nucleotide polymorphisms (SNPs): veranderingen in het genoom ter grootte van slechts 1 base. In een voortzetting van hoofdstuk 2 en 3, hebben wij daarom in de studie beschreven in **hoofdstuk 4** onderzocht hoe groot de bijdrage van de beschermende DRB1*13 allelen en verschillende SNPs is, aan de genetische variantie van de verschillende vormen van RA. Omdat het unieke RA-tweelingencohort dat voor deze studie noodzakelijk was, afkomstig was uit het Verenigd Koninkrijk, hebben wij eveneens data uit het Verenigd Koninkrijk gebruikt met betrekking tot het effect van DRB1*13. Hierbij bleek het effect van HLA DRB1*13 in het Engelse cohort onverwacht beduidend kleiner te zijn dan in de eerdere meta-analyse was gevonden. Logischerwijs leidde dit kleine genetische effect slechts tot een kleine bijdrage van HLA DRB1*13 aan de genetische variantie. Zelfs als de beschermende invloed van de HLA-DRB1*13 allelen groter zou zijn geweest, zoals werd aangenomen in een simulatie met behulp van Nederlandse data, dan nog bleek de bijdrage van deze allelen aan de genetische variantie zeer beperkt te zijn. Waarschijnlijk ligt de relatief lage prevalentie van HLA DRB1*13 ten grondslag aan het beperkte verklarende vermogen van deze allelen. De bijdrage van de verschillende SNPs die daarnaast in deze studie zijn onderzocht, was eveneens zeer klein. Dit was niet onverwacht gezien de bescheiden effecten van deze SNPs op het risico om RA te ontwikkelen. Deze bevindingen tonen aan dat het grootste gedeelte van de aanzienlijke invloed van genetische factoren op het ontstaan van RA, op dit moment niet verklaard kan worden.

Naast deze verschillende genetische risicofactoren, hebben wij ook het effect van een niet-erfelijke risicofactor onderzocht, namelijk alcohol. In het verleden is beschreven dat het drinken van alcohol mogelijk zou beschermen tegen de ontwikkeling van RA. In **hoofdstuk 5** tonen we aan dat alcohol inderdaad geassocieerd is met een significant lager risico op het ontstaan van RA, maar dat ditzelfde beschermende effect aanwezig is voor andere vormen van artritis. Daarnaast was er een opvallende inverse relatie tussen de mate van systemische ontstekingsactiviteit (gemeten door de bezinking) en de hoeveelheid geconsumeerde alcohol, wat suggereert dat alcohol mogelijk een effect heeft op, of in verband staat met, ontstekingsactiviteit in het algemeen, en niet specifiek met RA. Op dit moment is het daarom moeilijk om harde conclusies te trekken over het verband tussen alcohol en het ontstaan van RA. Prospectief onderzoek waarin het alcoholgebruik vóór het optreden van de klachten wordt onderzocht, en studies in andere aandoeningen die

net als RA gepaard gaan met systemische ontstekingsactiviteit zijn noodzakelijk om meer licht te werpen op dit mogelijke verband.

DEEL 2: EIGENSCHAPPEN VAN ACPA

Glycosylering

De ontdekking van ACPA heeft geleid tot grote veranderingen in de huidige hypothesen over de pathofysiologie van RA. Het feit dat zowel de aanwezigheid als de hoeveelheid van ACPA toeneemt vlak voordat de ziekte zich manifesteert, suggereert dat ACPA mogelijk pathogeen zouden kunnen zijn. Voor antilichamen in het algemeen geldt dat hun precieze eigenschappen bepalen of de binding aan hun antigen leidt tot weefselschade, of juist deel uitmaakt van een wenselijke afweerreactie. Om deze reden hebben wij besloten meerdere ACPA-karakteristieken nader te bestuderen.

