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Chapter 1

General Introduction

Rheumatoid Arthritis

The main characteristic of rheumatoid arthritis (RA) is a chronic inflammation of several synovial joints (polyarticular arthritis). Although all synovial joints may be involved, RA most commonly affects the small joints of hands and feet. The persistency of the synovitis can result in the destruction of cartilage and subchondral bone, eventually leading to malformations and disability. As RA is a systemic disease, symptoms such as fatigue, weight loss and fever as well as disorders of the heart, blood vessels, nerves and kidneys are also relatively common.

Because RA is a clinically heterogeneous condition and patients with RA do not share one common symptom that is specific for the disease, the diagnosis of RA is based on a combination of clinical, laboratory and radiological findings. To standardize epidemiologic studies and clinical trials, classification criteria were developed by the American College of Rheumatology in 1958 and were revised in 1987 [1] (Table 1).

The occurrence of RA varies among countries and areas over the world and varies over time [2]. With a prevalence of approximately 1% of the adult white population in northern Europe and North America [3], RA is the most common inflammatory joint disease. Women are affected by RA approximately two times more frequently than men in the Dutch population [4].

Table 1. American Collage of Rheumatology (ACR) 1987 revised criteria for the classification of Rheumatoid Arthritis

Criterion	Definition
1. Morning stiffness	Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement
2. Arthritis of 3 or more joint areas	At least 3 joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician. The 14 possible areas are right or left PIP, MCP, wrist, elbow, knee, ankle, and MTP joints
3. Arthritis of hand joints	At least 1 area swollen (as defined above) in a wrist, MCP, or PIP joint
4. Symmetric arthritis	Simultaneous involvement of the same joint areas (as defined in 2) on both sides of the body (bilateral involvement of PIPs, MCPs, or MTPs is acceptable without absolute symmetry)
5. Rheumatoid nodules	Subcutaneous nodules, over bony prominences, or extensor surfaces, or in juxtaarticular regions, observed by a physician
6. Serum rheumatoid factor	Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in <5% of normal control subjects
7. Radiographic changes	Radiographic changes typical of rheumatoid arthritis on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify)

* For classification purposes, a patient shall be said to have rheumatoid arthritis if he/she has satisfied at least 4 or these 7 criteria. Criteria 1 through 4 must have been present for at least 6 weeks. Patients with 2 clinical diagnoses are not excluded.

Undifferentiated arthritis

As a consequence of RA being a heterogeneous condition that shares characteristics with other diseases a delay in diagnosis and treatment is inevitable. Even when patient delay and referral delay have occurred, RA often cannot be directly diagnosed at the time a patient presents with arthritis to the rheumatologist for the first time. All cases of arthritis that cannot be classified in one of the accepted categories of rheumatic diseases are usually referred to as “undifferentiated arthritis” (UA). The disease course of UA is variable. Time eventually reveals whether an UA patient will develop a specific, chronic rheumatic disorder, for example RA, or whether symptoms will disappear. In the Leiden Early Arthritis Clinic, which provides an inception cohort of patients with recent onset arthritis, only 22% of patients were

diagnosed with RA within 2 weeks after their first visit and approximately 40% was defined as UA at that time point [5]. Spontaneous remission is reported in 13 to 55% of individuals with UA [6].

A problem with the expression "UA" is that it is a non-validated description of a phenotype. In clinical practice all arthritis that cannot be diagnosed into one of the categories will be referred to as *e causa ignota* or as "undifferentiated". In literature, various definitions and criteria are used for the early phase of arthritis. 'Early arthritis', 'early RA', and 'undifferentiated arthritis' are terms that are currently in use to describe either arthritis that has been recently diagnosed, arthritis that might evolve into RA or even arthritis early in the disease course of definite RA. Early after disease onset, patients with UA are in general seen as those patients with the potential for development of persistent inflammatory arthritis, including RA, but in whom a recognized clinical pattern does not (yet) exist. In 1958 the American Rheumatism Association (ARA) identified criteria for 'probable rheumatoid arthritis' [7] as a distinction from classical RA, but these criteria only define a subgroup of patients generally referred to as UA.

In this thesis all cases of arthritis that cannot be classified in one of the accepted categories of rheumatic diseases are referred to as UA. By definition, as soon as a patient does fulfil criteria for a certain category, the patient is reclassified in that other category. In case of fulfilling classification criteria for RA after initial classification as UA, it is often argued that the UA probably was misclassified and that the patient actually had had RA from the beginning. From a clinical point of view, of course it is difficult to argue that that certain patient has suddenly developed a completely different disease. More probably the expression of the disease shifted within the large scale of possible forms of clinical expressions. Assuming that the patient has had the same disease process the whole time, in this case one could consider the conditions rheumatoid arthritis and undifferentiated arthritis as stages of the same disease process. Although this distinction has the disadvantage of an arbitrary border, it allows the study whether the disease specific process acquires characteristics over time such as a change in isotype usage of antibody responses.

However, from a more etiologic point of view, as long as the aetiology of the development of RA is unknown, it is impossible to be sure that the initial UA diagnosis, in retrospective, represented one end of a continuum of the disease entity RA and that it just did not fulfil enough criteria for "full-blown" RA yet. The initial

UA may as well have represented a separate disease entity that required certain aetiological steps to develop into RA, especially since many patients with similar features of UA do not fulfil the criteria for RA at a later time point. As an example, this viewpoint can be compared with the distinction between the clinical diagnosis of tuberculosis and the mere presence of a mycobacterium in the body without any clinical consequences. A combination of both of the above described viewpoints is of course just as likely a possibility.

Furthermore, again from a clinical point of view, we can nowadays more accurately predict the chance that the condition at symptom onset referred to as UA eventually will be referred to as RA [8]. At onset, UA may therefore also be seen as a separate entity with either high or low potential to develop into RA. It is to be hoped for that the insufficient terminology of undifferentiated arthritis and rheumatoid arthritis for syndromes in which patients with different diseases are joined will in the future be separated into better definitions of diseases based on knowledge of the underlying etiopathogenesis.

In this thesis it is phrased "UA patients who develop RA (by fulfilling ACR criteria)", firstly, as it allows the possibility to speculate on aetiological factors being involved at this early symptomatic stage of the disease and secondly, as it describes certain previously defined groups of patients it facilitates easy comparisons between patient-groups and minimizes confusion. Hopefully the results of this thesis will be used with ongoing efforts of both the ACR and the EULAR (The European League Against Rheumatism) to arrive at better classification of patients.

Risk factors for development and progression of RA

Genetic risk factors

The aetiology of RA is, as in many other autoimmune disorders, complex and largely unknown. It is generally accepted that as well environmental as genetic factors, that probably interact with each other, are involved in the pathogenesis of the disease. All these factors result in a heterogeneous phenotype with a wide variety of clinical manifestations, severity in disease progression and differential response to therapy.
A strong genetic, inherited component in the development of RA is supported by familial and twin studies, which suggest that approximately 50 to 60% of the disease susceptibility is due to genetic factors [9;10].

The association of the human leukocyte antigen (HLA) region with RA was the first described, and certain HLA alleles remain the main characterized genetic risk factor contributing to the development of RA. Although the underlying mechanism of how this factor contributes to a higher risk is still not understood, it is estimated that genetic variation in the HLA complex accounts for approximately 35% of the heritability of RA [11].

The function of HLA molecules is to bind peptides and display them to the cell-surface for recognition by the appropriate effector cells. HLA antigens are encoded on the short arm of chromosome 6, within the major histocompatibility complex, which contains more than 200 genes, including for many proteins involved in antigen processing and presentation. Three loci encode for HLA class I molecules: HLA–A, HLA–B and HLA–C. Three other loci encode for HLA class II molecules: HLA–DR, HLA–DP and HLA–DQ. HLA class II molecules are composed of two transmembrane glycoprotein chains, α and β, each consisting of 2 domains: α1 and α2, β1 and β2. The α1 and β1 domains together form the peptide binding groove and contain most of the variation arising from genetic polymorphism. As an exception, the HLA-DRA locus encoding for the DRα chain is essentially invariant, whereas the HLA-DRB locus that codes for the DRβ chain is highly polymorphic (more than 300 allelic variants), especially in the β1 domain.

In 1978, Stastny first observed an association between HLA–DR4 (HLA–DRB1*04) and RA [12]. Since that time, the association has been studied extensively and it has been shown that several other HLA–DRB1 alleles were also associated with the disease, in many different populations. The products of these alleles (HLA–

DRB1*0101, *0102, *0104, *0401, *0404, *0405, *0408, *0410, *0413, *0416, *1001, *1402) are characterized by the shared presence of a conserved amino acid sequence (^{70}QKRAA^{74}, ^{70}QRRAA^{74} or ^{70}RRRAA^{74}) within the third hypervariable, peptide binding, region of the HLA–DRβ1 molecule [13]. Based on that observation, the shared epitope hypothesis was formulated, which proposed that the shared motif itself is directly involved in the pathogenesis of RA by allowing the presentation of the same arthritogenic peptide(s) to T–cells [13]. RA-inducing peptides have however never been identified. Refinements of and additions to the "SE hypothesis" have been proposed in recent years, concerning for example amino acid substitutions at positions 67-74 (instead of 70-74) and genes in linkage disequilibrium with HLA–DRB1 [14;15].

Apart from predisposing effects of HLA–DRB1 alleles, also protective effects have been reported. These protective effects are associated with HLA–DRB1 alleles that encode for another common aminoacid sequence in the third hypervariable region of the HLA–DRβ1 molecule: ^{70}DERAA^{74} (DRB1*0103, *0402, *1102, *1103, *1301, *1302, *1304) [16-18].

As genes within the HLA locus do not account for the entire genetic component of susceptibility, much recent research has focussed on identifying genetic risk factors outside of the HLA region. Candidate genes that have been identified include: PADI-4 [19-21], IL-10 [22;23], PTPN22 [24], CTLA-4 [20;25] and, most recently, TRAF1/C5 [26] and STAT4 [27].

Environmental and other non-genetic risk factors

Consistent information on environmental factors important for the development or the course of RA is relatively scarce. Apart from age and sex, smoking is one of the non-genetic risk factors that have been repeatedly associated with an increased risk to develop RA.

Because of the high female: male ratio in the occurrence of RA and because the severity of several auto-immune disorders tend to change with pregnancies, female sex hormones and reproductive issues have been frequently investigated and have been suggested to influence the development and the severity of RA. While investigating influences of oral contraceptive use on arthritis, in 1987 an unexpected association between smoking and referral to the hospital for RA was first reported by Vessey et al [28].

Since then, several studies investigating associations with and relative risks to develop RA have been published, confirming that smoking is a risk factor for RA. The association between cigarette smoking and RA in general seems to be stronger in men than in women [29;30]. A population based incident case-control study by Stolt et al. demonstrated that both current smokers and ex-smokers of both sexes displayed an increased risk for RF positive RA, not for RF negative RA [31]. A role for smoking in the pathophysiology of the disease is suggested by the fact that an increased cumulative dose of smoking increased the risk of developing RA in these subjects. After smoking cessation, it takes up to 20 years for the risk of RA to return to that of never smokers [31;32], suggesting that smoking does not affect the onset of clinical RA instantaneously.

Other non-genetic factors that have been reported to increase the risk for RA are occupational exposure to silica [33-35] or mineral oil [36]. Obesity and coffee consumption have also been observed to be associated with an increased risk for RA [37;38]. Furthermore, pathogenesis of viral origin has repeatedly been suggested for autoimmune chronic arthritis. Besides well-defined virus induced rheumatic diseases often resembling systemic autoimmune disorders such as RA, viruses may be able to contribute to disease pathogenesis by other mechanisms, such as molecular mimicry or impaired immune control. Several microbes (e.g. cytomegalovirus, Ebstein-Barr virus, parvovirus B19 and *Proteus*) have extensively been hypothesized to trigger autoimmunity in RA on basis of for example serologic data and studies that demonstrated viral DNA in synovial tissue [39-42]. However, microbial infections have as yet never been proven to initiate autoimmunity in RA.

Autoantibodies in RA

One of the reasons to consider the disease process in RA to have an autoimmune nature is the presence of autoantibodies. Many different autoantibodies have been described in RA, including antibodies against cartilage antigens (Type II collagen [43] and human cartilage glycoprotein-39 [44]), against glycolytic pathway enzymes (glucose-6 phosphate isomerase [45], alpha-enolase [46] and creatinine kinase [47]), against immunoglobulin antigens (rheumatoid factor [48] and advanced glycation end products [49]) and against citrullinated proteins or peptides (vimentin [50], fibrinogen [51] and filaggrin [52;53]). Some of these antibodies are specific for

RA, others are not. Two of the most extensively described groups of antibodies are discussed below.

Rheumatoid factor

Since the presence of rheumatoid factor (RF) is one of the ACR criteria for RA, the assay is the most commonly performed autoantibody assay in patients with arthritis. RF was first described to be a serum factor common in RA patients, causing agglutination of red blood cells coated with human or rabbit immunoglobulin G [54;55]. It later became clear that RF is an autoantibody of any immunoglobulin (sub)class (IgA, IgM, etc), directed against the Fc-part of immunoglobulin G (IgG) [48]. RF is not exclusively present in serum from RA patients; it is also commonly detected in patients with other autoimmune diseases (e.g. Sjögren's disease and SLE) or infectious diseases. In healthy individuals, the prevalence is higher in the elderly. The sensitivity and specificity of RF for RA depend highly on the population under study and are approximately 65% and 80% respectively [56].

Anti-citrullinated protein antibodies

In contrast to RF, antibodies against citrullinated peptides or proteins (ACPA) are highly specific for RA. Furthermore, ACPA have been demonstrated to be predictive for the development of RA in patients with UA. In the Leiden early arthritis clinic, RA had developed in 93% of the patients with ACPA, whereas only 25% of ACPA-negative UA patients had developed RA during the first 3 years [57]. The presence of ACPA in patients with RA has also been associated with the extent of joint destruction [58-61]. Furthermore, ACPA have been shown to be involved in the enhancement of disease activity in mice with experimental arthritis. Murine monoclonal antibodies specific to citrullinated fibrinogen enhanced arthritis in mice with collagen induced arthritis when the antibodies were co-administered with anti-collagen type II antibodies [62]. Taken together, these findings point towards a pivotal role of ACPA in the progression of RA. In two separate studies, ACPA has been detected in sera of patients predating the onset of first symptoms. In RA patients who had donated blood for disease registries in a Swedish study, the longest interval of ACPA detection predating the first symptoms of RA was 9 years [63]. In a Dutch population of RA patients who had had donated blood to the local Blood Bank in previous years, ACPA was detected in stored serum samples taken up to 14 years before disease onset [64]. Together with the finding that citrullinated proteins are expressed in the synovium of

inflamed joints [65-67], this has lead to the belief that ACPA play an important role in the pathophysiology of RA.

Citrulline is a non-standard amino acid, as it is not incorporated in proteins during translation. The process of post-translational modification of standard arginine residues into citrulline residues is called citrullination and is catalyzed by peptidylarginine deiminase (PAD) enzymes, which require Ca^{2+} for their activity (Figure 1). The possible effects of protein citrullination lay in electric charge changes, changes in ionic and hydrogen bond forming abilities and conformational changes (e.g. protein unfolding), and their possible consequences for inter- and intramolecular interactions [68]. Citrullination most probably occurs rather non-specifically during inflammation and citrulline is not uniquely present in RA synovium; it is the development of antibodies against citrulline containing peptides that is specific for RA [66].

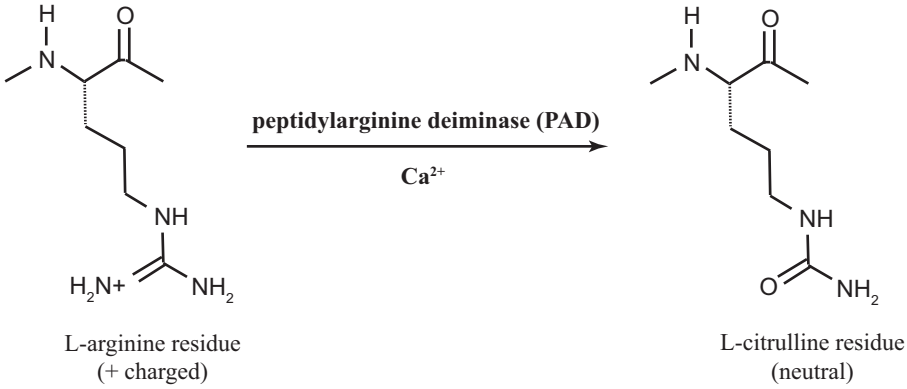

Figure 1. Enzymatic conversion of arginine residues to citrulline residues is catalyzed by PAD enzymes which require $Ca2^{+}$ for their activity.

In retrospective, the initial description of a specific autoantibody for RA, namely anti-perinuclear factor (APF) [69], is an anti-citrulline antibody. Both APF and an in 1979 described group of antibodies then called "anti-keratin antibodies" (AKA) [70] have been demonstrated to target the same antigen: the epithelial protein fillagrin [71]. Later, in 1999, it was shown that citrullination was essential for fillagrin to be recognized by these anti-fillagrin antibodies (AFA) [52;53]. Convenient, reliable

anti-citrulline antibody tests have been developed since, using synthetic cyclic, citrullinated peptides (CCP) as the capture antigen [72;73]. These tests reach a median specificity of 97% (range 81-100) with a sensitivity of approximately 68% [56].

At present, it is not clear which citrullinated antigens the anti-citrulline response in RA is initially directed against and which specific ACPA may be pathological. In the inflamed joint, several citrullinated proteins have been detected, for instance citrullinated fibrin [74] and vimentin [75]. Moreover, many different citrullinated peptides are recognized by different RA sera [53].

Whether ACPA indeed are of pathophysiological importance or whether they are merely an epiphenomenon is also still unknown and subject of investigation. One hypothesis on how ACPA could be involved in causing chronic arthritis in an otherwise transient joint inflammation is the following [76]: Monocytes and polymorphonuclear cells migrate into the synovium of the joint during inflammation. As a result of cell death, PAD enzymes that are expressed in these cells are released and activated by extracellular Ca^{2+}. Proteins become citrullinated by PAD and are picked up, processed and presented by antigen presenting cells (APCs) to T–cells in the context of HLA class II molecules. T–cells then provide help to B–cells that start producing ACPA. ACPA from locally producing B–cells or ACPA entering the joint from the serum is subsequently able to complex with citrullinated antigens in the joint and can attract and trigger several inflammatory cells and the complement system, resulting in a perpetuation of the process and chronicity of the disease.

Outline of the thesis

Without exact knowledge of the initiating and perpetuating factors of the disease, lots of progress has been achieved in the treatment of RA in the last decades. RA has become less often a severe disabling condition. Early treatment with new therapeutics and new treatment strategies are effective in the majority of the patients. Expectably, with a better understanding of the disease process and pathophysiology of several phenotypes within RA, treatment can be better targeted in the future, leading to an even better disease outcome. Although the presence of ACPA is only one property which could divide RA into two subpopulations, it may be a crucial one, as a pivotal role for these antibodies in the pathophysiology of the disease is suggested by many data although conclusive biological proof remains to be attained.

The present thesis concerns studies on several aspects of the ACPA response in patients with UA and patients with RA. One main objective is to investigate the effect ACPA has on the development of RA and in which way ACPA and other known risk factors for RA could collectively contribute to the risk to develop disease. The second aim of the thesis is to increase knowledge on the development of the ACPA response itself by describing characteristics of the ACPA response in several groups of patients.

Chapter 2 is a review of the present literature on the development of RA in patients with UA, to bring these syndromes into a broader context and give insight in the percentage of patients who develop RA according to the ACR classification criteria after they have presented to the rheumatologist with UA.

Chapter 3 describes the differences and similarities between ACPA-positive and ACPA-negative RA at the moment of first presentation to the rheumatologist and after follow-up.