Het eerste kenmerk dat in dit proefschrift in meer detail wordt beschreven, is antilichaam-glycosylering. Aan de eiwitstructuur van een antilichaam kunnen meerdere verschillende koolhydraatketens verbonden zijn. Deze zogenoemde glycanen zijn zeer belangrijk voor de stabiliteit van de antilichaamstructuur en voor de functies die het antilichaam uit kan oefenen. Uit eerder onderzoek was al bekend dat IgG-antilichamen van RA patiënten opvallend veel glycanen zonder galactose bevatten, die mogelijk zouden kunnen leiden tot meer weefselschade. In **hoofdstuk 6** wordt beschreven in hoeverre het glycosyleringsprofiel van ACPA verschilt van het profiel van andere antilichamen. In vergelijking met totaal serum IgG1 was ACPA IgG1 opvallend arm aan siaalzuur, waarvan wordt aangenomen dat het mogelijk een ontstekingsremmend effect heeft. Onderzoek van ACPA die waren geïsoleerd uit synoviaalvocht van RA patients toonde aan dat deze zeer weinig galactosehoudende glycanen bevatten. Binnen de ACPA-positieve RA patients verschilde de antilichaam-glycosylering afhankelijk van de aanwezigheid van reuma-factor (RF): RF-positieve patiënten hadden aanmerkelijk meer galactose-arme ACPA vergeleken met RF-negatieve patiënten. Omdat volgens de gangbare hypothesen de afwezigheid van galactose- en siaalzuurgroepen kenmerkend is voor antilichamen die veel weefselschade kunnen veroorzaken, suggereren deze bevindingen dat ACPA meer schade zouden kunnen veroorzaken dan andere antilichamen. Hiermee lijkt het glycosyleringsprofiel van ACPA compatibel met een pathogene rol van deze antistoffen.

Isotypes en fijnspecificiteit

Een andere eigenschap van antilichamen die van groot belang is voor hun functie, is het antilichaam-isotype. De verschillende humane isotypes (IgM, IgG, IgA, IgD en IgE) verschillen sterk in bijvoorbeeld hun binding aan Fc-receptoren of complement. Om te onderzoeken of het isotypeprofiel van de ACPA respons verandert in de loop van de tijd en

geassocieerd is met gewrichtsschade, wordt in de studie waarvan de resultaten zijn weergegeven in **hoofdstuk 7**, het isotypegebruik van de ACPA respons op meerdere tijdstippen tijdens het ziektebeloop beschreven. De uitkomsten van dit hoofdstuk kunnen het best worden geïnterpreteerd samen met de resultaten van het onderzoek naar de antilichaam-fijnspecificiteit in **hoofdstuk 8**. De fijnspecificiteit geeft de precieze antigenherkenning van een antilichaam weer. Daarnaast is het effect op het klinisch ziektebeloop van individuele verschillen in de antilichaam-fijnspecificiteit onderzocht in **hoofdstuk 9**. Deze drie hoofdstukken geven samen een overzicht over de ontwikkeling van ACPA-fijnspecificiteit en isotypegebruik, en over de voorspellende waarde van deze ACPA-eigenschappen voor het verdere ziektebeloop.

Voor deze studies is gebruik gemaakt van materiaal van patiënten die zich met ACPA-positieve ongedifferentieerde artritis (undifferentiated arthritis, UA) presenteerden bij de Leiden Early Arthritis Clinic (EAC). Een deel van deze patiënten heeft in het verdere beloop RA ontwikkeld, en een deel niet. Door baseline serummonsters van deze patiënten te vergelijken bleek dat de patiënten die later RA ontwikkelden, reeds bij presentatie significant meer gecitrullineerde peptiden herkenden en een breder ACPA-isotypegebruik hadden. Ondanks eenzelfde klinisch fenotype van UA waren daarmee de immunologische karakteristieken van de ACPA respons in dit stadium voorspellend voor het toekomstige ziektebeloop.

Na aanvang van de ziekte was er geen uitbreiding detecteerbaar van fijnspecificiteit- of isotype-repertoire, ondanks een toename van de ziekte-activiteit bij UA patiënten die RA ontwikkelden. Deze observaties werden bevestigd doordat bij patiënten met langdurige RA, geen verschil bleek te zijn in fijnspecificiteit- en isotypeprofiel bij vergelijking van serum afgenomen bij presentatie en na 7 jaar follow-up. Dit suggereert dat de evolutie van de ACPA-karakteristieken voornamelijk vóór aanvang van de ziekte plaatsvindt. Door gebruik te maken van een uniek cohort van bloeddonoren, die vóór en na het ontstaan van RA bloed hadden afgestaan, kon deze hypothese verder getoetst worden. Het aantal gecitrullineerde peptiden dat herkend werd, steeg inderdaad in de tijd voorafgaand aan de manifestatie van de ziekte. Hoewel het isotypeprofiel in dit cohort niet is onderzocht, is het aannemelijk dat de uitbreiding van het isotypegebruik eveneens vroeg in het beloop plaatsvindt, gezien de verschillen die reeds bij presentatie tussen UA patiënten konden worden gevonden. Een belangrijke conclusie die hieruit kan worden getrokken is dat de immunologische uitrijping van de ACPA-respons waarschijnlijk plaatsvindt vóór de klinische manifestatie van de ziekte. Dit betekent ook dat de behandeling waarmee wordt begonnen zodra er klachten zijn ontstaan, vermoedelijk geen invloed meer uit kan oefenen op de karakteristieken van de ACPA-respons.

Om de consequenties van de verschillen in fijnspecificiteit en isotypegebruik voor het verdere ziektebeloop te onderzoeken, werd de associatie tussen gewrichtsschade en deze twee karakteristieken in kaart gebracht. Hieruit kwam een verschil tussen de twee antili-

chaamkenmerken naar voren: hoewel een groter aantal isotypes bij presentatie gepaard ging met meer gewrichtsschade op de lange termijn, bleek deze associatie niet te bestaan met betrekking tot ACPA fijnspecificiteit.

Het effect van genetische risicofactoren en omgevingsfactoren op het ACPA fijnspecificiteitsrepertoire

Van de HLA SE allelen is niet alleen aangetoond dat zij geassocieerd zijn met een hoger risico op ACPA-positieve RA, maar ook dat zij een rol spelen bij het vormgeven van de ACPA-fijnspecificiteit. Met het oog op de bekende interactie tussen de HLA SE allelen en roken in de predispositie voor ACPA-positieve RA, hebben wij onderzocht in hoeverre deze interactie ook de herkenning van verschillende gecitrullineerde peptiden beïnvloedt. **Hoofdstukken 9, 10 en 11** bevatten de resultaten van ons onderzoek naar het effect van genetische risicofactoren en omgevingsfactoren op het ACPA fijnspecificiteitsrepertoire.

Het kwantitatieve effect van het aantal aanwezig HLA SE allelen op de herkenning van gecitrullineerde peptiden wordt beschreven in hoofdstuk 9. De herkenning van bepaalde, maar niet alle, gecitrullineerde peptiden was geassocieerd met het vóórkomen van SE, waarbij het aantal aanwezige HLA SE allelen geen verschil maakte. Dit betekent dat de aanwezigheid van één HLA SE allel voldoende is om het ACPA fijnspecificiteitsrepertoire te vormen. In de hoofdstukken 10 en 11 wordt ook de invloed van de belangrijkste omgevingsfactor voor het ontstaan van ACPA-positieve RA onderzocht: roken. In tegenstelling tot wat eerder was gepostuleerd, bleek de interactie tussen HLA SE allelen en roken niet te leiden tot de specifieke herkenning van een bepaald gecitrullineerd peptide, maar was deze interactie voor meerdere gecitrullineerde antigenen aantoonbaar, zoals voor gecitrullineerd vimentine (cVim) in hoofdstuk 10. Uit verdere analyse in hoofdstuk 11 kwam echter naar voren dat er geen interactie tussen HLA SE allelen en roken aanwezig was als ACPA-positieve cVim-positieve patiënten werden vergeleken met ACPA-positieve cVim-negatieve patiënten. Hieruit kan worden afgeleid dat de interactie tussen genetische risicofactoren en omgevingsfactoren weliswaar het risico op het ontstaan van ACPA-positieve RA verhoogt, maar niet leidt tot herkenning van een bepaald patroon van gecitrullineerde eiwitten. De in hoofdstuk 10 beschreven interactie die ook voor gecitrullineerd vimentine zou gelden, berustte in feite op enkel het effect van de HLA SE allelen en niet op de interactie tussen HLA SE allelen en roken. De belangrijkste bijdrage van deze hoofdstukken aan ons begrip van de pathofysiologie van RA, is de uitkomst dat de HLA SE allelen zeer nauw betrokken zijn bij het ontstaan en de vormgeving van de immuunrespons tegen gecitrullineerde antigenen, terwijl voor roken en de interactie tussen deze twee risicofactoren geen effect op de karakteristieken van de ACPA respons kon worden aangetoond.

De waarde van verschillende ACPA-tests in de klinische praktijk

In het laatste hoofdstuk van deel 2 van dit proefschrift worden verschillende ACPA-tests vergeleken die allen commercieel verkrijgbaar zijn. Naast de meest gebruikelijke tweede-generatie-test tegen cyclische gecitrullineerde peptiden (anti-CCP2), is voor dit onderzoek gebruik gemaakt van de anti-CCP3 test en de bepaling van antistoffen tegen gemodificeerd gecitrullineerd vimentine (anti-MCV). Deze ACPA-tests zijn bovendien ook vergeleken met de traditionele reumafactor (RF) IgM-test. De voorspellende waarde van de tests en van de combinaties van de verschillende bepalingen is onderzocht met betrekking tot drie uitkomstmaten: het voortschrijden van UA naar RA, de mate van gewrichtsschade, en aanhoudende medicatie-vrije remissie oftewel remissie zonder gebruik van disease-modifying antirheumatic drugs (DMARDs).