The strongest genetic risk factors identified many years ago are the HLA–DRB1 SE alleles. Recently, SE alleles were described to predispose only for ACPA-positive RA. In **Chapter 4**, it was investigated whether SE is a risk factor for ACPA-positive RA or whether it is a risk factor merely for the development of ACPA.

The contribution of HLA–DRB1 alleles to the development of ACPA-negative RA was investigated in **Chapter 5**.

In **Chapter 6**, it was determined whether HLA–DRB1 SE alleles interact with the known environmental risk factor tobacco exposure in the risk to develop either ACPA-positive or ACPA-negative RA and whether different subtypes of SE alleles interact differently with smoking in the risk to develop RA.

Chapter 7 evaluates whether tobacco exposure besides influencing the risk to develop (ACPA-positive) RA, also influences the constitution, the isotype usage of the ACPA response.

In **Chapters 8 and 9**, characteristics of the ACPA response are described, with respect to different isotypes of ACPA (Chapter 8) and the fine-specificity of the

ACPA response (Chapter 9). These characteristics may teach us about the status of the response in general, about differences between the ACPA response in RA and UA (Chapter 8) and about the possible role of SE alleles in the fine-specificity of the ACPA response (Chapter 9).

Finally, the results described in this thesis are summarized and discussed in **Chapter 10**.

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Chapter 2

Undifferentiated arthritis – Disease course assessed in several inception cohorts

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Abstract

The prognosis of patients with undifferentiated arthritis (UA) may vary from self-limited to severe destructive rheumatoid arthritis (RA). Because early aggressive treatment might offer an effective means to slow disease progression in RA, it is important to identify UA patients who will develop RA and treat them as early as possible. At the same time, inappropriate treatment of patients with a more benign disease course should be avoided. Here, an overview is given of the characteristics and numbers of patients with UA who evolve into RA.

UA is defined as any arthritis that has the potential for a persistent course, without fulfilling the classification criteria for specific rheumatic disorders. To compare endpoints in the different databases, the 1987 ACR criteria for RA were used.

In the nine databases employing a similar definition for undifferentiated arthritis, the proportion of patients with UA that evolved into RA within 1 year varied from 6% to 55%. These differences arise in large part from differences in the inclusion criteria and in the definitions used for UA and RA. The data from the various cohorts support a hypothesis that a considerable proportion of UA patients are actually patients with RA in a very early stage. Controlled intervention studies with early antirheumatic treatment in these patients are mandatory in order to provide further insight into the natural course of UA and to define a treatment strategy that will successfully slow or prevent disease progression.

Chapter
2

Introduction

Several studies have indicated a beneficial effect of the early treatment of rheumatoid arthritis (RA) to achieve a less severe disease course or even to induce remission [1-3]. The possible extra therapeutic benefit attainable in this early period in the disease has been called the "window of opportunity". Since the presentation pattern of RA varies widely, it has been suggested that the treatment should be started as early as possible, even before patients fulfil the American College of Rheumatology (ACR) criteria for RA [4]. Ideally, knowledge of prognostic factors in patients with undifferentiated arthritis (UA) will allow the identification of those patients who will develop RA, so that the inappropriate treatment of patients who will not develop RA can be avoided. For this it is also necessary to know the natural course of UA. The present review will attempt to describe the natural course of UA as reported in early arthritis cohorts.

The first problem encountered in the search for the percentage of patients presenting with UA who will develop RA is the fact that UA is a non-validated description of a phenotype. In clinical practice, all cases of arthritis that cannot be classified in one of the accepted categories are referred to as *e causa ignota* or "undifferentiated". For inclusion in early arthritis cohorts, various definitions and criteria have been used for the early phase of arthritis, which makes it difficult to compare the composition of the different study groups. 'Early arthritis', 'early RA', and 'undifferentiated arthritis' are terms that are currently in use to describe either arthritis that might evolve into RA or that has been diagnosed early after onset of arthritis or even early in the disease course of definite RA. Therefore, patients with UA are in general seen as those patients with the potential for development of persistent inflammatory arthritis, including RA, but in whom a recognized clinical pattern does not (yet) exist. In 1958 the American Rheumatism Association (ARA) identified criteria for 'probable rheumatoid arthritis' [5] as a distinction from classical RA, but these criteria only define a subgroup of patients generally referred to as having UA.

In this review, defining RA according to the classification criteria also has disadvantages from a scientific viewpoint. The ACR criteria for RA were developed to identify patients with established RA, and not for diagnostic purposes. In clinical practice, it is of great relevance to distinguish patients on prognostic items such as persistent arthritis or destructive arthritis. On the other hand, all intervention studies to date have been based on fulfilment of the ACR criteria, and evidence that adequate

treatment changes the course of disease as well as the prognosis is available only in patients who meet the ACR criteria. Therefore, notwithstanding the imperfect definitions of the phenotype for clinical practice, it is important to assess what proportion of UA cases progress to RA, as defined by the ACR criteria.

Inception cohorts

Early RA databases and their inclusioncriteria are listed in Table 1. The databases marked by an asterisk have included and described patients with UA. Only the latter databases will be discussed. The other databases include 'early RA' patients who fulfilled the1987 ACR criteria for established RA. In Finland an early arthritis cohort was started in 1975 [6]. Adults with one or more swollen joints and a symptom duration of less than 6 months were referred to the hospital in Heinola. Forty-three percent of the patients from this cohort had non-specific arthritis, defined as probable RA according to the 1958 ARA criteria or arthritis not falling within any specific diagnostic group [7]. The percentage of UA patients who developed RA was not mentioned. After 3 years 58% of the UA patients had no symptoms. Twenty-eight percent of the patients in this cohort met the 1987 ACR classification criteria for RA at inclusion.

From the same cohort, 32 patients were described with the diagnosis of non-classified monoarthritis, defined as swelling of a peripheral joint not due to trauma, degenerative joint diseases or any other specific joint disease [8]. Of those 32 patients, 2 (6%) had rheumatoid factor (RF)-positive definite RA after a 3-9 year follow-up. In 29 patients the diagnosis remained "non-classified" arthritis during follow-up.

In the Finnish cohort a group of 47 patients with recent onset RF-negative oligoarthritis was also described [9]. After 23 years of follow-up, reclassification of the diagnoses revealed 1 patient with RA, 7 patients with erosions in the hands or feet, 1 patient with systemic lupus erythematosus (SLE), 1 patient with ankylosing spondylitis, 2 patients with "post-traumatic arthritis", 4 patients with osteoarthritis, and 6 patients with reactive arthritis. The other 25 patients presumably still did not fulfil the criteria for a rheumatic disease.

In the UK the Norfolk Arthritis Registry (NOAR) has been following patients with early inflammatory polyarthritis who had been referred by general practitioners (GPs) and local rheumatologists since January 1990, as described by Symmons et al. [10]. All adults with two or more swollen joints, lasting for at least 4 weeks, could be included. The proportion of UA patientswho developed RA was not mentioned in

the published data. However, Wiles et al. [11] described a study in which the ACR criteria were applied cumulatively, meaning that once a criterion was fulfilled, this criterion was regarded as positive in all subsequent assessments. In this study, 55% of the patients with a symptom duration of less than 2 years satisfied the criteria for RA at inclusion as described above. Sixty-seven percent fulfilled these criteria after one year.

Also from the UK, Quinn et al. [12] recently described a cohort of 97 patients with early undifferentiated arthritis of the hands and a disease duration of less than 12 months who were followed for 12 months. RA developed in 14% of the 97 UA patients. Thirty-six percent had persistent synovitis (defined as the presence of 2 or more of the following: joint swelling, joint tenderness or decreased range of motion) after 12 months, whereas 13% were in clinical remission. Only 54% of the patients could be diagnosed with a specific rheumatic disease after a 12-month follow-up.

Initially these patients were included in a cohort of 1877 patients in the Leeds early arthritis clinic of whom 56% had an inflammatory arthritis at inclusion; 50% of these patients had RA and 23% had UA. Patients with UA were classified as having an inflammatory disorder where a specific rheumatic disease could not be diagnosed. It should be noted that patients were eligible for inclusion in the study if they had a history suggestive of inflammatory arthritis, but clinically detectable synovitis was not required. This resulted in the observation that 47% of patients with UA had no synovitis at the time of inclusion.

In Germany Huelsemann et al. [13] described a two-year prospective cohort study of patients with "rheumatic symptoms" for less than 1 year's duration who were investigated in an early arthritis clinic in Duesseldorf. The patients were sent to the tertiary referral centre by general practitioners, internists and orthopaedic physicians. Of 320 patients who were investigated, 217 were classified as having inflammatory rheumatic diseases. Of these 217 patients, 117 (54%) could not be diagnosed definitely and were thus considered undifferentiated, and 39 (19%) were diagnosed as having RA. Sixty-eight percent of the patients with UA presented with oligoarticular joint manifestations, while 14% had a monoarticular and 18% had a polyarticular disease (5 or more joints). Follow-up data 4 to 38 months after the initial symptoms were available for 28 patients with UA. Fifteen (54%) of them had a complete remission, 8 patients had unchanged or progressive unclassified disease and 2 (7%) were diagnosed with RA according to the ACR 1987 criteria.

Table 1. Early RA databases

Study group	Inclusion criteria	Study strategy and characteristics	N	Reference
Heinola Cohort/ Rheumatism Foudation Hospital Cohort (Finland) *	≥1 swollen joints Disease duration ≤6 months Age ≥16 years	Prospective cohort Referred by phycisians of several health centres and hospitals Follow-up after 1, 3, 8, 15, 20 and 25 years	442	(6)
Norfolk Arthritis Register (UK) *	early inflammatory polyarthritis Age ≥ 6 years ≥2 swollen joints Symptom duration ≥4 weeks Onset after January 1989	Referred from GP and local rheumatologists Yearly follow-up for at least 5 yrs Patient visited at home		(10;22)
Leeds (UK) *	Undifferentiated arthritis of the hands Symptom duration < 12 months	Patients from the Leeds Early Arthritis Clinic (n=1877) Pyramid treatment strategy	97	(12)
Duesseldorf (Germany)*	Rheumatic symptoms Duration ≤1year Age >15 years	2-year prospective cohort study Referred by GPs, internist, orthopaedic physicians	320	(13)
Austrian Early Arthritis Registry *	Inflammatory arthritis with ≥2 clinical criteria and ≥1 laboratory criterion Duration of symptoms <12 weeks	Referred by GPs and internists to participating rheumatologists Multi-centre (country-wide) Every 3 months questionnaires		(14;16)
Wichita Arthritis Centre (USA) *	Undifferentiated polyarthritis syndrome or RA (ACR'87 criteria) Disease duration ≤2 years	Half of patients self-referred Follow-up at least 13 months	506 (RA) 638 (UA)	(17)

Study group	Inclusion criteria	Study strategy and characteristics	N	Reference
ESPOIR Cohort Study (France) *	Certain or probable clinical diagnosis of RA UA that may develop into RA Duration of symptoms <6 months Age 18-70 years ≥2 inflammatory joints for the past 6 weeks No DMARD use prior to inclusion	800 patients from the community 10 yrs follow-up		(18)
Amsterdam (The Netherlands) *	≥2 swollen joints Disease duration <3 years	Patients from an early arthritis clinic	203	(19)
Leiden Early Arthritis Clinic (The Netherlands) *	Any arthritis confirmed by rheumatologist Symptom duration < 2 years No DMARD use prior to inclusion	Referred by GPs Follow-up at 2 weeks, 3 months and yearly		(20)
EURIDISS-Oslo (Norway)	RA (ACR'87 criteria) Age 20-70 years Disease duration ≤4 yrs	Norwegian part of international collaborative research effort Follow-up at 1, 2 and 5 years	238	(23)
French Early Arthritis Cohort	RA (ACR'87 criteria) RA diagnosis < 1 year No DMARD use prior to inclusion	Multi-centre Referred from primary care Follow-up 10 year		(18)
GIARA Registry Study Group (Italy)	RA (ACR'87 criteria)	Aggressive RA registry	706	(24)
Jyäskylä Cohort (1983-1985) (Finland)	Newly diagnosed RA (ARA'58 criteria)	Follow-up 18-24 months	58	(6;25)
Jyäskylä Cohort (1988-1989) (Finland)	Definite RA (ARA'58 criteria) and ≥2 criteria (ESR>20mm/hour, ≥6 joints with active RA, duration morning stiffness >45 minutes) Age 18-80 years Symptom duration <1 year	Randomised, double blind, placebo controlled study on treatment with sulfasalazine Follow-up at 4, 8, 12, 24 and 48 weeks	80	(6;26)

Study group	Inclusion criteria	Study strategy and characteristics	N	Reference
Central Finland RA database	(Newly) diagnosed RA according to physician	All new patients with RA are referred to Jyäskylä Central Hospital	>2000	(6)
Helsinki Cohort (Finland)	RA (ACR'87 or revised ACR'87 criteria) symptom duration <2 year no DMARD use prior to inclusion	Prospective study on early aggressive therapy Referred from primary care or private outpatients clinics	150	(6;27)
FIN-RACo study (Finland)	RA (ACR'87 criteria) Symptom duration <2 year Age 18-65 year, ≥3 swollen joints and three of: ESR>28, CRP>19, morning stiffness.>29min, >5 swollen joints, >10 tender joints	Multi-centre Randomised trial on treatment strategies	199	(28)
CLEAR Registry (USA)	Early RA Disease duration < 2 years African-American		500	(29)
German early RA inception cohort	RA (ACR'87 criteria) Age 21-75 years Disease duration < 1 year	Prospective, multi-centre study Referred by GP, rheumatologist, arthritis care units Follow-up at least 3 years		(30;31)

The Austrian early arthritis registry (Austrian Early Arthritis Action, EAA) [14] follows patients with inflammatory arthritis whose symptoms began less than 12 weeks before presentation and who fulfil at least 2 clinical criteria (absence of trauma, joint swelling in at least 1 joint, joint pain in at least 1 joint, morning stiffness >60 minutes) and at least 1 laboratory criterion (positive RF, ESR >20 mm/hour, CRP >5 mg/L, leucocytes > upper limit of normal). Approximately 15% of the patients after 1 year still had no established diagnosis and were classified as having UA. Sixty-five percent of the patients had RA after 1 year, using the ACR 1987 criteria cumulatively as described in the NOAR (15).

In another paper, Machold et al. [16] describe 108 patients who had been followed for at least 1 year. At inclusion, 31 patients (29%) had UA and 50 patients (46%) were diagnosed with RA. After 1 year, 17 of the UA patients (55%) were diagnosed with RA. The diagnosis of RA was made if patients fulfilled the ACR 1987 criteria, or if clinical examination revealed a polyarthritis of at least 6 weeks duration without evidence of other inflammatory rheumatic diseases. In cases in which the diagnosis could not be ascertained by the rheumatologist, the disease was classified as UA.

Wolfe et al. [17] followed 532 patients with UA at the Wichita Arthritis Center who at presentation had a symptom duration of at least 2 years. Synovitis was not required if the patient had other clinically suspected characteristics of RA in the history, at physical examination or in laboratory results. 100% were followed up for ≥13 months, 93% for ≥2 years and 87% for ≥ 3 years. Twenty-two percent of the patients had no joint swelling, and 6% had questionable swelling at the time of inclusion. Fifty-four percent of the cases resolved, while 17% evolved into RA.

A French multi-centre cohort study [18] that includes patients with early arthritis with a maximum duration of 6 months has recently been started. No data on this ESPOIR cohort have been published yet. The study includes RA patients, probable RA patients and patients with a clinical diagnosis of UA that may potentially develop into RA and with at least two inflammatory joints for the past 6 weeks. UA patients with “no potential to develop into RA” are excluded.

In a Dutch study by Jansen et al. [19], a group of patients from the Amsterdam early arthritis clinic with peripheral arthritis involving at least 2 joints and a disease duration of less than 3 years was followed in order to identify variables that could predict an outcome of progressive disease after 1 year. In this study 27% (n=77) of the patients were clinically diagnosed as having UA at inclusion and 72% (n=203) as

RA. Forty-two percent of the UA patients had oligoarthritis and 58% had polyarthritis. After one year 42% of the patients with UA were categorized as progressive and 58% as mild, using radiographic parameters and the HAQ score as criteria. Thirty-one percent of the progressive UA group (n=10) fulfilled the ACR criteria for RA after one year. From the total UA group, 17% were classified as having RA at 1 year.

The other Dutch cohort is the Leiden Early Arthritis Clinic, which includes patients with any form of arthritis confirmed by a rheumatologist except gout, and a symptom duration of 2 years or less [20]. Out of 936 patients at inclusion, 346 (37%) were categorized as having UA and 22% were diagnosed with RA. After one year of follow-up 32% of the UA patients fulfilled the ACR 1987 criteria for RA. The percentage had increased to 40% at 3 years of follow-up [21].

Discussion

We have reviewed inception cohorts with monoarthritis and polyarthritis to evaluate what proportion of patients with UA progress to RA. In the various cohorts these proportions varied considerably. This may be explained by the differences in referral and recruitment procedures, inclusion criteria and, most notably, disease criteria between the various cohorts. The reported proportions of patients with UA who progressed to RA one year after inclusion range between 6% and 55%. However, in the cohorts that required arthritis to be present at inclusion and that defined RA according to the ACR 1987 criteria, the proportions range from 17% to 32%.

The part of the Finnish early RA cohort in which only 6% of the patients with UA progressed to RA after a follow-up period of 3 to 9 years [8] probably represents a subgroup of UA, defined as non-classified monoarthritis and RF negative oligoarthritis, and consequently, a small group of patients is concerned (n=32). Huelsemann et al. reported that 7% of their patients with UA developed RA [13]. However, at inclusion patients were diagnosed based on clinical expertise and were not classified according to ACR criteria. As only 18% of the UA patients at inclusion had a polyarticular disease, it is possible that a certain proportion of the patients with polyarthritis at inclusion were prematurely diagnosed as having RA. Therefore the proportion of UA patients who progressed to RA might have been underestimated. Also, only 24% of the 117 patients with UA at inclusion were followed. This suggests that these patients represent a subgroup of UA that more often than not has a mild or self-limiting disease course.

Wolfe et al. reported that 17% of their UA patients progressed to RA after 3 years [17]. The inclusion of patients without synovitis in this cohort could have led to an underestimation of this value however. The same is true for the cohort described by Quinn et al. [12]. Jansen et al. [19] described a cohort of oligo- or polyarthritis patients, and found a 17% progression from UA to RA. In a mixed population of mono- and polyarthritis patients, van Gaalen et al. [21] reported that 32% progressed from UA to RA (diagnosis according to the ACR 1987 criteria) within one year. An even higher rate of 55% was described by Machold et al. [16]. However, in that study not only patients who fulfilled the ACR criteria were diagnosed as having RA, but also patients with polyarthritis for more than 6 weeks without evidence of other inflammatory rheumatic diseases upon investigation. Therefore, the value of 55% could be an overestimation of RA in comparison with other studies that focused only on the ACR criteria for diagnosing RA.

The findings of these cohort studies support the hypothesis that many patients with UA are actually in the first stages of RA. Unpublished observations in the Leiden EAC cohort indicate that patients whose UA evolved into RA within one year have, on average, the same prognosis as patients who presented with RA at baseline, as measured by the rate of joint destruction, disease activity and functional status. Early treatment may moderate the disease progression, possibly to the point that fewer patients develop RA as defined by the ACR 1987 criteria. Ideally, patients with UA who will progress to RA should be identified at presentation in order to receive early aggressive treatment. Decisions to treat UA patients will depend on the likelihood that a patient will develop RA. When this is high, it is worthwhile to start disease modifying anti-rheumatic drug (DMARD) therapy immediately. Our review shows there is a 17-32% pre-test probability that a patient with UA actually has RA. The question is what tests are available to obtain a substantially higher post-test probability.

A great deal of research has already been carried out to try to identify predictors that could be used for such a test. At present the most promising diagnostic tool appears to be a test for anti-cyclic citrullinated peptide (CCP) autoantibodies. Van Gaalen et al. [21] reported that in the Leiden EAC 93% of the patients with UA who were anti-CCP–positive fulfilled the ACR 1987 criteria for RA within 3 years. The negative predictive value was 75%. Furthermore, anti-CCP antibody testing was of little value in UA patients who fulfilled none of the ACR 1987 criteria for RA, but had a significant additional value in predicting the progression to RA in UA patients

fulfilling one or more of these criteria at presentation. As anti-CCP antibodies can be detected several years before the onset of disease, Holers and Majka [32] proposed a model in which the development of anti-CCP antibodies in genetically predisposed individuals initiates the autoimmune process in a preclinical phase. The presence of anti-CCP antibodies could therefore be used as prediction criterion for the development of RA in patients with UA.

Another more intuitive approach rather than an analytical one is to treat all UA patients with a relatively safe drug regardless of their post-test probability in the event of new predictive tests. This would prevent that "false-negative" patients would not receive aggressive therapy. It is however not (yet) clear how aggressive such a – at the same time safe – therapy could be. It is unclear if such a therapy should be, for example, MTX, corticosteroids or NSAIDs. Current research is focusing on these treatments and on whether patients with UA will benefit from early treatment with DMARDs to a similar extent as RA patients. In Leiden a double-blind placebo-controlled randomised trial (Probaat) with 110 patients who fulfill the ACR 1958 criteria for probable RA and with a symptom duration of less then 2 years is now underway. The aim of the study is to determine whether early treatment can prevent progression into RA or even induce remission. The patients are being treated with either placebo or MTX. After one year the medication will be tapered and then stopped.

The study 'Stop Arthritis Very Early' (SAVE) is another placebo-controlled study that has just started and will try to modify the disease course of UA patients whose complaints began less than 16 weeks earlier, with a single injection of methylprednisolone i.m. Subgroup analyses may reveal whether all UA patients need to be treated or if only a proportion of these patients will benefit from early treatment.

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Chapter 3

Antibodies to citrullinated proteins and differences in clinical progression of rheumatoid arthritis

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Abstract

Antibodies to citrullinated proteins (anti-cyclic-citrullinated peptide [anti-CCP] antibodies) are highly specific for rheumatoid arthritis (RA) and precede the onset of disease symptoms, indicating a pathogenetic role for these antibodies in RA. We recently showed that distinct genetic risk factors are associated with either anti-CCP–positive disease or anti-CCP–negative disease. These data are important as they indicate that distinct pathogenic mechanisms are underlying anti-CCP–positive disease or anti-CCP–negative disease. Likewise, these observations raise the question of whether anti-CCP–positive RA and anti-CCP–negative RA are clinically different disease entities. We therefore investigated whether RA patients with anti-CCP antibodies have a different clinical presentation and disease course compared with patients without these autoantibodies. In a cohort of 454 incident patients with RA, 228 patients were anti-CCP–positive and 226 patients were anti-CCP–negative. The early symptoms, tender and swollen joint count, and C-reactive protein level at inclusion, as well as the swollen joint count and radiological destruction during 4 years of follow-up, were compared for the two groups. There were no differences in morning stiffness, type, location and distribution of early symptoms, patients' rated disease activity and C-reactive protein at inclusion between RA patients with and without anti-CCP antibodies. The mean tender and swollen joint count for the different joints at inclusion was similar. At follow-up, patients with anti-CCP antibodies had more swollen joints and more severe radiological destruction. Nevertheless, the distribution of affected joints, for swelling, bone erosions and joint space narrowing, was similar. In conclusion, the phenotype of RA patients with or without anti-CCP antibodies is similar with respect to clinical presentation but differs with respect to disease course.

Introduction

Autoantibodies directed to citrullinated proteins (e.g. anti-cyclic-citrullinated peptide [anti-CCP] antibodies) are highly specific serological markers for rheumatoid arthritis (RA) that are thought to be directly involved in the disease pathogenesis [1]. Citrullinated proteins are not exclusively located in synovial tissue of RA patients, but can also be found in synovium samples of patients with other inflammatory joint diseases [2] – suggesting that the specificity of anti-CCP antibodies for RA is not due to the expression of citrullinated proteins, but might be the result of an abnormal humoral response. Intriguingly, this antibody response may occur years before any clinical symptoms, as shown by the presence of anti-CCP antibodies several years before the clinical onset of arthritis [3,4]. Furthermore, a proportion of RA patients do not harbour anti-CCP antibodies, suggesting that the presence of anti-CCP antibodies is not obligatory for the development of arthritis or that the pathogenic mechanisms underlying anti-CCP–positive RA and anti-CCP–negative RA are different.

These observations inspired subsequent research addressing the question of whether RA patients with anti-CCP antibodies are different from those who are anti-CCP–negative. We very recently demonstrated in two independent Caucasian populations that the shared epitope encoding HLA-DBR1 alleles associated with RA in patients with anti-CCP antibodies but not in patients without these antibodies (unpublished data, [5]). These findings are important as they indicate that the shared epitope alleles are not associated with RA as such, but rather with a particular phenotype of the disease.

Given the findings suggesting a pathophysiological role for anti-CCP antibodies in RA and the reported immunogenetic differences between anti-CCP–positive and anti-CCP–negative patients, it is conceivable that anti-CCP–positive RA and anti-CCP–negative RA are different disease entities and thus have different phenotypical properties. Anti-CCP antibodies have been suggested to be associated with more severe radiological outcome [5,6]. To our knowledge, however, a detailed description of the distribution and degree of early symptoms and signs in both patient groups has not been published. Nevertheless, such an analysis is relevant as it might provide novel insight into the putative pathogenic role of anti-CCP antibodies in the aetiology of the disease.

In this study, therefore, we set out to determine whether anti-CCP–positive RA patients and anti-CCP–negative RA patients differ in different aspects of their

phenotype: the early symptoms of disease, the findings of physical examination at initial presentation, or the acute phase reactant C-reactive protein at initial presentation. Moreover, we expanded the data on the influence of anti-CCP antibodies on the disease course during 4-year follow-up for the distribution and extent of both inflammation (swollen joints) and radiological joint destruction. We show that the phenotype of RA patients with or without anti-CCP antibodies is similar with respect to clinical presentation but differs with respect to disease course.

Patients and methods

Patients

An Early Arthritis Clinic was started in 1993 at the Department of Rheumatology of the Leiden University Medical Center, the only referral centre for rheumatology in a health care region of about 400,000 inhabitants in the western part of The Netherlands [7]. General practitioners were encouraged to refer patients directly when arthritis was suspected. Referred patients could be seen within 2 weeks and were included in the programme when the physician's examination of the patients revealed arthritis and the symptoms had lasted less than 2 years.

At the first visit the rheumatologist answered a questionnaire inquiring about the initial symptoms as reported by the patient (type of initial joint symptoms, localization and distribution of initial joint symptoms, presence of morning stiffness). Patients rated their global assessment of disease activity on a visual analogue scale (0-100). The Health Assessment Questionnaire, a self-assessed questionnaire asking about the ability of the patient to perform several daily activities over the past week, was used to obtain an index of disability. A tender joint count and a swollen joint count [8,9] were performed on entering the study and yearly thereafter. For the tender joint count, each joint was scored on a 0-3 scale with 3 being maximal tenderness (0=no tenderness, 1=pain on pressure, 2=pain and winced, and 3=winced and withdrew). For the swollen joint count, the individual joints were scored on a 0-1 scale (0=no swelling, and 1=swelling).

At inclusion, blood samples were taken from every patient for routine diagnostic laboratory screening including C-reactive protein and were stored to determine antibodies to CCP2 at a later time point. The anti-CCP2 antibody ELISA (Immunoscan RA Mark 2; Euro-diagnostica, Arnhem, The Netherlands) was

performed according to the manufacturer's instructions with a cut-off value of 25 units.

More than 1600 early arthritis patients are presently included in the Early Arthritis Clinic cohort and have a follow-up of at least 1 year. A total of 454 patients fulfilled the diagnosis of RA according to criteria of the 1987 American College of Rheumatology 1 year after inclusion in the study. The treatment of the patients in our longitudinal cohort study is characterized by a secular trend. The 122 RA patients (61 anti-CCP–negative and 61 anti-CCP–positive) included between 1993 and 1995 were treated initially with analgetics and subsequently with chloroquine or salazopyrine if they had persistent active disease (delayed treatment). The 135 (70 anti-CCP–negative and 65 anti-CCP–positive) RA patients included between 1996 and 1998 were promptly treated with either chloroquine or salazopyrine (early treatment) (for further description, see [10]). The 197 RA patients (97 anti-CCP–negative and 100 anti-CCP–positive) included after 1998 were promptly treated with either methotrexate or salazopyrine (early treatment).

The rheumatologists that treated the patients were not aware of the anti-CCP status of their patients because anti-CCP antibodies were not routinely determined at inclusion but were assessed for research purposes years after inclusion using stored serum samples. Patients gave their informed consent and the local Ethical Committee approved the protocol.

Radiographic progression

Radiographs of the hands and feet were made at baseline, at 1 year and yearly thereafter. For 138 patients a complete radiological follow-up was available for 4 years. Inherent to an inception cohort, not all included patients had already completed 4 years of follow-up. Radiographs were scored using the Sharp-van der Heijde method [11]. The rheumatologist that scored the radiographs was blinded to the clinical data and was unaware of the study question. The distribution of radiological destruction of the small joints was studied by comparing the erosion score and joint space narrowing score of the metacarpophalangeal (MCP) and proximal interphalangeal (PIP) joints of the hands.

Statistical analysis

Differences in means between groups were analysed with the Mann-Whitney test or the *t* test when appropriate. Proportions were compared using the chi-square

test. In the analysis of the tender joint count and the swollen joint count, the scores for the left and right joints were summed for each joint location. Furthermore, the scores for the individual MCP joints were summed, as well as the scores for the metatarsophalangeal joints and the interphalangeal joints of the hands and feet. For the 138 RA patients with complete 4-year radiological follow-up, the swollen joint count, the erosion score and the joint space narrowing score were determined for the individual MCP and PIP joints of the hands at inclusion and at 2 and 4 years follow-up, and are expressed as the mean with the 95% confidence interval (CI).

The distribution and degree of radiological destruction and swelling of these joints was studied by comparing the variance of these scores for the individual joints. The 95% CI was used as a measure of variance; as the number of observations in this study is constant (138 patients at all time points during 4 years of follow-up), the extent of the CI reflects the degree of variance. Correlations between joint swelling and erosion score or joint space narrowing score were determined for each MCP and PIP joint of the hands using the Spearman correlation test. The Statistical Package for Social Sciences, version 12.0.1 (SPSS Institute, Chicago, IL, USA) was used to analyse the data. In all tests, $P<0.05$ was considered significant.

Results

Early symptoms of disease

In total 454 patients fulfilled the American College of Rheumatology criteria for RA; 228 of these patients had anti-CCP antibodies and 226 patients had no anti-CCP antibodies at inclusion. Patient characteristics and the type, localization and distribution of initial disease symptoms are presented in Table 1. In both groups, 13% of patients reported no morning stiffness. In the patients that did experience morning stiffness, the mean duration in the anti-CCP–negative patients and anti-CCP–positive patients was similar at 118 min and 123 min, respectively. In both groups symptoms started with pain and swelling, predominantly symmetrical and in the small joints of the hands and feet.

In the statistical analysis without correction for multiple testing, one difference in initial presentation between the two groups was observed: in anti-CCP–positive patients symptoms started more often at both upper and lower extremities than in anti-CCP–negative patients (20% vs 11%, respectively; $P<0.05$). Given the marginal P

value, which was not significant after correction for multiple testing, this finding was not considered a relevant difference. The mean patients' rated global disease activity on a visual analogue scale was not significantly different between the two groups. Likewise, the functional ability measured by the Health Assessment Questionnaire score was similar in both groups. In conclusion, there are no fundamental differences in the early symptoms of disease between anti-CCP–positive RA patients and anti-CCP–negative RA patients.

Table 1. Characteristics of the early symptoms in rheumatoid arthritis patients with and without anti-cyclic-citrullinated peptide (anti-CCP) antibodies

	Anti-CCP–negative (n=228)	**Anti-CCP–positive (n=226)**
Female [n (%)]	147 (64%)	150 (66%)
Age at inclusion (mean ± standard deviation)	57 ± 17	55 ± 16
Morning stiffness	30 (13%)	30 (13%)
No [n (%)]	118 ± 138	123 ± 128
Yes (min) (mean±standard deviation)		
Type of initial joint symptoms [n (%)] #		
Pain	208 (91%)	205 (91%)
Swelling	146 (64%)	135 (60%)
Stiffness	106 (46%)	85 (38%)
Function loss	64 (28%)	57 (25%)
Redness or increased surface temperature of joints	19 (8%)	26 (12%)
Localization of initial joint symptoms [n (%)]		
Small joints of hands and/or feet	105 (46%)	112 (50%)
Large joints	54 (24%)	50 (22%)
Both small and large joints	63 (28%)	59 (26%)
Unknown	6 (2%)	5 (2%)
Localization of initial joint symptoms [n (%)]		
Upper limbs	114 (50%)	86 (38%)*
Lower limbs	72 (32%)	77 (34%)
Both upper and lower limbs	25 (11%)	45 (20%)*
Unknown	18 (8%)	18 (8%)
Localization of initial joint symptoms [n (%)]		
Symmetric	145 (64%)	130 (58%)
Asymmetric	71 (31%)	83 (37%)
Unknown	10 (4%)	13 (6%)
VAS patients' rated global disease activity (0-100)	51.3 ± 39.9	46.7 ± 28.2
HAQ-score (mean ± SD)	1.0 ± 0.7	1.0 ± 0.7

VAS, visual analogue scale. HAQ, Health Assessment Questionnaire
Patients can have both swelling and pain at the start of the symptoms and therefore total can add to more than 100%.
* $P<0.05$, anti-CCP–positive versus anti-CCP–negative

Findings at physical examination at initial presentation

In each of the 454 patients a tender joint count and a swollen joint count were performed at inclusion. The mean tender joint count per joint is presented in Table 2. There were no significant differences between RA patients with and without anti-CCP antibodies. Table 3 presents the mean scores for joint swelling for both anti-CCP–positive and anti-CCP–negative patients, showing no statistical significant differences between the two groups. Anti-CCP–positive RA patients and anti-CCP–negative RA patients therefore cannot be distinguished at presentation by physical examination.

Table 2. Tender joint count at inclusion in rheumatoid arthritis patients with and without anti-CCP antibodies

	Anti-CCP–negative (n=228)	**Anti-CCP–positive (n=226)**
Temporomandibular joints	0.01 ± 0.41	0.08 ± 0.36
Sternoclavicular joints	0.23 ± 0.76	0.12 ± 0.47
Acromioclavicular joints	0.31 ± 0.63	0.55 ± 0.79
Shoulder joints	0.85 ± 1.5	0.86 ± 1.4
Elbow joints	0.42 ± 0.99	0.35 ± 0.81
Wrist joints	0.94 ± 0.94	0.80 ± 0.93
Metacarpophalangeal joints	4.3 ± 4.3	3.5 ± 3.4
Proximal interphalangeal joints of the hands	3.2 ± 3.6	3.3 ± 3.4
Distal interphalangeal joints of the hands	1.3 ± 2.4	1.2 ± 2.2
Hip joints	0.18 ± 0.73	0.11 ± 0.54
Knee joints	0.54 ± 0.88	0.59 ± 0.90
Ankle joints	0.41 ± 0.92	0.53 ± 1.1
Subtalar joints	0.31 ± 0.72	0.52 ± 0.76
Midtarsal joints	0.21 ± 0.40	0.18 ± 0.58
Metatarsophalangeal joints	4.2 ± 3.4	4.1 ± 3.7
Interphalangeal joints of the feet	0.91 ± 1.8	1.4 ± 3.2
Total Ritchie articular index score	10.4 ± 8.2	10.2 ± 8.0

Tenderness was scored per joint on a 0-3 scale: 0=no tenderness, 1=pain at pressure, 2=pain and winced and 3=winced and withdrew. The scores for the metacarpophalangeal joints were summed, as were the scores for metatarsophalangeal joints and the interphalangeal joins of the hands and feet. The scores for the left joints and the right joints were summed. The summed scores were divided by the total numbers of patients; the resulting mean ± standard deviation is presented. There were no statistical differences between patients with and without anti-CCP antibodies.

Acute phase reactant at initial presentation

The mean C-reactive protein level was 29.5 mg/l (standard deviation [SD], 31.5) in the anti-CCP–negative RA patients and was 35.6 mg/l (SD, 37.8) in the anti-CCP–positive RA patients. The mean C-reactive protein level was not significantly different between the two groups (P=0.08).

Table 3. Joint swelling at inclusion in rheumatoid arthritis patients with and without anti-CCP antibodies

	Anti-CCP–negative (n=228)	**Anti-CCP–positive (n=226)**
Temporomandibular joints	0.01 ± 0.10	0.02 ± 0.18
Sternoclavicular joints	0.08 ± 0.34	0.04 ± 0.22
Acromioclavicular joints	0.06 ± 0.24	0.03 ± 0.17
Shoulder joints	0.08 ± 0.30	0.12 ± 0.40
Elbow joints	0.22 ± 0.54	0.20 ± 0.49
Wrist joints	1.0 ± 0.89	1.0 ± 0.90
Metacarpophalangeal joints	3.2 ± 3.0	2.2 ± 2.2
Proximal interphalangeal joins of the hands	2.6 ± 3.1	2.0 ± 1.8
Distal interphalangeal joints of the hands	0.32 ± 0.60	0.21 ± 0.60
Knee joints	0.46 ± 0.74	0.49 ± 0.74
Ankle joints	0.31 ± 0.67	0.34 ± 0.63
Subtalar joints	0.24 ± 0.61	0.21 ± 0.55
Metatarsophalangeal joints	1.6 ± 2.2	1.8 ± 2.4
Interphalangeal joints of the feet	0.06 ± 0.24	0.18 ± 0.58
Total number of swollen joints	10.0 ± 7.2	8.6 ± 5.5

Swelling was scored for each joint on a 0-1 scale: 0=no swelling, and 1=swelling. The scores for the metacarpophalangeal joints were summed, as were the scores for metatarsophalangeal joints and the interphalangeal joins of the hands and feet. The scores for the left joints and the right joints were summed. The summed scores were divided by the total numbers of patients; the resulting mean ± standard deviation is presented. There were no statistical differences between patients with and without anti-CCP antibodies.

Swollen joints at follow-up

The swollen joint count was assessed yearly in the 138 early arthritis patients with complete radiological follow-up for 4 years. These patients had a mean age at inclusion of 53.7 ± 13.9 years, 67% (93 patients) were women, and 54% (74 patients) were anti-CCP–positive. The total number of swollen joints decreased during follow-

up. In the anti-CCP–negative patients at inclusion the mean ± SD number of swollen joint was 10.0 ± 7.2; at 2 years and 4 years follow-up the mean ± SD numbers of swollen joints were, respectively, 4.1 ± 6.7 and 3.1 ± 4.2. The mean ± SD number of swollen joints in the anti-CCP–positive group at inclusion was 8.6 ± 5.5; this decreased to 5.2 ± 7.5 and 5.3 ± 6.8 at 2 years and 4 years follow-up, respectively. At 4 years follow-up the total number of swollen joints was significantly higher in the RA patients with anti-CCP antibodies ($P = 0.01$).