In **hoofdstuk 12** wordt aangetoond dat de vier bepalingen vergelijkbaar presteerden. Geen van de tests had een significant hogere positieve of negatieve voorspellende waarde dan de andere bepalingen. Daarnaast had het uitvoeren van twee of meer tests geen toegevoegde waarde boven een enkele test, met name als de eerste bepaling een CCP2- of CCP3-test was. Patiënten met positieve uitslagen bij twee of meer van deze auto-antilichambepalingen hadden een slechtere prognose dan patiënten bij wie slechts één auto-antilichaam aangetoond kon worden. Desondanks bleek het aanvragen van meerdere bepalingen niet zinvol, omdat juist de patiënten met twee of meer auto-antilichamen nagenoeg altijd een positieve uitslag van de anti-CCP2- of anti-CCP3-test hadden. Daarom was de voorspellende waarde van een dergelijke test alleen, even groot als die van meerdere bepalingen samen.

DEEL 3: REMISSIE

Het laatste deel van dit proefschrift bestaat uit twee studies over de meest gunstige uitkomst van RA: aanhoudende DMARD-vrije remissie. Hoewel RA traditioneel werd beschouwd als een chronische, levenslang aanhoudende ziekte, hebben recente verbeteringen in de therapeutische mogelijkheden ertoe geleid dat remissie voor steeds meer patiënten binnen handbereik komt. Tegelijkertijd is remissie als klinische uitkomstmaat het onderwerp van talrijke studies geworden. In trials van nieuwe geneesmiddelen wordt remissie vaak als maat voor de werkzaamheid genoemd, maar tot nu toe was onbekend hoeveel patiënten in remissie komen na behandeling met conventionele medicijnen zoals methotrexaat. Een analyse van welke klinische patiëntenkarakteristieken geassocieerd zijn met remissie, was bovendien van grote waarde om een beter begrip van de prognose van de individuele patiënt en van de mogelijk hieraan ten grondslag liggende biologische mechanismen te verwerven.

In **hoofdstuk 13** zijn daarom twee grote, onafhankelijke cohorten van conventioneel behandelde patiënten gebruikt om de prevalentie en voorspellende factoren voor aanhoudende DMARD-vrije remissie vast te stellen. Uit lange termijn data van de Leidse EAC en de Britse Early RA Study (ERAS) kwam naar voren dat 9 tot 15% van de RA-patiënten deze uitkomst behaalt. Twee factoren waren in beide cohorten geassocieerd met het bereiken van aanhoudende DMARD-vrije remissie: een korte klachtenduur voorafgaand aan presentatie bij de reumatoloog en de afwezigheid van IgM RF- en anti-CCP-antilichamen.

Om deze data te kunnen vergelijken met gegevens van patiënten die zijn behandeld volgens een moderne strategie gericht op het bereiken van een zo laag mogelijke ziekte-activiteit, zijn in **hoofdstuk 14** de EAC en de BeSt (behandelstrategieën) studie naast elkaar gelegd. In de BeSt trial werd iedere 4 maanden de ziekte-activiteit met behulp van de Disease Activity Score (DAS) gemeten, waarbij de behandeling volgens protocol werd aangepast met het doel om een $DAS_{44} \leq 2.4$ te bereiken. In vergelijking met de EAC hadden de patiënten die deelnamen aan de BeSt een hogere ziekte-activiteit bij aanvang van de studie, ten gevolge van de inclusie-criteria van de BeSt die een bepaalde mate van ziekte-activiteit vereisten. Desalniettemin was de prevalentie van aanhoudende DMARD-vrije remissie vergelijkbaar in beide cohorten (9.8% versus 10.6%). ACPA-positieve patiënten bereikten vaker remissie als zij waren behandeld met DAS-gestuurde in plaats van met niet-DAS-gestuurde therapie (5.4% versus 2.1%, Odds Ratio 2.68, 95%CI 0.97-7.43). In beide cohorten waren de afwezigheid van ACPA en een korte klachtenduur voorafgaand aan presentatie onafhankelijke voorspellers voor aanhoudende DMARD-vrije remissie.