In addition, the scores for the individual MCP and PIP joints of the hands were compared. Overall the pattern of inflammation of the individual small joints is similar in anti-CCP–negative RA and in anti-CCP–positive RA, as is depicted by the mean and 95% CI of the swollen joint count in Figure 1. Several individual joints had significantly higher scores in the anti-CCP–positive patients compared with the anti-CCP–negative patients; at inclusion this concerned the first MCP joint on the right side, at 2 years follow-up this concerned the fifth PIP joint on the left side, and at 4 years follow-up this concerned the first MCP, third PIP, fourth PIP and fifth PIP joints on the left side and the third PIP, fourth PIP and fifth PIP joints on the right side ($P< 0.05$). Furthermore, Figure 1 shows that in both anti-CCP–positive RA patients and anti-CCP–negative RA patients, the second and third MCP joints were more frequently swollen than the other MCP joints. Likewise, in both groups the second and third PIP joints were more frequently affected than the other PIP joints. In conclusion, the pattern of inflammation of the individual small joints of the hand seems similar in anti-CCP–positive and anti-CCP–negative patients; however, particularly at 4 years follow-up some MCP and PIP joints are significantly less frequently swollen in anti-CCP–negative RA patients.

Radiographic progression

In the 138 RA patients with a complete 4-year radiological follow-up, the total Sharp-van der Heijde scores were compared between the RA patients with and without anti-CCP antibodies (Figure 2). At 2 years and 4 years follow-up, anti-CCP–positive patients had significantly higher radiological scores than anti-CCP–negative patients ($P<0.001$).

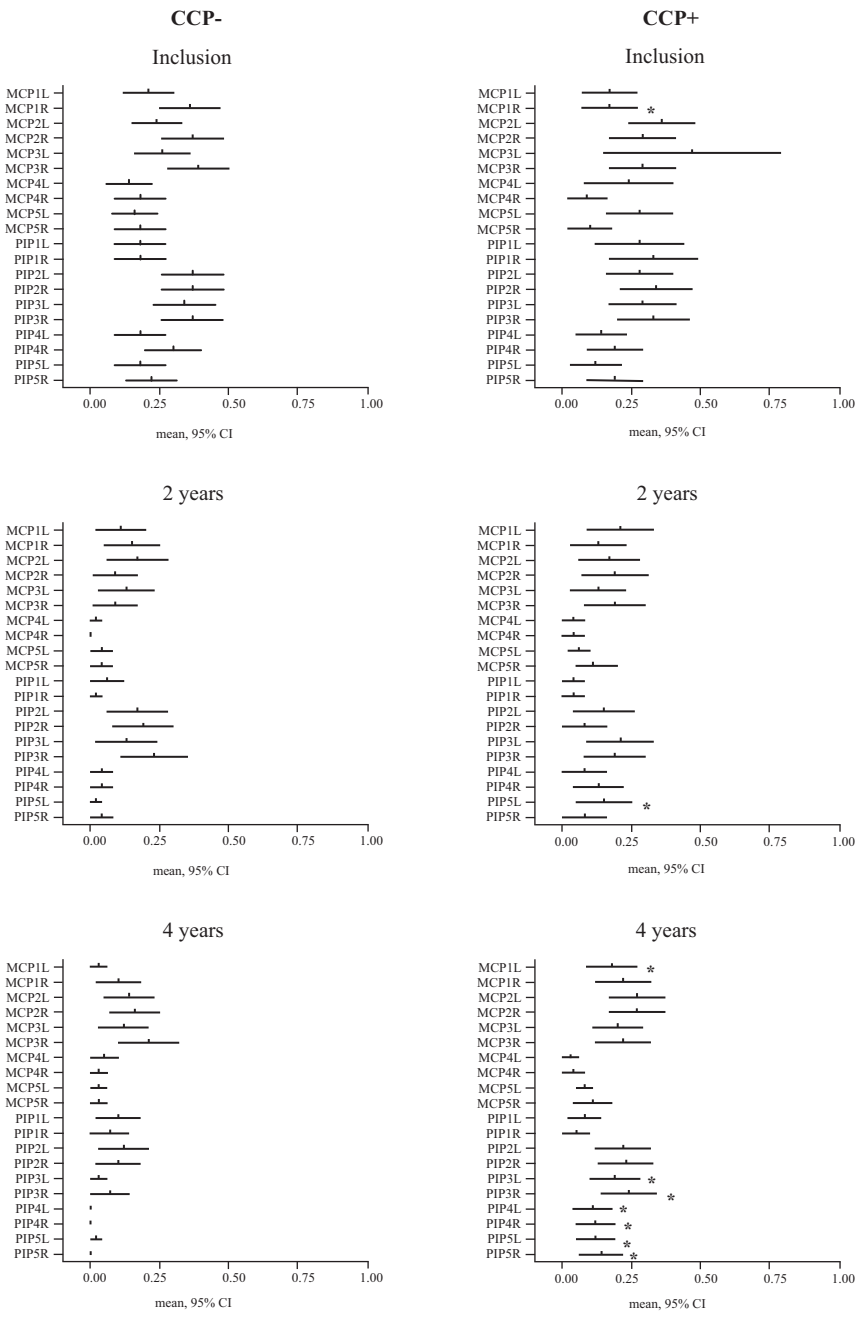

Figure 1. Joint swelling (mean and 95% confidence interval [CI]) of the metacarpophalangeal (MCP) and proximal interphalangeal (PIP) joints of the hands a inclusion and at 2 and 4 years follow-up in rheumatoid arthritis patients with (CCP+) and without (CCP-) anti-cyclic-citrullinated peptide antibodies. L, left; R, right.

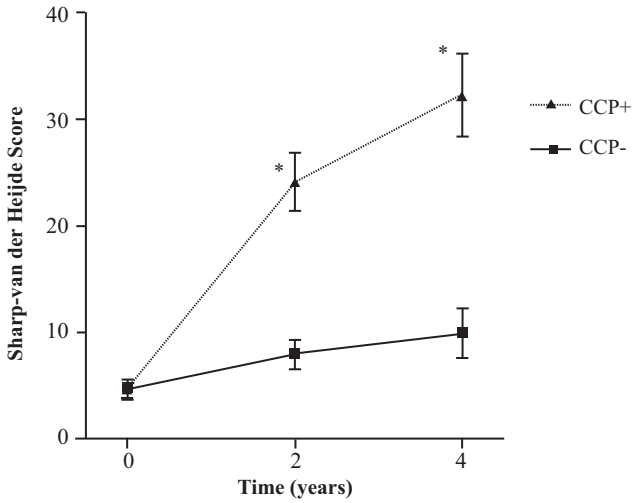

Figure 2. Radiological destruction as measured by total Sharp-van der Heijde scores (mean ± standard error of the mean) at inclusion and at 2 and 4 years follow-up in rheumatoid arthritis patients with (CCP+) and without (CCP-) anti-cyclic-citrullinated peptide antibodies.

The distribution of the radiological destruction in the MCP and PIP joints of the hands was further investigated. The erosion scores and joint space narrowing scores of the MCP and PIP joints are depicted in Figure 3. As the most pronounced radiological destruction was present in anti-CCP–positive patients, the erosion scores and joint space narrowing scores are shown for the RA patients with anti-CCP antibodies. Figure 3 shows that at all time points, of all the MCP joints, the second MCP joints had the highest erosion score, followed by the third MCP joints. Concerning the PIP joints, the highest erosion scores were present in the third and fourth PIP joints. Figure 3 further reveals that the second and third MCP joints are the MCP joints with the highest joint space narrowing scores at all time points during follow-up. The joint space narrowing scores of the PIP joints differ less, but there are slightly higher scores for the third and fourth PIP joints.

The erosion scores and joint space narrowing scores for the patients without anti-CCP antibodies revealed the same distribution as for the anti-CCP–positive RA patients (data not shown). In the anti-CCP–negative patients the values for the mean and 95% CI were lower than in the anti-CCP–positive patients, which is in concordance with the finding of lower total Sharp-van der Heijde scores in anti-CCP–negative RA patients. Correlations between joint swelling and the erosion score and between joint

swelling and the joint space narrowing score were determined for each MCP and PIP joint at 4 years follow-up. For all PIP joints and for all MCP joints, except the fourth MCP joints, the erosion score was significantly correlated with joint swelling ($P<0.05$). The joint space narrowing scores were significantly correlated with joint swelling in all MCP joints except the fourth MCP joint ($P<0.05$). This implies that at that time point the joints that were the most swollen were also the joints with the most severe radiological destruction.

Discussion

This study shows that the phenotype of RA patients with or without anti-CCP antibodies does not differ at clinical presentation. In a large, prospective, early arthritis cohort we observed neither a significant difference in the reported first symptoms nor in the signs found in the physical examination at initial presentation between anti-CCP–positive patients and anti-CCP–negative patients. During follow-up, however, anti-CCP–positive RA patients have more swollen joints and show more radiological destruction than anti-CCP–negative RA patients. It is remarkable that at follow-up, in spite of the difference in magnitude of the disease characteristics, the distribution of swollen joints and the distribution of radiological joint space narrowing and bone erosions remains similar for RA patients with and without anti-CCP antibodies. This implies that although different associations with known risk factors are reported for anti-CCP–positive and anti-CCP–negative RA patients, the presence or absence of anti-CCP antibodies is not associated with a distinguishable clinical phenotype at presentation of disease.

Pathophysiologically, this may have implications. It was recently observed that the prominent genetic risk factor HLA class II alleles only associate with susceptibility to RA in the presence of anti-CCP antibodies but not with RA in the absence of these antibodies (unpublished data, [5]). It has been shown in mice that citrullination of arginine in a peptide can lead to a higher binding affinity of that peptide for HLA–DRB1*0401, an important shared epitope allele [12], allowing peptide-specific T-cell induction. It can be speculated that also in humans citrullination may improve antigen presentation to CD4-positive T–cells and that the genetic background (presence of shared epitope alleles) provides the basis for a citrulline-specific immune reaction.

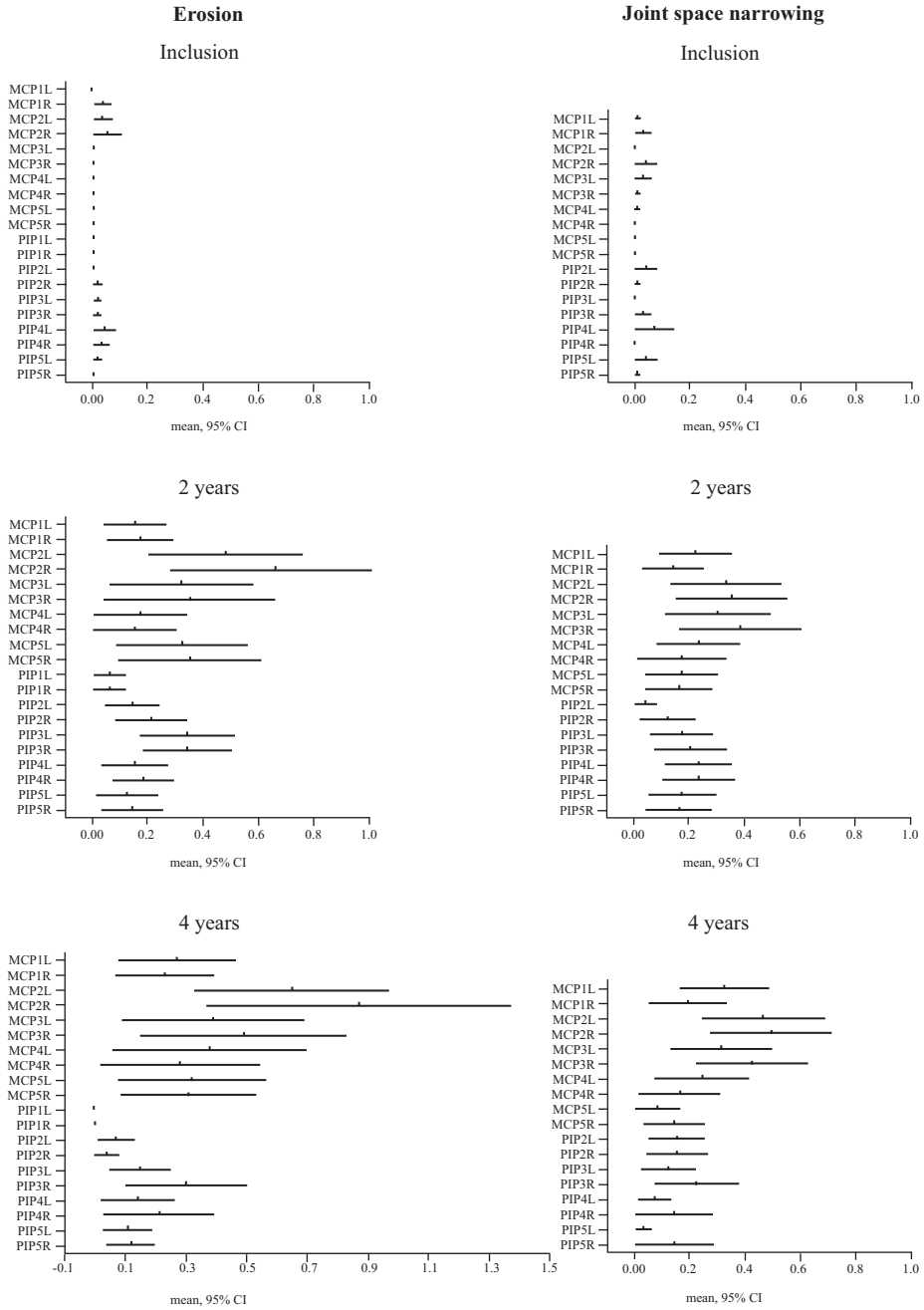

Figure 3. Erosion and joint space narrowing scores of the metacarpophalangeal (MCP) and proximal interphalangeal (PIP) joints of the hands (means and 95% confidence interval [CI]) at inclusion and at 2 and 4 years follow-up in rheumatoid arthritis patients with anti-cyclic-citrullinated peptide antibodies. L, left; R, right.

It has been demonstrated that anti-CCP antibodies occur years before disease onset [3,4]. This observation suggests that the induction of disease in anti-CCP–positive RA patients occurs years before clinical presentation. The current study, however, shows that the age of onset of clinical disease is similar in RA patients with and without anti-CCP antibodies.

The risk factors such as HLA alleles differ between anti-CCP–negative RA and anti-CCP–positive RA [5]. Although differences in risk factors presume different pathophysiological pathways for anti-CCP–positive RA and anti-CCP–negative RA, the initial phenotypical presentation of both patient groups is similar and is characterized by a symmetric polyarthritis of the same small joints. At follow-up the clinical phenotype remains comparable with regard to joint distribution, but the anti-CCP–positive patients have more inflamed joints and once there is inflammation also have more rapid joint destruction.

This leads to a pathophysiological model in which one or more triggers lead to arthritis in similar joints in anti-CCP–positive patients and anti-CCP–negative patients. Antigens are subsequently citrullinated during inflammation; in the presence of anti-CCP antibodies the inflammation is aggravated, resulting in more severe radiological destruction. Further studies are needed to add insight into the pathogenic role of circulating anti-CCP antibodies in anti-CCP–positive RA and to unravel the risk factors associated with anti-CCP–negative RA.

In a study by Kastbom and colleagues [13] several baseline disease characteristics of anti-CCP–positive RA patients and anti-CCP–negative RA patients were compared. This study observed no significant differences in baseline total swollen joint count, in C-reactive protein levels or in the Disease Activity Score (DAS)28 between RA patients with and without anti-CCP antibodies, but showed a positive correlation between the number of fulfilled American College of Rheumatology criteria and the frequency of anti-CCP positivity [13]. Furthermore, in that study anti-CCP–positive individuals were more often treated with disease-modifying antirheumatic drugs than were anti-CCP–negative patients [13].

Although in the present study secular trends in the initial treatment strategies with disease-modifying antirheumatic drugs were present, these trends yielded the same effect for the anti-CCP–positive and anti-CCP–negative RA patients. Furthermore, the rheumatologists that treated the patients were not aware of the anti-CCP status of their patients. The more severe disease course in patients with anti-CCP antibodies is therefore probably not due either to a more delayed treatment of

these patients or to confounding by treatment adapted to the anti-CCP status. We cannot exclude the fact that during follow-up the anti-CCP–positive patients that had more inflamed joints received more aggressive treatment. In the case of a more aggressive treatment during follow-up in anti-CCP–positive patients, however, this did not prevent the development of more severe radiological destruction in the RA patients with anti-CCP antibodies. The finding that the swollen joint count decreased during follow-up is probably due to the fact that patients were not treated with disease-modifying antirheumatic drugs at inclusion.

The sensitivity of anti-CCP2 antibodies for RA is reported to vary between 39% and 80% [14,15]. The present study measured anti-CCP2 levels at inclusion (a very early stage of the disease) and reports a relatively low percentage (50%) of RA patients with anti-CCP antibodies. As cyclic-citrullinated peptide measurements were not repeated during follow-up, we cannot exclude that some RA patients that were anti-CCP–negative at inclusion have become anti-CCP–positive at a later stage in the disease. A relatively low prevalence of anti-CCP antibodies in early arthritis patients has been described previously [14].

The present study shows that the second and third MCP joints have the highest erosion scores as well as the highest joint space narrowing scores and are, of all the MCP joints, the most frequently swollen. Although the present study was not designed to study the correlation between inflammation and destruction, the observed similarity in joints that are affected by swelling, erosions and joint space narrowing supports the concept that, in general, the mechanisms leading to clinical inflammation and radiological destruction are related.

The present study includes a detailed description on the distribution of affected joints in RA and shows that the MCP joints of the second and the third digits are most frequently inflamed and destroyed. Although to our experience rheumatologists generally feel that the joints of the second and third digits are more frequently inflamed than other joints of the hands, to our knowledge this phenotypic characterization has not been frequently described.

Conclusion

The present study shows that, although separate risk factors for anti-CCP–positive RA and anti-CCP–negative RA have been recently described, the clinical presentation of RA patients with or without anti-CCP antibodies is not different. Patients with anti-CCP antibodies develop a more severe disease course with more radiological destruction compared with RA patients without these autoantibodies. Nonetheless, the distribution of affected joints is also similar at follow-up.

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Chapter 4

The HLA–DRB1 shared epitope alleles are primarily a risk factor for anti-cyclic citrullinated peptide antibodies and are not an independent risk factor for development of rheumatoid arthritis

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Abstract

Objective

The shared epitope (SE)-containing HLA–DRB1 alleles represent the most significant genetic risk factor for rheumatoid arthritis (RA). Recent studies indicate that the SE alleles are associated only with RA that is characterized by the presence of anti-cyclic citrullinated peptide (anti-CCP) antibodies, and not with anti-CCP–negative disease. In this study we investigated whether the SE alleles contribute to the development of anti-CCP–positive RA, or whether they are associated solely with the presence of anti-CCP antibodies. We therefore determined the influence of the SE alleles and anti-CCP antibodies on the progression from recent-onset undifferentiated arthritis (UA) to RA.

Methods

Patients with recent-onset UA at the 2-week visit (n=570) were selected from the Leiden Early Arthritis Cohort. SE alleles, rheumatoid factor (RF) status, and anti-CCP antibody levels were determined. Progression to RA or other diagnoses was monitored.

Results

One hundred seventy-seven patients with UA developed RA during the 1-year followup, whereas the disease in 393 patients remained unclassified or was given other diagnoses. The SE alleles correlated with the presence of anti-CCP antibodies, but not with the presence of RF. Both in SE-positive and in SE-negative patients with UA, the presence of anti-CCP antibodies was significantly associated with the development of RA. More intriguingly, however, no apparent contribution of the SE alleles to the progression to RA was found when analyses were stratified according to the presence of anti-CCP antibodies. In patients with anti-CCP–positive disease, the presence of SE alleles was associated with significantly higher levels of anti-CCP antibodies, suggesting that the SE alleles act as classic immune response genes.

Conclusion

The SE alleles do not independently contribute to the progression to RA from UA, but rather contribute to the development of anti-CCP antibodies.