Uit deze resultaten kan worden afgeleid dat een aanzienlijk deel van RA patiënten na behandeling met conventionele therapie, aanhoudende DMARD-vrije remissie bereikt. De geringe kans van ACPA-positieve patiënten om remissie te bereiken, vestigt eens te meer de aandacht op het ongunstige ziektebeloop bij deze patiëntengroep en de grote verschillen tussen ACPA-positieve en ACPA-negatieve patiënten. In de toekomst zal hopelijk meer duidelijkheid ontstaan over hoe het best met de ACPA-status van een individuele patiënt rekening kan worden gehouden bij het maken van beslissingen omtrent de behandeling.



CURRICULUM VITAE

Diane van der Woude werd in 1979 geboren in Groningen. Van 1985 tot 1987 woonde zij twee jaar in Minneapolis in de Verenigde Staten, waar zij de eerste twee jaar van de basisschool bezocht. Vervolgens verhuisde zij naar Leiden waar zij in 1997 slaagde voor het eindexamen gymnasium. In datzelfde jaar begon zij aan haar studie geneeskunde aan de universiteit van Heidelberg in Duitsland. Tijdens haar studie deed zij een jaar lang onderzoek in het immunologisch laboratorium van de afdeling pathologie van de Harvard Medical School in Boston. Met de resultaten van dit onderzoek behaalde zij haar Duitse doctorstitel met het predicaat “summa cum laude”. Tevens won zij de Wilma Moser Prijs van de universiteit Heidelberg voor het beste medische proefschrift. Na haar co-schappen aan onder andere de Mayo Clinic in Rochester, Minnesota te hebben afgerond, studeerde zij in 2004 af met de hoogste onderscheiding.

In 2005 begon zij aan haar specialistenopleiding interne geneeskunde aan het Medisch Centrum Haaglanden te Den Haag. Op basis van haar score bij de kennistoets voor internisten in opleiding werd zij door de Nederlandse Internisten Vereniging uitgenodigd om deel te nemen aan de European School of Internal Medicine in Portugal. Bij de afdeling reumatologie van het Leids Universitair Medisch Centrum (LUMC) begon zij in 2007 aan haar promotieonderzoek waarvoor zij een AGIKO subsidie van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek ontving. Tijdens haar onderzoeksperiode won zij diverse reisbeurzen evenals een posterprijs en voordrachtsprijs van de Nederlandse Vereniging voor Reumatologie. Op voordracht van de Koninklijke Nederlandse Akademie van Wetenschappen heeft zij in 2011 deelgenomen aan de ontmoetingsdagen met Nobelprijswinnaars in Lindau, Duitsland.

Vanaf 2010 heeft zij haar specialistenopleiding voortgezet in het LUMC, allereerst bij de afdeling interne geneeskunde en vanaf september 2011 bij de afdeling reumatologie. Zij woont in Leiden samen met Ivo Barends.

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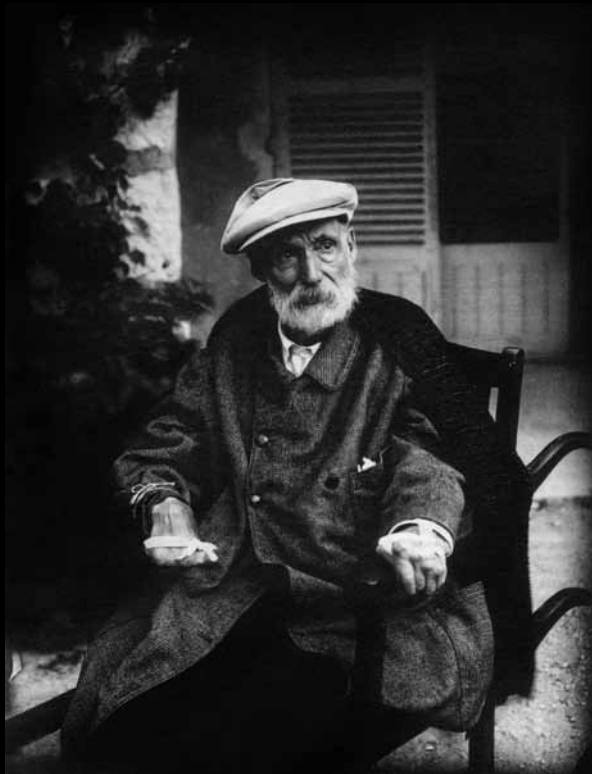
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The painter Pierre-Auguste Renoir
with joint deformities due to
severe rheumatoid arthritis.