Introduction

The most important genetic risk factor for rheumatoid arthritis (RA) is the HLA-class II alleles. In particular, the HLA–DRB1 alleles encoding for the shared epitope (SE) confer a higher risk for the development of RA [1]. The SE hypothesis postulates that the SE motif (a conserved amino acid sequence in the peptide binding pocket of the DRβ1 molecule) is directly involved in the pathogenesis of RA by allowing the presentation of an arthritogenic peptide to T–cells [2]. Recently, it was observed by 2 different methods (linkage and association analyses) that the SE alleles are a risk factor for only RA that is characterized by the presence of anti-cyclic citrullinated peptide (anti-CCP) antibodies, and not for anti-CCP–negative RA [3].

Anti-CCP antibodies are highly specific for RA, can be detected years before the first clinical manifestation of RA [4], and are reported to be a good predictor for the development of RA [5]. Because the contribution of the SE-containing HLA alleles to the pathogenesis of RA is not well understood, the novel information on the association of SE alleles with anti-CCP–positive disease [3] led us to evaluate the hypothesis that the SE alleles are mainly a risk factor for anti-CCP antibodies, rather than for (anti-CCP–positive) RA. To this end, we took advantage of a well-characterized inception cohort and studied the patients with an early arthritis that, at presentation, could not be classified according to the 1987 American College of Rheumatology (ACR; formerly, the American Rheumatism Association) criteria [6] (referred to as undifferentiated arthritis [UA]). Analysis of the clinical evolution in conjunction with the genetic and serologic risk factors in these patients who are prone to develop RA allows insight into the factors that are associated with progression to RA. Accordingly, this permits analysis of the contribution of the SE alleles to the development of RA after stratification for the influence of anti-CCP antibodies.

Patients and methods

Study population

For this study, patients were evaluated at the Leiden Early Arthritis Clinic (EAC), which was started in 1993 (for description, see ref. 7). Patients were referred to the EAC when arthritis was suspected, and were included in the cohort when arthritis was diagnosed at physical examination. At baseline, blood samples were obtained. More than 1,900 patients are currently included in the cohort.

Two weeks after inclusion, 313 patients received the diagnosis of RA according to the ACR 1987 criteria and 570 patients had an arthritis that could not be classified according to the ACR criteria and were therefore classified as having UA. After 1 year of followup, the disease status of all patients with UA was examined to determine whether they had developed RA according to the ACR criteria. It might be possible that some patients did not fulfill the ACR criteria for RA at 1 year but developed RA at a later time point. Inherent to the design of an inception cohort, the duration of followup will differ within the study population. However, at the time of this analysis, the majority of the patients (94%) had been followed up for more than 1 year (mean followup 8 years, SD 3 years), and only 9% of the patients who were not classified as having RA at 1 year developed RA later on in the disease course.

Laboratory investigations

Baseline laboratory parameters (determined using the blood samples that were obtained at inclusion) included IgM-rheumatoid factor (RF) by enzyme-linked immunosorbent assay (ELISA) and anti-CCP-2 antibodies by ELISA (Immunoscan RA Mark 2; Euro-Diagnostica, Arnhem, The Netherlands). The cutoff level for anti-CCP antibody positivity was set at 25 arbitrary units, according to the manufacturer's instructions. The HLA–DRB1 subtyping was performed by polymerase chain reaction using specific primers and hybridization with sequence-specific oligonucleotides. The SE alleles were HLA–DRB1*0101, *0102, *0401, *0404, *0405, *0408, *0410, and *1001. For 438 of the 570 patients with UA, both data on SE alleles and data on anti-CCP antibodies were available.

Statistical analysis

Odds ratios (ORs) were calculated and proportions were compared by chi-square test. Differences in mean values between groups were analyzed using the Mann-Whitney

test or *t*-test when appropriate. The question as to whether SE alleles and anti-CCP antibodies both independently contribute to progression to RA was investigated with a stratification procedure, as well as with logistic regression analysis. In this logistic regression analysis, the disease outcome was entered as the dependent variable and anti-CCP antibodies and SE alleles were possible explanatory variables. Using a backward selection procedure, the significant independent variables were selected. For all tests, P values less than 0.05 were considered significant.

Results

Outcome in patients with UA

Of the 570 patients with UA at the 2-week visit, 177 developed RA during the first year of followup, 99 patients developed other rheumatic diseases (reactive arthritis, psoriatic arthritis, and systemic lupus erythematosus, among others), and 294 patients remained unclassified (having persistent UA). For further analysis, the patients with persistent UA and those with other diagnoses of rheumatic disease were described as the non-RA group. Characteristics of the patients who developed RA and of the patients in the non-RA group are given in Table 1. In univariate analysis, the presence of SE alleles, RF, and anti-CCP antibodies were all associated with significantly higher ORs for the likelihood of developing RA (ORs of 1.8, 6.3, and 8.5, respectively) (Table 1).

Association between SE alleles and presence of autoantibodies

To determine whether the SE alleles are correlated with RF positivity, with anti-CCP antibodies, or with both types of autoantibodies, the associations between the SE alleles and anti-CCP antibodies and between the SE alleles and RF were investigated in the 570 patients with UA. In univariate analysis, the SE alleles were associated both with RF and with anti-CCP antibodies (OR 1.7, 95% confidence interval [95% CI] 1.1-2.7, P=0.01 and OR 3.1, 95% CI 2.1-5.3, P<0.001, respectively).

Since anti-CCP positivity is correlated with RF positivity, the association between the SE alleles and anti-CCP antibodies was assessed in groups of patients stratified according to RF-positive and RF-negative disease. In patients with RF-negative disease, the presence of the SE alleles was associated with an increased likelihood of developing anti-CCP antibodies (OR 2.9, 95% CI 1.2-6.9, P<0.01).

Similarly, in patients with RF-positive disease, the presence of the SE alleles conferred an increased likelihood of having anti-CCP antibodies (OR 5.6, 95% CI 2.1-14.6, P<0.001). These data indicate that the SE alleles are associated with the presence of anti-CCP antibodies independent of the RF status.

Table 1. Baseline characteristics of patients with undifferentiated arthritis at 2 weeks who did and those who did not develop RA during the first year of followup*

	RA (n=177)	non-RA (n=393)	P	OR (95%CI)
Age, mean ± SD years	56.3 ± 15.3	48.6 ± 16.9	<0.001	-
Sex, no. female/no. male	121/56	208/185	0.001	1.9 (1.3-2.8)
SE positive, no. (%) †	100 (63)	158 (49)	0.005	1.8 (1.2-2.6)
Anti-CCP positive, no. (%) ‡	83 (51)	38 (11)	<0.001	8.5 (5.2-13.7)
RF positive, no. (%)	84 (47)	56 (14)	<0.001	6.3 (4.1-9.7)

*RA = rheumatoid arthritis; OR = odds ratio; 95% CI = 95% confidence interval; RF = rheumatoid factor.
† Data on shared epitope (SE) alleles were missing in 17 of the patients with undifferentiated arthritis (UA) progressing to RA and in 68 of the non-RA patients with UA.
‡ Anti-cyclic citrullinated peptide (anti-CCP) antibody data were missing in 15 of the patients with UA progressing to RA and in 49 of the non-RA patients with UA.

We next assessed whether the SE alleles are associated with the presence of RF independent of the presence or absence of anti-CCP antibodies. In both the anti-CCP–positive and anti-CCP–negative patient groups, the SE alleles were not associated with the presence of RF (P=0.9 and P=0.2, respectively), indicating that after correction for the presence or absence of anti-CCP antibodies, the SE alleles do not confer a risk of RF positivity. Therefore, the SE alleles are primarily correlated with the presence of anti-CCP antibodies, but not with the presence of RF.

SE alleles and anti-CCP antibodies in progression from UA to RA

Subsequently, the influence of the SE alleles on the progression from UA to RA was examined. Univariate analysis assessing the association between patient characteristics and disease outcome revealed that the presence of the SE alleles and the presence of anti-CCP antibodies at baseline were both associated with the development of RA (see Table 1). However, because the presence of the SE alleles and the presence of anti-CCP antibodies are correlated, the individual effect of the SE alleles on the development of RA was determined after stratification for the presence or absence of anti-CCP antibodies. Both in the anti-CCP-positive and in the anti-

CCP–negative patients with UA, the presence of the SE alleles was not associated with the development of RA (Table 2). These data are important because they indicate that the SE alleles are not correlated with progression to RA in patients with UA when corrections are made for the presence or absence of anti-CCP antibodies.

Table 2. Comparison of patients with undifferentiated arthritis who, during 1 year of followup, did not and those who did develop RA, stratified for baseline anti-CCP antibodies and SE alleles*

	non-RA, no. (%)	RA, no. (%)	P	OR (95%CI)
Stratification for anti-CCP antibodies				
anti-CCP -				
SE -	142 (55)	37 (53)		
SE +	118 (45)	33 (47)	0.8	1.1 (0.6-1.9)
anti-CCP+				
SE -	8 (26)	21 (27)		
SE +	23 (74)	56 (73)	0.9	0.9 (0.3-2.6)
Stratification for SE alleles				
SE -				
anti-CCP -	142 (95)	37 (64)		
anti-CCP +	8 (5)	21 (36)	<0.001	10.1 (3.9-27.1)
SE +				
anti-CCP -	118 (84)	33 (39)		
anti-CCP +	23 (6)	56 (61)	<0.001	8.7 (4.5-17.0)

*RA = rheumatoid arthritis; OR = odds ratio; 95% CI = 95% confidence interval; RF = rheumatoid factor.
Data on shared epitope (SE) alleles were missing in 17 of the patients with undifferentiated arthritis (UA) progressing to RA and in 68 of the non-RA patients with UA.
Anti-cyclic citrullinated peptide (anti-CCP) antibody data were missing in 15 of the patients with UA progressing to RA and in 49 of the non-RA patients with UA.

To assess the effect of anti-CCP antibodies independent of the SE alleles, the risk of developing RA was determined in SE-positive and SE-negative patients with UA in a separate analysis (Table 2). This analysis showed that both in the SE-positive and in the SE-negative patients with UA, the presence of anti-CCP antibodies was significantly associated with the development of RA (OR 8.7 and OR 10.1, respectively).

In a logistic regression analysis with a backward selection procedure, with the disease outcome (RA versus non-RA) entered as the dependent variable and the SE

alleles and anti-CCP antibodies as possible explanatory variables, the presence of anti-CCP antibodies was the only independent factor that was significantly associated with the development of RA, with an OR of 9.2 ($P<0.001$). This result obtained from multivariate analysis was not substantially different from that obtained by univariate analysis in determining the influence of anti-CCP antibodies on the development of RA (OR 8.5) (see Table 1).

Thus, these data show that after stratification for the influence of the SE alleles, the presence of anti-CCP antibodies confers a high risk for the development of RA, whereas after stratification for the presence or absence of anti-CCP antibodies, the SE alleles are not associated with progression to RA. Taken together, these data indicate that the SE alleles primarily predispose to the presence of anti-CCP antibodies, and are not an independent risk factor for the development of RA.

Association between SE alleles and anti-CCP antibody level

In classic studies, performed in mice, on the genetic background associated with antibody production, it has been shown that major histocompatibility complex (MHC) alleles act as immune response genes that control the magnitude and specificity of antibody production in a dominant manner [8]. In mice, the magnitude of the antibody response in the first generation offspring was comparable with the magnitude of response in the high-responding parent, denoting that in mice, homozygosity for MHC genes did not improve the level of antibody production compared with that in a heterozygous background [8].

Because the results of the present study revealed that the presence of the SE alleles is associated with positivity for anti-CCP antibodies, we wished to investigate whether the characteristics of the SE alleles resemble those of a classic immune response gene. We therefore analyzed whether the level of anti-CCP antibodies present in serum was correlated with the presence of the SE alleles. To this end, the correlation between the presence of the SE alleles and the level of anti-CCP antibodies was assessed in all anti-CCP–positive patients who, at the 1-year followup, had progressed to having RA. Of a total of 490 RA patients (313 with RA diagnosed at 2 weeks' followup and 177 patients whose condition progressed from UA to RA during the first year of followup), 233 patients had anti-CCP antibodies, of whom 73% carried SE alleles.

The anti-CCP antibody levels in the anti-CCP–positive, SE-positive and anti-CCP–positive, SE-negative patients are shown in Figure 1. SE-positive patients had a

significantly higher level of anti-CCP antibodies (n=169, mean 1,032 arbitrary units, SEM 72) than did SE-negative patients (n=46, mean 652 arbitrary units, SEM 86) (P=0.001). Patients carrying 1 SE allele displayed a significantly higher level of anti-CCP antibodies (n=123, mean 1,029 arbitrary units, SEM 86) compared with patients without SE alleles (P=0.002). Patients with 2 SE alleles did not have a significantly higher anti-CCP level (n=46, mean 1,041 arbitrary units, SEM 134) compared with patients carrying 1 SE allele (P=0.94).

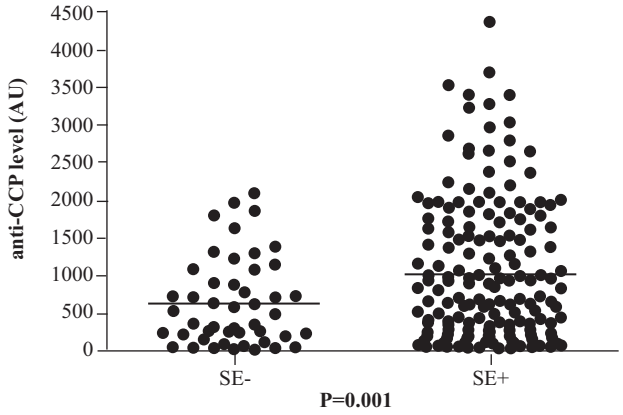

Figure 1. Levels of anti–cyclic citrullinated peptide (anti-CCP) antibodies (in arbitrary units [AU]) in anti-CCP–positive patients with rheumatoid arthritis (RA) without and those with shared epitope (SE) alleles. Bars indicate the median anti-CCP antibody level. The mean anti-CCP antibody levels in the anti-CCP–positive RA patients were 1,041 (SEM 134) for those carrying 2 SE alleles (n=46), 1,029 (SEM 86) for those carrying 1 SE allele (n=123), and 652 (SEM 86) for those carrying no SE alleles (n=46). In the subgroup of anti-CCP– positive patients with undifferentiated arthritis that progressed to RA, the median anti-CCP antibody levels were 699 (interquartile range [IQR] 278-1,282) for those carrying 2 SE alleles (n=13), 927 (IQR 251-1,970) for those carrying 1 SE allele (n=43), and 358 (IQR 169-1,424) for those carrying no SE alleles (n=21).

Thus, the current data show that in anti-CCP– positive patients, the presence of SE alleles is associated with higher levels of anti-CCP antibodies, and indicate that the presence of 1 or 2 SE alleles does not result in an apparent difference in anti-CCP antibody level. This observation is compatible with the notion that the SE alleles function as immune response genes in the development of anti-CCP antibodies.

Discussion

We recently reported [3] that the SE alleles were only associated with anti-CCP–positive RA and not with anti-CCP–negative disease, indicating that the SE alleles are not associated with RA as such, but rather with a distinct phenotype of the disease. We now extend these findings by showing that the SE alleles are not an independent risk factor for the development of RA after correction for anti-CCP antibody status. The SE alleles were, however, associated with the presence of anti-CCP antibodies. Moreover, the presence/absence of SE alleles was correlated with the levels of anti-CCP antibodies, suggesting that the SE alleles act as classic immune response genes for the development of anti-CCP antibodies.

Although no formal conclusions on causality can be drawn from this association study, these findings suggest that anti-CCP antibodies mediate the association between SE alleles and RA. It would be of interest to replicate the findings of the present study by following the development of anti-CCP antibodies and RA in healthy asymptomatic persons with and without SE alleles. Nevertheless, the present findings constitute an important refinement of the long-known association between HLA and RA by indicating that the SE alleles are not primarily associated with RA, but rather with anti-CCP antibody positivity.

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Chapter 5

Association of HLA–DR3 with anti-cyclic citrullinated peptide antibody-negative rheumatoid arthritis

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Abstract

Objective

Recent data have shown that the most prominent and longest known genetic risk factors for rheumatoid arthritis (RA), HLA–DRB1 shared epitope alleles, are only associated with RA that is characterized by the presence of antibodies against cyclic citrullinated peptide (anti-CCP antibodies) and not with anti-CCP–negative RA. We undertook this study to investigate whether anti-CCP–negative RA is associated with other HLA–DRB1 alleles.

Methods

HLA typing was performed for 377 patients from the Leiden Early Arthritis Clinic who were diagnosed as having RA within the first year of followup (206 anti-CCP–positive patients and 171 anti-CCP–negative patients), 235 patients who, after 1 year, had undifferentiated arthritis (UA) (28 anti-CCP–positive patients and 207 anti-CCP–negative patients), and 423 healthy control subjects. Odds ratios (ORs) with 95% confidence intervals (95% CIs) for HLA–DRB1 allele frequencies were determined for all patient groups compared with the healthy control group.

Results

HLA–DR3 was more frequently present in the anti-CCP–negative RA group than in the control group (OR 1.84, 95% CI 1.26-2.67). This was not the case for anti-CCP–positive RA (OR 0.92, 95% CI 0.60-1.40). HLA–DR3 was also more frequently present in anti-CCP–negative UA patients (OR 1.59, 95% CI 1.10-2.28), but not in anti-CCP–positive UA patients (OR 0.68, 95% CI 0.17-1.92).

Conclusion

HLA–DR3 is associated with anti-CCP–negative arthritis and not with anti-CCP–positive arthritis. These data show that distinct genetic risk factors are associated with the presence of anti-CCP antibodies in RA and indicate that different pathogenetic mechanisms underlie anti-CCP–positive and anti-CCP–negative RA.

Introduction

Rheumatoid arthritis (RA) is a multifactorial autoimmune disease with a complex genetic background. As in other autoimmune diseases, an association between RA and the HLA complex has long been observed in many different populations and is thought to account for approximately one-third of the genetic component of RA susceptibility [1]. There is extensive evidence for the association between certain frequently occurring HLA–DRB1 alleles, the so-called "shared epitope" (SE)-encoding alleles (DRB1*0101, *0102, *0104, *0401, *0404, *0405, *0408, *0413, *0416, and *1001), and susceptibility to RA [2]. These SE alleles encode for a common amino acid sequence in the third hypervariable region of the DRß1 molecule (QKRAA, QRRAA, or RRRAA).

In recent years, many studies on antibodies against cyclic citrullinated peptide (CCP) showed that these antibodies are highly specific and predictive for RA [3], that they can be detected years before onset [4], and that they are associated with joint destruction [5]. Furthermore, the presence or absence of these antibodies seems to be a stable trait [6]. Anti-CCP antibodies are detected in SE-positive as well as in SE-negative RA patients. Carriership of SE alleles in RA is associated with the presence of anti-CCP antibodies [7]. Interestingly, when we recently compared anti-CCP–positive and anti-CCP–negative RA patients with healthy controls, we found that HLA–DRB1 alleles encoding the SE were only associated with RA in the presence of anti-CCP antibodies and were not associated with anti-CCP–negative RA [8]. These data indicate that the SE-encoding alleles are not associated with RA as such, but rather with anti-CCP–positive RA. These observations indicate that distinct phenotypic manifestations of the disease are associated with distinct genetic risk factors. They also raise the question of whether anti-CCP–negative RA is associated with HLA–DRB1 alleles other than SE-encoding alleles. We therefore investigated the possible associations of particular HLA–DRB1 alleles with anti-CCP–positive RA and anti-CCP–negative RA. To verify the results and to study whether the results were specific for RA, we also performed the same analysis in a group of patients with undifferentiated arthritis (UA).

Patients and methods

Study population

In 1993 an Early Arthritis Clinic (EAC) was started at the Department of Rheumatology of the Leiden University Medical Center, as described previously [9]. The population studied here consisted of 377 patients who, within the first year of followup, fulfilled the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) 1987 revised criteria for RA [10] and 235 patients who, after 1 year of followup, could not be properly classified according to one of the ACR criteria sets and were therefore categorized as having UA. After 3 years of followup, the disease in most of the UA patients was classified or the patients were no longer receiving followup care at the EAC. The latter most likely resulted from the fact that patients who had had no signs of arthritis in the absence of treatment with disease-modifying antirheumatic drugs for ≥1 year had been discharged from the outpatient clinic. For every patient within the cohort, routine diagnostic laboratory screening was performed, including measurements of IgM-rheumatoid factor (IgM–RF). Informed patient consent was obtained, and the study was approved by the local medical ethics committee. Four hundred twenty-three healthy Dutch individuals served as controls. The control subjects were normal healthy donors of both sexes who were randomly selected and were ages 55 years and younger.

HLA genotyping

HLA class II alleles were determined in all patients and controls. The HLA–DRB1 (sub)typing was performed by polymerase chain reaction using specific primers and hybridization with sequence-specific oligonucleotides.

Anti-CCP autoantibodies

Serum antibodies directed against CCP were assessed with a commercial enzyme-linked immunosorbent assay (Immunoscan RA, Mark 2; Euro-Diagnostica, Arnhem, The Netherlands). Anti-CCP antibodies were measured in serum collected within 4 months after the first visit (94%) or, when serum was not available within this time period, in the first stored serum sample available thereafter.

Statistical analysis

Odds ratios (ORs) were calculated using the Epi Info Statcalc computer program (Centers for Disease Control and Prevention, Atlanta, GA) to compare HLA–DR allele frequencies between the patient groups and the control population. ORs were reported with 95% confidence intervals (95% CIs), which excluded the value of 1 in case of statistical significance. Exact confidence limits were used as described by Mehta et al [11]. For HLA–DR3, both allele frequencies and genotype frequencies were compared between the patient groups and the control population using the same methods described above.

Results

To find possible associations of HLA–DRB1 alleles with anti-CCP–positive or anti-CCP–negative RA, we analyzed HLA–DRB1 allele frequencies and the presence of anti-CCP antibodies in 377 RA patients of the Leiden EAC. Two hundred six of the RA patients had anti-CCP antibodies and 171 were anti-CCP–negative. Other patient characteristics are presented in Table 1. After we determined HLA–DRB1 allele frequencies, we calculated ORs and 95% CIs for both patient groups compared with a control group of 423 healthy individuals (see the table of supplementary data).

Table 1. Baseline characteristics of the 377 RA patients and 235 UA patients in the study*

	RA	**UA**
Age, mean (range) years	57 (14-92)	48 (16-88)
Female, %	66	58
IgM-RF positive, %	55	14
Anti-CCP antibody positive, %	55	12
Presence of erosions on radiographs of hands and feet**, %	35	18
Duration of symptoms, median (range) weeks	19 (0.6-104)	23 (0.14-104)

* RA=rheumatoid arthritis; UA=undifferentiated arthritis; IgM-RF=IgM rheumatoid factor; anti-CCP=anti-cyclic citrullinated peptide.
** radiographic data were available for 58% of the 377 RA patients and for 51% of the 235 UA patients

Table 2. Association of HLA–DR3 alleles with anti-CCP–positive or anti-CCP–negative RA and UA*

	anti-CCP–positive RA patients (n=206)		anti-CCP–negative RA patients (n=171)		anti-CCP–positive UA patients (n=28)		anti-CCP–negative UA patients (n=207)		controls (n=423)
DR3	**No. (%)**	**OR (95 % CI)**	**No. (%)**	**OR (95% CI)**	**No. (%)**	**OR (95 % CI)**	**No. (%)**	**OR (95 % CI)**	**No. (%)**
Genotype frequency									
DR3/DR3	0 (0)	-	5 (2.9)	3.64 (0.77-18.62)	0 (0)	-	4 (1.9)	2.3 (0.42-12.52)	4 (0.9)
DR3/x	39 (18.9)	1.02 (0.65-1.59)	49 (28.7)	1.83 (1.18-2.82)	4 (14.3)	0.73 (0.18-2.21)	55 (26.6)	1.62 (1.07-2.45)	78 (18.4)
x/x	167 (81.1)	1.0	117 (68.4)	1.0	24 (85.7)	1.0	148 (71.5)	1.0	341 (80.6)
DR3/DR3 or DR3/x	39 (18.9)	0.97 (0.62-1.51)	54 (31.6)	1.92 (1.25-2.92)	4 (14.3)	0.69 (0.17-2.10)	59 (28.5)	1.66 (1.10-2.48)	82 (19.4)
Allele frequency	39 (9.5)	0.92 (0.60-1.40)	59 (17.3)	1.84 (1.26-2.67)	4 (7.1)	0.68 (0.17-1.92)	63 (15.2)	1.59 (1.10-2.28)	86 (10.2)

*Odds ratios (ORs) were calculated comparing double-dose DR3 carriers (DR3/DR3), single-dose DR3 carriers (DR3/x), or carriers of at least a single dose of DR3 (DR3/DR3*or DR3/x) with carriers of no DR3 alleles (x/x) in patients versus controls and comparing allele frequencies in all groups versus controls. 95% CI = 95% confidence interval (seeTable 1 for other definitions).

As described previously, the SE alleles DRB1*0401, *0404, *0405, and *0408 were associated with predisposition for anti-CCP–positive RA, as were DR9 and DR10. Interestingly, HLA–DR3 was associated only with predisposition for anti-CCP–negative RA (OR 1.84, 95% CI 1.26-2.67) (Table 2). This association was not found for anti-CCP–positive RA, indicating that HLA–DR3 is only associated with RA in the absence of anti-CCP antibodies.

To confirm these findings in another group of patients and to address the question of whether the association is only found in anti-CCP–negative RA or whether it is also present in another form of arthritis, we also analyzed the association of HLA–DR3 with UA. In a group of 235 patients who, 1 year after their first visit, were categorized as having UA (Table 1), HLA–DR3 was more frequently present in anti-CCP–negative patients (n=207) than in healthy controls (OR 1.59, 95% CI 1.10-2.28), suggesting that HLA–DR3 is not specifically associated with anti-CCP–negative RA, but rather with anti-CCP–negative arthritis. No association was observed between HLA–DR3 and anti-CCP–positive UA (n=28) (OR 0.68, 95% CI 0.17-1.92) (Table 2). Analysis of HLA–DR3 allele frequencies in UA patients thus confirmed the results found in RA patients and indicated that association with HLA–DR3 also occurs in anti-CCP–negative UA. Analysis of whether HLA–DR3 is associated with development of RA in patients who presented initially with anti-CCP–negative UA did not show that HLA–DR3 increased the risk for developing RA (data not shown), indicating that HLA–DR3 is not a prognostic risk factor for the development of RA in this group of patients.

Since the presence of anti-CCP antibodies is linked to the presence of RF and since RF is associated with the SE [12], we next compared the frequencies of HLA–DR3 in IgM-RF-positive and IgM-RF-negative RA patients with that in controls. Indeed, HLA–DR3 was also associated with RF-negative RA (OR 1.66, 95% CI 1.13–2.43). However, after dividing the study population into those with and without anti-CCP antibodies, the association was lost for the anti-CCP–positive group (Table 3). Anti-CCP–negative, RF-positive RA patients and anti-CCP–negative, RF-negative RA patients both harbored HLA–DR3 significantly more frequently than did control subjects (OR 2.44, 95% CI 1.25–4.53 and OR 1.69, 95% CI 1.11–2.54, respectively). In contrast, anti-CCP–positive, RF-positive RA patients and anti-CCP–positive, RF-negative RA patients did not (OR 0.91, 95% CI 0.58–1.42 and OR 0.98, 95% CI 0.37–2.24, respectively) (Table 3). These data indicate that anti-CCP status, rather than RF status, is the predominant disease trait associated with HLA–DR3.

Table 3. HLA–DR3 allele frequencies in RA patients with and without anti-CCP antibodies and with and without IgM-RF*

	No. of HLA–DR3 alleles/total no. of alleles (%)	OR (95% CI)
RA patients		
anti-CCP + ; RF +	32 / 342 (9.4)	0.91 (0.58 – 1.42)
anti-CCP + ; RF -	7 / 70 (10.0)	0.98 (0.37 – 2.24)
anti-CCP - ; RF +	16 / 74 (21.6)	2.44 (1.25 – 4.53)
anti-CCP - ; RF -	43 / 268 (16.0)	1.69 (1.11 – 2.54)
Controls	86 / 846 (10.2)	1.0

* Odds ratios (ORs) were calculated comparing the allele frequencies in groups of RA patients with the allele frequency in healthy controls. 95% CI = 95% confidence interval (see Table 1 for other definitions).

Discussion

The data presented herein show that distinct genetic risk factors are associated with distinct subtypes of RA as defined by the presence of anti-CCP antibodies. SE-encoding alleles are associated with anti-CCP–positive RA and not with anti-CCP–negative disease. In contrast, anti-CCP–negative disease is associated with HLA–DR3, while this association is not found in anti-CCP–positive RA. Although HLA–DR3 or SE expression is not required for the development of anti-CCP–negative or anti-CCP–positive RA, respectively, our findings are important because they indicate that distinct pathogenic mechanisms may underlie anti-CCP–positive and anti-CCP–negative RA.

In a previous study of a relatively small number of patients (n=44), it was found that HLA–DR3 frequencies in RA patients differ from the frequencies observed in controls [13]. Likewise, in a group of 85 Arab RA patients, HLA–DR3 conferred a risk for RA susceptibility [14], and a more recent study showed a significantly increased frequency of HLA–DR3 in patients with synovitis of recent onset [15]. In a Caucasian population of 167 RA patients, an association with HLA–DR3 was observed after excluding SE alleles from the analysis [16]. Our findings represent an extension of those findings by establishing that HLA–DR3 is associated only with a particular subset of RA. Our analysis also confirmed that particular HLA–DRB1 alleles, such as DR8, DR11, and DR13, protect against RA [17]. We now show that these alleles tend to be associated with protection against both anti-CCP–positive

and anti-CCP–negative disease. Therefore, our data indicate that the HLA alleles conferring protection do so independently of the anti-CCP status, while the alleles that predispose to RA are associated with distinct RA phenotypes (anti-CCP–positive or anti-CCP–negative RA).

It is debatable, however, whether the association of HLA–DR3 with anti-CCP–negative RA (and UA) is attributable to the HLA–DR3 gene itself. Other genetic factors in high linkage disequilibrium with HLA–DR3 may also underlie the observed association. HLA–DR3 is known to be part of a conserved ancestral haplotype (A1;B8;DRB1*03, also known as the 8.1 haplotype [18]) that occurs frequently in Caucasian individuals and has been reported to be associated with RA [19]. Jawaheer et al described an additional genetic risk factor present within the major histocompatibility complex (MHC) that is part of this conserved haplotype [20]. Their finding concerned a certain allelic combination of tumor necrosis factor (TNF) polymorphisms and another polymorphism on the HLA class III region of chromosome 6. Likewise, the class III MHC TNF–lymphotoxin region was described as appearing to influence susceptibility to RA separately from the HLA–DR region [21], and a microsatellite marker (MIB*350) that is also part of an ancestral haplotype associated with DRB1*0301 was described as a risk factor for RA independently of DRB1*0301 [22].

In summary, HLA–DR3 is associated with anti-CCP–negative RA and UA and not with anti-CCP–positive RA or UA. The data presented herein indicate that separate genetic risk factors are associated with different phenotypes, which suggests that various pathogenetic mechanisms underlie anti-CCP–positive and anti-CCP–negative disease.

Supplementary data

Tabel 1. HLA–DRB1 allele frequencies in 206 anti-CCP–positive RA patients, 171 anti-CCP– negative RA patients and 423 healthy controls

DRB1	anti-CCP–positive RA (n=412)			anti-CCP–negative RA (n=342)			Controls (n=846)	
	n	%	OR (95% CI)	n	%	OR (95% CI)	n	%
DR1								
01(00)	33	8.0		31	9.1		6	0.7
0101	28	6.8		13	3.8		92	10.9
0102	0	0		3	0.9		2	0.2
0103	0	0		1	0.3		1	0.1
Total	*61*	*14.8*	*1.28 (0.89-1.83)*	*48*	*14.0*	*1.20 (0.81-1.76)*	*101*	*11.9*
DR3								
03(00)	0	0		0	0		2	0.2
0301/ [a]	9	2.2		5	1.5		0	0
0301	30	7.3		53	15.5		84	9.9
0302/ [b]	0	0		1	0.3		0	0
Total	*39*	*9.5*	*0.92 (0.60-1.40)*	*59*	*17.3*	***1.84 (1.26-2.67)***	*86*	*10.2*
DR4								
04(00)	9	2.2		2	0.6		0	0
0401	84	20.4	**2.56 (1.80-3.63)**	33	9.6	1.07 (0.67-1.66)	77	9.1
0402	0	0		1	0.3		2	0.2
0403	2	0.5	0.34 (0.04-1.53)	2	0.6	0.41 (0.04-1.85)	4	0.5
0403/ [c]	0	0		0	0		8	0.9
0404/ [d]	0	0		0	0		18	2.1
0404	25	6.1	**3.02 (1.69-5.49)**	12	3.5	1.89 (0.89-3.94)	2	0.2
0408	7	1.7		3	0.9		3	0.4
0405	5	1.2	**10.4 (1.15-492)**	0	0	-	1	0.1
0406	1	0.2		0	0		0	0
0407	1	0.2		4	1.2		8	0.9
Total	*134*	*32.5*	***2.83 (2.12-3.79)***	*57*	*16.7*	*1.18 (0.82-1.67)*	*123*	*14.5*
DR7								
07(00)	6	1.5		5	1.5		91	10.8
0701	28	6.8		27	7.9		0	0
Total	*34*	*8.3*	*0.75 (0.48-1.14)*	*32*	*9.4*	*0.86 (0.54-1.33)*	*91*	*10.8*
DR8								
08(00)	2	0.5		2	0.6		16	1.9
0801	3	0.7		1	0.3		10	1.2
0803	0	0		0	0		1	0.1
Total	*5*	*1.2*	***0.37 (0.11-0.99)***	*3*	*0.9*	***0.27 (0.05-0.88)***	*27*	*3.2*
DR9								
09(00)	2	0.5		1	0.3		10	1.2
0901	11	2.7		5	1.5		0	0
Total	*13*	*3.2*	***2.72 (1.09-7.00)***	*6*	*1.8*	*1.49 (0.44-4.57)*	*10*	*1.2*

DR10								
10(00)	8	1.9		2	0.6		4	0.5
1001	12	2.9		1	0.3		0	0
Total	*20*	*4.9*	***10.7 (3.56-43.4)***	*3*	*0.9*	*1.86 (0.27-11.1)*	*4*	*0.5*
DR11								
11(00)	8	1.9		6	1.8		5	0.6
1101/ [e]	2	0.5		6	1.8		79	9.3
1101	8	1.9	**0.29 (0.14-0.55)**	6	1.8	**0.41 (0.21-0.75)**	0	0
1104	2	0.5		2	0.6		0	0
1102/ [f]	0	0		1	0.3		6	0.7
1102	1	0.2	0.34 (0.01-2.82)	2	0.6	1.24 (0.20-5.84)	0	0
Total	*21*	*5.1*	***0.45 (0.26-0.75)***	*23*	*6.7*	***0.61 (0.36-0.99)***	*90*	*10.6*
DR12								
12(00)	6	1.5		4	1.2		7	0.8
1201	1	0.2		1	0.3		16	1.9
1202	0	0		1	0.3		4	0.5
Total	*7*	*1.7*	*0.52 (0.19-1.25)*	*6*	*1.8*	*0.54 (0.18-1.36)*	*27*	*3.2*
DR13								
13(00)	0	0		3	0.9		6	0.7
1301/ [g]	2	0.5		9	2.6		116	13.7
1301	4	1.0		13	3.8		0	0
1302	13	3.2		14	4.1		1	0.1
1303	2	0.5		4	1.2		3	0.4
Total	*21*	*5.1*	***0.31 (0.18-0.50)***	*43*	*12.6*	*0.82 (0.55-1.20)*	*126*	*14.9*
DR14								
14(00)	6	1.5		9	2.6		0	0
1401	0	0		1	0.3		27	3.2
1404	1	0.2		0	0		0	0
Total	*7*	*1.7*	*0.52 (0.19-1.25)*	*10*	*2.9*	*0.91 (0.39-1.97)*	*27*	*3.2*
DR15								
15(00)	33	8.0		33	9.6		128	15.1
1501	16	3.9		17	5.0		1	0.1
Total	*49*	*11.9*	*0.75 (0.52-1.08)*	*50*	*14.6*	*0.95 (0.65-1.37)*	*129*	*15.2*
DR16								
16(00)	1	0.2		2	0.6		5	0.6
Total	1	0.2	0.41 (0.01-3.68)	2	0.6	0.99 (0.09-6.08)	5	0.6
Total alleles	412	100		342	100		846	100

RA=rheumatoid arthritis; UA=undifferentiated arthritis; CCP=cyclic citrullinated peptide; OR=odds ratio; 95% CI=95% confidence interval.

ORs were calculated comparing allele frequencies of the diseased group with allele frequencies in the healthy control group. HLA typings without subtyping are presented as x(00). Subtypings without conclusive result are presented as [a] 0301/ for subtyping HLA–DRB1*0301, *0304 or *0305; [b] 0302/ for subtyping HLA–DRB1 *0302 or *0303 or *0307; [c] 0403/ for subtyping HLA-DRB1*0403, *0406 or *0407; [d] 0404/ for subtyping HLA–DRB1*0404, *0408 or *0419; [e] 1101/ for subtyping HLA–DRB1*1101 or *1104; [f] 1102/ for subtyping HLA–DRB1*1102 or *1103; [g] 1301/ for subtyping HLA–DRB1*1301 or *1302.

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Chapter 6

The HLA–DRB1 shared epitope alleles differ in the interaction with smoking and predisposition to antibodies to cyclic citrullinated peptide

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Abstract

Objective

The HLA shared epitope (SE) alleles are primarily a risk factor for the presence of antibodies to cyclic citrullinated peptide (anti-CCP antibodies) rather than for the development of rheumatoid arthritis (RA). The SE alleles interact with the environmental risk factor tobacco exposure (TE) for predisposition to anti-CCP–positive RA. The objectives of this study were to determine 1) whether different SE subtypes contribute differently to the presence of anti-CCP antibodies, 2) whether different SE subtypes all interact with TE for the development of anti-CCP antibodies, and 3) the effect of TE in relation to the SE alleles and anti-CCP antibodies on the risk of progression from undifferentiated arthritis (UA) to RA.

Methods

We assessed the effect of SE subtypes and TE on the presence and level of anti-CCP antibodies and on the risk of progression from UA to RA in 977 patients with early arthritis who were included in the Leiden Early Arthritis Clinic.

Results

The HLA–DRB1*0401, *0404, *0405, or *0408 SE alleles conferred the highest risk of developing anti-CCP antibodies (odds ratio [OR] 5.0, compared with an OR of 2.0 for the HLA–DRB1*0101 or *0102 SE alleles and an OR of 1.7 for the HLA–DRB1*1001 SE allele). Conversely, the TE–SE allele interaction was the strongest for the HLA–DRB1*0101 or *0102 SE alleles and the HLA–DRB1*1001 SE allele. TE in SE–positive, anti-CCP–positive patients correlated with higher levels of anti-CCP antibodies and with progression from UA to RA. In logistic regression analysis, only the presence and level of anti-CCP antibodies were associated independently with RA development.

Conclusion

The HLA–DRB1 SE subtypes differ in their interaction with smoking and in their predisposition to anti-CCP antibodies. TE contributes to the development of RA in SE–positive, anti-CCP–positive patients, which is explained by its effect on the level of anti-CCP antibodies.

Introduction

Since antibodies to cyclic citrullinated peptide (anti-CCP antibodies) are highly specific for rheumatoid arthritis (RA) [1], precede the development of RA [2,3], and are also associated with a more severe disease course [4,5], it has been suggested that these antibodies have a causative role in the pathogenesis of RA [6,7]. Furthermore, antibodies against a citrullinated protein have been shown to enhance experimental arthritis in mice [8]. The most important genetic risk factors for RA, which were identified almost 30 years ago, are the HLA–DRB1 alleles that encode for a common amino acid sequence called the shared epitope (SE) [9,10]. We recently reported that the SE alleles are not primarily a risk factor for RA, but rather, they predispose to the presence of anti-CCP antibodies (11).

The SE alleles are HLA–DRB1*0101, *0102, *0401, *0404, *0405, *0408, *1001, and *1402. HLA–DRB1*0101, *0102, *0404, *0405, *0408, and *1402 encode for the amino acid sequence QRRAA at positions 70–74 of the third hypervariable region (HVR3) of the DRB1 molecule, while HLA–DRB1*0401 encodes for the sequence QKRAA and HLA–DRB1*1001 encodes for the sequence RRRAA. Interestingly, there are differences in the strength of the association between different SE alleles and RA. The HLA–DRB1*04 alleles represent a considerably stronger susceptibility factor than the other SE alleles [12]. Moreover, these alleles are associated with a higher level of joint destruction [13], and the HLA–DRB1*0401 alleles are particularly associated with bone erosions [14] and rheumatoid vasculitis [15].

Given these differences between the various SE alleles and the correlations with RA susceptibility and severity, as well as our recent observation that the SE alleles are primarily a risk factor for anti-CCP antibodies and the fact that these antibodies are associated with (severe) RA, in the present study we tested the hypothesis that the various SE alleles differ in their predisposition to the presence of anti-CCP antibodies. Notably, the HLA–DRB1*0401 alleles were expected to confer a high risk for the presence of anti-CCP antibodies, since these alleles display the strongest association with disease susceptibility and severity.

Recently, 2 independent studies showed that tobacco exposure (TE) predisposes to anti-CCP–positive RA only in the presence of SE alleles [16,17]. Thus, the second aim of our study was to examine the gene-environment interaction in more detail by investigating whether the interaction between SE alleles and TE

varied for the different SE subtypes. In order to further increase our comprehension of the contribution of TE to the development of RA, the third aim of our study was to determine the effect of TE, in relation to the presence or absence of SE alleles and anti-CCP antibodies, on the risk for patients who presented with undifferentiated arthritis (UA) to have their disease progress to RA during 1 year of followup.

Patients and methods

Study population

For this study, the Leiden Early Arthritis Clinic (EAC) cohort was used (for a description, see ref. 18). Briefly, the EAC is an inception cohort that started in 1993. Patients were referred to the EAC when arthritis was suspected, and they were included in the cohort when arthritis was found at physical examination. At present, ~1,700 patients with early arthritis are included and have been followed up for at least 1 year. At inclusion, blood samples were obtained and the smoking history (cigarettes, cigars) was assessed with the help of questionnaires. Current and past smokers were classified as TE+ and those who had never smoked were classified as TE-. We selected patients for whom baseline data on smoking, anti-CCP antibodies, and HLA–DRB1 subtypes were available. This concerned 977 patients with early arthritis, all of whom were Caucasians.

To study the contribution of TE to the progression from UA to RA in the 977 patients with early arthritis, we selected a subset of 421 patients who had a form of arthritis (UA) at presentation that could not be classified as RA according to the 1987 revised criteria of the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) [19]. After 1 year of followup, we determined whether the disease in these patients with UA had or had not progressed to RA, and we assessed the association of the genetic (SE alleles), environmental (TE), and serologic (anti-CCP antibodies) risk factors with the disease course. It might be possible that some patients did not fulfill the ACR criteria for RA at 1 year but developed RA at a later time point. It is inherent in the design of an inception cohort that the duration of followup differs within the study population. However, at the moment of analysis, the majority of patients had been followed up for >1 year, and <10% of the patients who were not classified as having RA at 1 year developed RA later in their disease course.

Anti-CCP antibodies

The anti-CCP antibodies were assessed using an enzyme-linked immunosorbent assay to detect and quantitate anti-CCP–2 antibodies (Immunoscan RA Mark 2; Euro-Diagnostica, Arnhem, The Netherlands). The cutoff level for anti-CCP antibody positivity was set at 25 arbitrary units (AU) in accordance with the manufacturer's instructions.

HLA–DRB1 subtyping

The HLA–DRB1 subtyping was performed by polymerase chain reaction using specific primers and hybridization with sequence-specific oligonucleotides. The SE alleles were HLA–DRB1*0101, *0102, *0401, *0404, *0405, *0408, *1001, and *1402. In order to study the effect of different SE alleles, they were subdivided into subtypes. SE DR1 indicates the presence of HLA–DRB1*0101 or *0102, but no other SE allele. SE DR10 indicates the presence of HLA–DRB1*1001, but no other SE allele. SE DR4 indicates the presence of HLA–DRB1*0401, *0404, *0405, or *0408, but no other SE allele. Since HLA–DRB1*0401 encodes for an amino acid sequence different from that encoded by the other HLA–DRB1*04 SE alleles (QKRAA versus QRRAA), the effects of HLA–DRB1*0401 were investigated separately from those of the other HLA–DRB1*04 SE alleles. SE DR0401 indicates the presence of HLA–DRB1*0401, but no other SE allele. Patients homozygous for 1 SE subtype (e.g., *0101/0101) were counted once. Patients who carried 2 SE alleles of a different subtype (e.g., *0101/*0401) were not assigned to one of the SE subgroups.

Statistical analysis

Odds ratios (ORs) were calculated to investigate the contribution of the different SE subtypes to the presence of anti-CCP antibodies. Proportions were compared by the chi-square test. Differences in mean or median values between groups were analyzed using the Mann-Whitney U test.

A gene-environment interaction is defined as a different effect of an environmental exposure on disease risk in persons with different genotypes [20]. The assessment of an interaction critically depends on the definition of (the model of) interaction, and either an additive or multiplicative model can be applied [20–22]. Since Rothman et al and Ottman have pointed out that the choice of the measurement scale depends on the goal of the investigation, and that to unravel disease etiology

a multiplicative model is more appropriate than an additive model [20,21], the presence of a gene-environment interaction was measured on a multiplicative scale. For this analysis, TE, SE (subtype) and the interaction term TE–SE (subtype) were entered in a logistic regression analysis with the presence of anti-CCP antibodies as the dependent variable.

To determine whether the ORs for the effects of SE alleles and TE on the presence of anti-CCP antibodies were different between the 3 SE subtypes, a likelihood ratio test was used, comparing 2 logistic regression models [21]. In the first model, the presence of anti-CCP antibodies was entered as the dependent variable, and this model assumed that the effect of TE, SE and the interaction between SE and TE was similar for the 3 SE subtypes. The second model allowed variation in the effects of TE, SE and the TE–SE interaction. We compared the 2 models using a chi-square test with 4 degrees of freedom, and we tested the hypothesis that the effects of the risk factors were not different between the 3 SE subtypes. To investigate which of the genetic and environmental risk factors were associated independently with progression from UA to RA, a logistic regression analysis with a backward selection procedure ($P>0.05$ as the removal criterion, using the likelihood ratio test) was performed in the UA patients, with the disease outcome (not RA or RA) as the dependent variable and the presence of TE, SE alleles, SE subtypes, TE–SE interaction term, anti-CCP antibodies, and level of anti-CCP antibodies in case of anti-CCP positivity as possible explanatory variables.

The Statistical Package for the Social Sciences, version 10.0 (SPSS, Chicago, IL) was used. In all tests, P values less than 0.05 were considered significant.

Results

Association between SE subtypes and anti-CCP antibodies

The association between the different SE subtypes and the presence of anti-CCP antibodies was analyzed in 977 patients with early arthritis. The mean ± SD age of these patients was 52 ± 17 years, 588 patients (60%) were women, 438 patients (45%) were past or current smokers, 540 patients (55%) were SE+ (homozygous or heterozygous), and 270 patients (28%) had anti-CCP antibodies. After 1 year of follow-up, 405 patients were diagnosed as having RA, 272 as having UA, and 300 as having other rheumatic diseases.

SE alleles were associated with the presence of anti-CCP antibodies both in the patients with RA and in the patients with UA or other diagnosed diseases (OR 3.0, 95% confidence interval [95% CI] 1.6–5.6 and OR 3.3, 95% CI 1.4–8.1, respectively). SE alleles were distributed as follows: 145 patients (15%) with HLA– DRB1*0101 or *0102; 140 patients (14%) with HLA–DRB1*1001; 303 patients (31%) with HLA–DRB1*0401, *0404, *0405, or *0408; and 9 patients (0.9%) with HLA–DRB1*1402. After exclusion of the patients who carried 2 different SE subtypes, the SE subtype distributions were as follows: 116 patients (12%) had SE DR1, 262 patients (27%) had SE DR4, 175 patients (18%) had SE DR0401, and 114 patients (12%) had SE DR10. Because of the low number of patients carrying HLA–DRB1*1402, the association between this SE subtype and the presence of anti-CCP antibodies could not be properly addressed.

In the absence of TE, the presence of the SE DR1 alleles was associated with an OR of 2.0 (95% CI 1.0–4.6; P<0.05), the SE DR4 alleles with an OR of 5.0 (95% CI 2.9–8.5; P<0.0001), and the SE DR10 alleles with an OR of 1.7 (95% CI 0.8–3.4; P not significant [NS]) for the presence of anti-CCP antibodies (see Table 1). Subsequently, the HLA–DRB1*0401 alleles were analyzed separately from the other SE DR4 alleles. The presence of HLA–DRB1*0401 conferred an OR of 5.8 (95% CI 3.2–10.6; P<0.0001) for anti-CCP positivity, and the presence of HLA–DRB1*0404, *0405, or *0408 conferred an OR of 3.8 (95% CI 1.8–8.1; P<0.0001) for anti-CCP positivity. A log-likelihood comparison revealed that the ORs conferred by the SE subtypes DR1, DR4, and DR10 for anti-CCP positivity differed significantly (see below for description and data). Thus, these data show that the SE DR4 alleles, and HLA– DRB1*0401 in particular, confer the highest risk for anti-CCP–positive arthritis.

TE–SE gene–environment interaction for predisposition to anti-CCP antibodies

Comparing the distributions of SE alleles and TE between patients with early arthritis with and those without anti-CCP antibodies revealed that TE did not result in a higher OR for anti-CCP antibodies in SE– patients, in contrast to the effect of TE in SE+ patients (P=0.14 for interaction) (Table 1). The effect of the number of SE alleles was investigated. Using TE-,SE- as the referent category, the results for having 1 SE dose were as follows: for TE+,SE-, OR 1.0 (P NS); for TE-,SE+, OR 2.8 (95% CI 1.7–4.5, P<0.0001); and, for TE+,SE+,OR 4.5 (95% CI 2.8–7.2; P<0.0001), with a significant

difference between TE-,SE+ and TE+,SE+ (P<0.05). Using TE-,SE- as the referent category, the results for patients who were homozygous for SE alleles were as follows: for TE+,SE-, OR 1.1 (P NS); for TE-,SE+, OR 6.2 (95% CI 3.1–12.5; P<0.0001); and, for TE-,SE+, OR 14.9 (95% CI 6.7–33.4; P<0.0001), with a significant difference between TE-,SE+ and TE+,SE+ (P<0.05). These data suggest that TE alone does not significantly increase the risk of antibodies, but that the presence of TE interacts with the SE dose to increase the risk of anti-CCP antibodies.

Since we showed that the SE subtypes differed significantly in the strength of the association with anti-CCP antibodies, we further explored the gene– environment interaction and examined whether interaction between SE alleles and TE was different for the various SE subtypes. TE significantly interacted with the DR1 and DR10 SE alleles for the presence of anti-CCP antibodies (Table 1). The addition of TE to the presence of SE DR1 alleles increased the OR for anti-CCP positivity from 2.0 to 4.4, and the addition of TE to the presence of SE DR10 alleles increased the OR for anti-CCP positivity from 1.7 to 4.9 (P<0.05 for interaction, for both SE subtypes). The presence of TE in patients with early arthritis carrying SE DR4 alleles did not significantly increase the OR for having anti-CCP antibodies.

To investigate whether the effect of TE and SE on the development of anti-CCP antibodies was significantly different between the 3 SE subtypes, the log likelihoods of the following 2 logistic regression models were compared. The first model assumed that the effect of TE and SE on the predisposition to anti-CCP antibodies was equal for the 3 SE subtypes, whereas the second model allowed variation between the effects of SE subtypes and TE on the predisposition to anti-CCP antibodies. Comparison of the log likelihoods revealed a significant difference (P=0.004), indicating that the ORs for the effects of TE and SE on the presence of anti-CCP antibodies were different for the 3 SE subtypes.

In summary, the HLA–DRB1 SE subtypes differed significantly in their predisposition to anti-CCP antibodies that they conferred and in their interaction with TE. A larger significant gene-environment interaction between SE alleles and TE was observed for SE DR1 and SE DR10 alleles, while the interaction term between the SE DR4 alleles and TE was small and not significant.

Table 1. ORs for developing anti-CCP antibodies in the presence of SE alleles (any or specific SE subtype) and/or a history of smoking in patients with early arthritis*

SE subtype, SE status/TE status†	No. of anti-CCP–negative patients	No. of anti-CCP–positive patients	Total	OR (95% CI)	P
Any SE‡					
-/-	218	35	253	1	–
-/+	158	26	184	1.0 (0.6–1.8)	NS
+/-	187	99	286	3.3 (2.1–5.2)	<0.0001
+/+	135	119	254	5.5 (3.5–8.7)	<0.0001
SE DR1§					
-/-	218	35	253	1	–
-/+	158	26	184	1.0 (0.6–1.8)	NS
+/-	49	16	65	2.0 (1.0–4.6)	0.04
+/+	30	21	51	4.4 (2.1–8.9)	<0.0001
SE DR4					
-/-	218	35	253	1	–
-/+	158	26	184	1.0 (0.6–1.8)	NS
+/-	69	55	124	5.0 (2.9–8.5)	<0.0001
+/+	69	69	138	6.2 (3.7–10.5)	<0.0001
SE DR0401					
-/-	218	35	253	1	–
-/+	158	26	184	1.0 (0.6–1.8)	NS
+/-	41	35	76	5.8 (3.2–10.6)	<0.0001
+/+	45	51	96	7.1 (4.0–12.5)	<0.0001
SE DR10¶					
-/-	218	35	253	1	–
-/+	158	26	184	1.0 (0.6–1.8)	NS
+/-	56	15	71	1.7 (0.8–3.4)	NS
+/+	24	19	43	4.9 (2.3–10.5)	<0.0001

*OR = odds ratio; anti-CCP antibodies = antibodies to cyclic citrullinated peptide; SE = shared epitope; TE = tobacco exposure (current or past smoker); 95% CI = 95% confidence interval; NS = not significant.
† Any SE indicates the presence of the SE alleles HLA–DRB1*0101, *0102, *0401, *0404, *0405, *0408, *1001, or *1402. SE DR1 indicates the presence of the SE alleles HLA–DRB1*0101 or *0102, but no other SE allele. SE DR4 indicates the presence of the SE alleles HLA–DRB1*0401, *0404, *0405, or *0408, but no other SE allele. SE DR0401 indicates the presence of the SE allele HLA–DRB1*0401, but no other SE allele. SE DR10 indicates the presence of the SE allele HLA–DRB1*1001, but no other SE allele.
‡ P=0.004 for SE+,TE- versus SE+,TE+; P NS (0.14) for interaction. § P=0.06 for SE+,TE- versus SE+,TE+; P=0.02 for interaction. ¶ P=0.009 for SE+,TE- versus SE+,TE+; P=0.003 for interaction

Contribution of TE to development of RA in relation to SE alleles and anti-CCP antibodies

To further investigate the possible contribution of TE to the pathogenesis of RA, the effect of TE on the progression from UA to RA was evaluated in relation to the presence or absence of SE alleles and the presence, level, and absence of anti-CCP antibodies in 421 patients with early arthritis who presented with UA. After 1 year of followup, 142 of these patients (34%) had developed RA, whereas 279 patients (66%) had (persistent) UA, had achieved remission, or had developed other rheumatic diseases (together classified as non-RA). The UA patients whose disease had progressed to RA were significantly older, were more often women, were more often SE DR4+, more often carried anti-CCP antibodies, and when anti-CCP+, had significantly higher levels of anti-CCP antibodies compared with the UA patients who did not develop RA (see Table 2). TE was equally distributed among the UA patients whose disease did or did not progress to RA (Table 2).

To study the effect of TE on the risk of progression from UA to RA in relation to SE and anti-CCP status, the UA patients were stratified according to the presence or absence of SE alleles. Subsequently, we assessed whether the presence of TE increased the risk of developing RA in the absence and presence of anti-CCP antibodies (Table 3). In the SE– patients with UA, the presence of anti-CCP antibodies was associated with an OR of 7.5 for progression to RA. The additional presence of TE did not result in a significantly higher risk of developing RA. Conversely, in the SE+, anti-CCP+ UA patients, the presence of TE significantly increased the OR for developing RA from 3.3 (anti-CCP+,TE-) to 8.0 (anti-CCP+,TE+)($P=0.003$; $P=0.002$ for interaction) (Table 3). The effect of TE on the risk of developing RA in the presence and absence of anti-CCP antibodies could not be adequately addressed for the different SE subtypes, since this yielded subgroups containing small numbers of patients.

We next analyzed whether TE influences the level of anti-CCP antibodies, since this might explain the effect of TE in the presence of anti-CCP antibodies on the risk of developing RA. To this end, we compared the anti-CCP antibody levels in anti-CCP+ patients with and those without SE alleles in relation to TE (see Figure 1). Anti-CCP+,SE+,TE+ patients had a significantly higher level of anti-CCP antibodies compared with anti-CCP+,SE+,TE- patients ($P<0.01$). Together, the finding that a higher level of anti-CCP antibodies is associated with progression from UA to RA (Table 2), combined with the observation that the presence of TE in SE+, anti-CCP+

patients is associated with a higher risk of developing RA (Table 3) and a higher level of anti-CCP antibodies (Figure 1) suggests that the increased risk conferred by TE for the development of RA in SE+, anti-CCP+ UA patients is mediated by an increased level of anti-CCP antibodies.

Table 2. Characteristics of the 421 patients who presented with UA and who did or did not develop RA after 1 year of followup*

	Non-RA patients (n=279)	RA patients (n=142)	P
Age, mean ± SD years	49 ± 17	57 ± 16	<0.0001
Women	150 (54)	95 (67)	0.01
SE subtype†			
SE DR1	42 (15)	18 (13)	NS
SE DR4	58 (21)	49 (35)	<0.01
SE DR0401	38 (14)	34 (24)	<0.01
SE DR10	29 (10)	10 (7)	NS
Current or past smoker	133 (48)	70 (49)	NS
Anti-CCP+	34 (12)	72 (51)	<0.0001
Anti-CCP level if anti-CCP+, median (IQR) AU	173 (44–1,082)	665 (243–1,684)	<0.01

* Except where indicated otherwise, values are the number (%). UA = undifferentiated arthritis; RA = rheumatoid arthritis; IQR = interquartile range; AU = arbitrary units (see Table 1 for other definitions).
† SE DR1 indicates the presence of the SE alleles HLA–DRB1*0101 or *0102, but no other SE allele. SE DR4 indicates the presence of the SE alleles HLA–DRB1*0401, *0404, *0405, or *0408, but no other SE allele. SE DR0401 indicates the presence of the SE allele HLA–DRB1*0401, but no other SE allele. SE DR10 indicates the presence of the SE allele HLA–DRB1*1001, but no other SE allele.

Subsequently, to determine which of the above-mentioned factors is independently associated with the development of RA, a logistic regression analysis was performed. This analysis revealed that only the presence of anti-CCP antibodies (OR 4.7, P<0.0001) and the level of anti-CCP antibodies in the case of anti-CCP positivity (OR 1.1 per 100 AU, P<0.05) were independently associated with progression from UA to RA, whereas TE and SE status, SE subtypes, and TE–SE interaction were not. In conclusion, these data indicated that TE in the presence of SE alleles was primarily associated with both the presence and the level of anti-CCP antibodies, and that the correlation between TE and the development of RA in SE+, anti-CCP+ patients was explained by the effect of TE on the level of anti-CCP antibodies.

Table 3. Risk of developing RA in UA patients in the presence/ absence of TE and/or anti-CCP antibodies after stratification for SE alleles*

	Non-RA patients (n=279)	RA patients (n=142)	OR (95% CI)	P
SE-				
Anti-CCP-				
TE-	72	21	1	–
TE+	62	18	1 (0.5–2.2)	NS
Anti-CCP+				
TE-	5	11	7.5 (2.1–28.5)†	<0.001
TE+	4	11	9.4 (2.4–39.8)†	<0.001
SE+				
Anti-CCP-				
TE-	54	21	1	–
TE+	57	10	0.5 (0.2–1.2)	NS
Anti-CCP+				
TE-	15	19	3.3 (1.3–8.3)‡	<0.01
TE+	10	31	8.0 (3.1–21.1)‡	<0.0001

*RA = rheumatoid arthritis; UA = undifferentiated arthritis (see Table 1 for other definitions).
† P NS for anti-CCP+,TE- versus anti-CCP+,TE+; P NS for interaction.
‡ P<0.01 for anti-CCP+,TE- versus anti-CCP+,TE+; P<0.01 for interaction.

Since we observed that TE was associated with a higher level of anti-CCP antibodies in the presence of SE alleles (Figure 1) and that the TE–SE interaction for the presence of anti-CCP antibodies differed for the different SE subtypes (Table 1), we explored whether the effect of TE on the level of anti-CCP antibodies was also different for the 3 SE subtypes. Although no significant differences were observed, possibly due to the low numbers of patients per subgroup, this analysis indicated that TE in the presence of SE DR1 and SE DR10 alleles had a stronger effect on the level of anti-CCP antibodies compared with the effect of TE in the presence of the SE DR4 alleles (Figure 2).

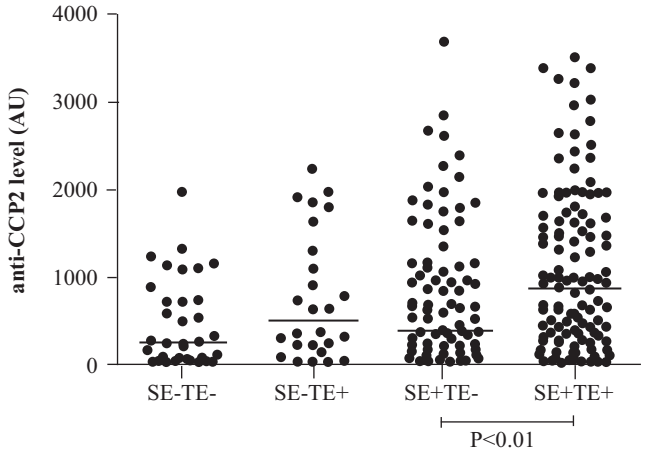

Figure 1. Levels of antibodies to cyclic citrullinated peptide (anti-CCP antibodies) in anti-CCP+ patients with or without shared epitope (SE) alleles and with or without tobacco exposure (TE). SE alleles included HLA–DRB1*0101, *0102, *0401, *0404, *0405, *0408, *1001, or *1402. Horizontal lines indicate median values in arbitrary units (AU). P not significant (NS) for SE-,TE- versus SE-,TE+; P=0.06 (NS) for SE-,TE- versus SE+,TE+; P=0.005 for SE+,TE- versus SE+,TE+.

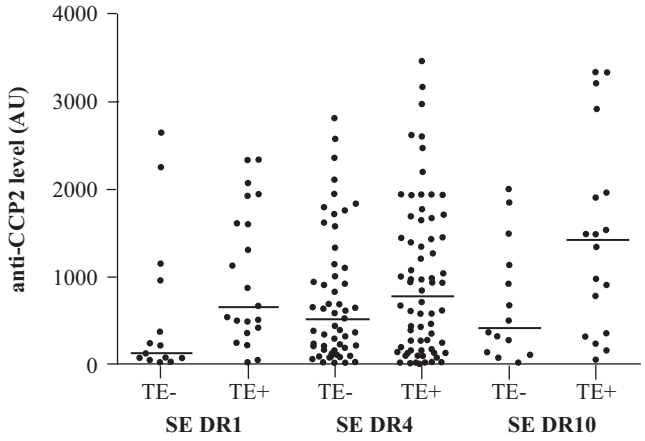

Figure 2. Anti-CCP antibody levels in anti-CCP+,SE+ patients with or without TE, among patients with different SE subtypes. SE DR1 indicates the presence of the SE alleles HLA–DRB1*0101 or *0102; SE DR4 indicates the presence of the SE alleles HLA–DRB1*0401, *0404, *0405, or *0408; SE DR10 indicates the presence of the SE allele HLA–DRB1*1001. Horizontal lines indicate median values in AU. See Figure 1 for other definitions.

Discussion

The association between RA and the HLA–DRB1 alleles encoding for the common amino acid sequence referred to as the SE was discovered almost 30 years ago, and these SE-encoding alleles represent the most important genetic risk factor for RA [9,10]. Differences in the strength of the association between various SE alleles and both susceptibility to RA and severity of RA have been described [12–15]. Recently, we have shown that the SE alleles are primarily a risk factor for anti-CCP antibodies rather than for RA [11]. In addition, 2 independent reports suggested that the most important environmental risk factor for RA, smoking, is related to the pathway in which the SE alleles and anti-CCP antibodies interact, since it was described that TE was only associated with RA in SE+ patients [16] and that this gene–environment interaction was restricted to anti-CCP+ RA [17].

The current data further refine our understanding of the contribution of the HLA SE subtypes, smoking, and anti-CCP antibodies to the pathogenesis of RA. We have now shown that 1) the SE DR4 alleles, and HLA–DRB1*0401 in particular, confer the highest risk for having anti-CCP antibodies; 2) the SE DR1 and SE DR10 alleles in particular, but not the SE DR4 alleles, significantly interact with TE for the development of anti-CCP antibodies; and 3) the presence of TE in SE+, anti-CCP+ patients correlates with a higher level of anti-CCP antibodies, and both the presence and the level of anti-CCP antibodies are associated with the risk of disease progressing from UA to RA.

The observation that HLA–DRB1*0401 conferred the highest risk for developing anti-CCP antibodies is interesting, since the amino acid sequence encoded by HLA–DRB1*0401 (QKRAA) is different from those encoded by the other SE alleles (QRRAA or RRRAA). This could imply that position 71 of the HVR3 of the DRß1 molecule forms a critical part of the antigen-presenting binding site. Although lysine (K) and arginine (R) both have a positive electric charge, differences in size and structure between these amino acids may influence the peptide binding specificity of the pocket. Carrying a lysine at position 71 might hypothetically be associated with a higher affinity for antigens that are instrumental to the development of anti-CCP antibodies, leading to more robust T cell activation and anti-CCP antibody production by B–cells. Of note, Hill et al showed that in mice transgenic for major histocompatibility complex (MHC)–DRB1*0401, a vimentin peptide citrullinated in a region with contact to the SE binding site harbored a higher affinity for the

MHC– DRB1*0401 molecule, resulting in a better T cell activation compared with its noncitrullinated counterpart [23].

In the present study, a significant multiplicative gene-environment interaction for the presence of anti-CCP antibodies was shown for TE and the SE DR1 and SE DR10 alleles, but not for TE and the SE DR4 alleles. From the current data, it appeared that the effect of the environmental factor (TE) depended on the strength of the association between the genetic factor (SE) and the disease outcome: the stronger the association of the SE alleles with the development of anti-CCP antibodies, the weaker the contribution of TE to the additional development of anti-CCP antibodies. Recently, Klareskog et al proposed a disease model in which long-term exposure to tobacco smoke induces long-term posttranslational citrullination, which in the presence of SE alleles, leads to the presentation of citrullinated antigens and subsequent T cell activation, ultimately precipitating an autoimmune disease [17]. On the basis of the current data, this hypothesis can be extended as follows. The HLA–DRB1*0401 alleles have a relatively high binding affinity for citrullinated peptides; therefore, immunity to citrullinated peptides is generated more easily, resulting in the production of anti-CCP antibodies. In the presence of other SE alleles that have a lower binding affinity for citrullinated peptides, the effect of TE leads to an increased amount of citrullinated antigens that reaches the threshold required to activate T–cells.

To increase our comprehension of the contribution of TE to the development of RA, the present study investigated the effect of TE in relation to the SE alleles and anti-CCP antibodies on the risk of disease progression from UA to RA. Although numbers were relatively small in some stratified subgroups of patients, the presence of TE in SE+, anti-CCP+ patients was associated with increased levels of anti-CCP antibodies as well as with an increased risk of developing RA. Additionally, the present study showed that the level of anti-CCP antibodies was correlated with the progression from UA to RA. Importantly, in a logistic regression analysis, only the presence and level of anti-CCP antibodies were independently associated with the development of RA, indicating that the observed association between TE and RA development in SE+, anti-CCP+ patients is explained by the correlation between TE and anti-CCP antibody levels. Together, these findings further point to the relevance of both the presence and the level of anti-CCP antibodies (or a factor associated with this) in the development of RA.

In conclusion, the data presented in this report show that the HLA–DRB1*04(01) alleles confer a higher risk for anti-CCP antibodies compared with the HLA–DRB1*01 and DRB1*10 SE alleles. This might be due to a difference in antigen binding affinity caused by the composition of the amino acids at positions 70 and 71 of the DRß1 molecule. Additionally, the present study showed that TE increases the risk of anti-CCP antibodies, particularly in the presence of the HLA– DRB1*01 and DRB1*10 SE alleles, thereby refining the association between HLA, anti-CCP antibodies, and TE.

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Chapter 7

Association of smoking with the constitution of the anti-cyclic citrullinated peptide response in the absence of HLA–DRB1 shared epitope alleles

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Abstract

Objective

Smoking is a risk factor for anti-cyclic citrullinated peptide (anti-CCP) antibody-positive rheumatoid arthritis (RA) in patients with HLA–DRB1 shared epitope (SE) alleles. It is unknown whether smoking influences not only the presence of these antibodies, but also other characteristics of the anti-CCP response, such as isotype usage. The aim of this study was to determine the influence of smoking on anti-CCP isotypes in RA patients, and to determine whether this influence is observed in the presence and/or absence of SE alleles.

Methods

IgA, IgM, and IgG subclasses of anti-CCP antibodies were measured by enzyme-linked immunosorbent assay in serum obtained at the first visit to the Leiden Early Arthritis Clinic from 216 patients with anti-CCP–positive RA whose smoking habits were also assessed. HLA genotyping data were available for 202 of these patients.

Results

IgA and IgM anti-CCP were more frequent in RA patients who were smokers than in those who were nonsmokers (odds ratio 2.8 and 1.8, respectively). In addition, levels of all isotypes of anti-CCP, except IgG3, were significantly higher ($P<0.05$) in smokers. The number of anti-CCP isotypes was higher in smokers compared with nonsmokers, both in SE–negative RA ($P=0.04$) and in SE-positive RA ($P=0.07$).

Conclusion

Patients with anti-CCP–positive RA who have a current or former tobacco exposure display a more extensive anti-CCP isotype usage in general, and IgA and IgM in particular, compared with patients with anti-CCP–positive RA who have never smoked. In contrast to its influence on the incidence of anti-CCP positivity, the influence of tobacco exposure on the constitution of the anti-CCP response is significant in SE-negative RA. These findings suggest a differential effect of tobacco exposure on the induction as compared with the propagation of the anti-CCP antibody response.

Introduction

Antibodies against citrullinated proteins are thought to play a pivotal role in the progression of rheumatoid arthritis (RA) because they are highly specific and predictive of RA [1], are associated with the extent of joint destruction [2], and have been shown to enhance disease severity in mice with experimental arthritis [3]. The most prominent genetic risk factors for RA, the HLA–DRB1 shared epitope (SE) alleles, encode for a common amino acid sequence in the peptide presenting part of the HLA class II molecule. These SE alleles have been described recently to be a risk factor for the development of anti–cyclic citrullinated peptide (anti-CCP) antibodies, rather than for anti-CCP–positive RA per se [4].

Smoking is a well-known environmental risk factor for the development of RA [5] and has been reported to influence the severity of RA in terms of disease expression, disease activity, and radiologic joint damage [6,7]. However, tobacco exposure has been associated with anti-CCP–positive RA only, as opposed to RA in general. This association was only observed in the context of SE alleles, and not with SE-negative RA, thus demonstrating a gene–environment interaction between the HLA–SE and smoking [8,9]. Together, these observations were the basis for the hypothesis, first postulated by Klareskog et al [9], that smoking may trigger RA-specific immune reactions to citrullinated proteins, possibly by inducing citrullination of damaged, dying cells in the bronchoalveolar tract.

The observation that the gene-environment interaction between HLA–SE alleles and smoking was only present in anti-CCP–positive disease [9] makes it attractive to speculate that smoking may affect not only the presence, but also the "nature" of the anti-CCP response. For example, it is conceivable that the contribution of HLA–SE alleles to the association between smoking and anti-CCP–positive disease is routed through CD4+ T helper cells, which influence the magnitude and/or quality of the citrullinated protein- directed B cell responses and thereby the overall anti-CCP response.

We have recently shown that the levels of anti-CCP antibodies in patients with SE-positive, anti-CCP–positive arthritis who smoked were higher compared with those in patients with SE-positive, anti-CCP–positive arthritis who never smoked [10]. However, no information is available on the constitution of the anti-CCP response with respect to, for example, isotype usage as a characteristic of the anti-CCP response. This information could be of relevance because it might provide

novel details on the relationship between anti-CCP antibodies, the HLA–SE, and smoking in patients with RA, and subsequently may increase the understanding of how tobacco exposure contributes to the development and progression of RA.

Smoking is associated with a higher prevalence of citrullinated proteins in cells obtained by bronchoalveolar lavage [9], presumably caused by abundant protein citrullination in damaged cells. Therefore, the effect of smoking on the anti-CCP response could be mediated through modulation of citrulline-directed immune responses in the bronchus-associated lymphoid tissue (BALT). We hypothesized that the prevalence of IgA anti-CCP, and possibly other isotypes, would differ between RA patients with and those without tobacco exposure, since IgA is an isotype that is typically, although not exclusively, produced in mucosa-associated lymphoid tissue such as BALT.

Levels of total IgG anti-CCP are commonly measured in studies and in daily clinical practice. However, little information on the IgA, IgM, and subclasses of IgG anti-CCP antibodies is available, in contrast to the extensive study findings on the isotypes used by rheumatoid factor (RF)-producing B–cells. IgA-RF has been reported to be associated with a more severe disease outcome, and smokers have been reported to produce more IgM-RF and IgA-RF as compared with the levels of these isotypes in nonsmokers [7].

In this study we first addressed whether the isotype usage in patients with anti-CCP–positive RA who were smokers differed from the isotype usage in patients with anti-CCP–positive RA who were nonsmokers, focusing especially on the participation of IgA in the anti-CCP response. We then analyzed whether this influence of tobacco exposure on isotype usage depended on the presence of HLA–DRB1 SE alleles, as was recently described with respect to the influence of tobacco exposure on the presence of IgG anti-CCP antibodies in RA patients. Our data demonstrate that smoking influences the pattern of isotype usage in the anti-CCP response, and that this effect is not limited to SE-positive RA.

Patients and methods

Study population and serum samples

Patients having received a diagnosis of RA within the first year after their initial clinic visit were selected from the Leiden Early Arthritis Clinic (EAC), which provides an inception cohort of patients with recent-onset arthritis (duration of symptoms <2 years). The EAC was started at the Department of Rheumatology of the Leiden University Medical Center in 1993 and is described in detail by van Aken et al [11]. RA was diagnosed according to the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) 1987 revised criteria for the classification of RA [12].

At the first EAC visit, serum samples were obtained and smoking history (all sorts of active tobacco exposure) was assessed by means of patient questionnaires. Patients who were current smokers and those with a history of smoking were classified as smokers, while those who had never smoked were classified as nonsmokers. Patients for whom baseline serum samples and smoking history were available were selected for the present study (n=416). Anti-CCP antibody isotypes were assessed in IgG anti-CCP–positive patients only, resulting in a cohort of 216 patients for inclusion in the present study. Among the 216 patients with anti-CCP–positive RA, 202 had data available on the HLA genotype. Patients provided their informed consent, and the study was approved by the local review board of medical ethics.

Isotypes of anti-CCP antibodies

Total IgG anti-CCP antibodies were assessed by an enzyme-linked immunosorbent assay (ELISA) (Immunoscan RA Mark 2; Euro-Diagnostica, Arnhem, The Netherlands). The cutoff for IgG anti-CCP positivity was set at a level of 25 units/ml, according to the manufacturer's instructions.

Levels of the IgG subclasses of anti-CCP as well as levels of IgA and IgM anti-CCP were determined by a sandwich ELISA technique as described previously [13]. Briefly, microtiter plates coated with CCPs (Immunoscan RA Mark 2; Euro-Diagnostica) were incubated with the patients' serum. The next incubation step was performed with conjugated polyclonal antibodies for the detection of IgM and IgA (AHI 0605 and AHI 0105; BioSource International, Camarillo, CA), and unconjugated mouse monoclonal antibodies followed by conjugated rabbit anti-mouse Ig for the detection of the IgG subclasses. A series of successive dilutions of

pooled patient sera was used as a reference standard in all plates. Microtiter plates coated with uncitrullinated control peptide (Euro-Diagnostica) were used as a control for citrulline specificity of the antibodies.

Cutoff values for the presence of the different isotypes of anti-CCP antibodies were defined as the mean plus 2 SD in serum samples of a group of 50 IgG anti-CCP–negative control subjects, and were corrected for a high background level of response against the control peptide, as described previously [13]. The cutoff values for positivity were as follows: 25 units/ml for IgA anti-CCP, 32 units/ml for IgM anti-CCP, 2 units/ml for IgG1 anti-CCP, 20 units/ml for IgG2 anti-CCP, 52 units/ml for IgG3 anti-CCP, and 0.1 units/ml for IgG4 anti-CCP.

HLA genotyping

The HLA–DRB1 alleles were determined in 202 patients with anti-CCP–positive RA. HLA– DRB1 (sub)typing was performed by polymerase chain reaction using specific primers and hybridization with sequence-specific oligonucleotides as previously described [14]. The SE alleles were DRB1*0101, *0102, *0104, *0401, *0404, *0405, *0408, *1001, and *1402.

Statistical analysis

Odds ratios (ORs) were calculated by comparing patients whose serum was positive and patients whose serum was negative for the different anti-CCP isotypes. Differences in levels of anti-CCP isotypes and differences in the number of anti-CCP isotypes were analyzed using the Mann-Whitney U test. SPSS software, version 12.0 (SPSS, Chicago, IL) was used for all statistical analyses. In all tests, P values less than 0.05 were considered significant.

Results

Different classes and subclasses of anti-CCP antibodies were determined in 216 patients with anti-CCP–positive RA to determine whether tobacco exposure influences the usage of the different isotypes, and in particular the presence of IgA anti-CCP. We found that IgA anti-CCP was more frequently present in smokers than in nonsmokers, with an OR of 2.8 (95% confidence interval [95% CI] 1.60–5.04). IgM anti-CCP was also more frequent in smokers than in nonsmokers (OR 1.8, 95%

CI 1.03–3.15), whereas the subclasses of IgG anti-CCP were not significantly more frequent among smokers (Table 1).

Table 1. Distribution of isotypes of anti-CCP in 117 smokers versus 99 nonsmokers*

anti-CCP isotype	Nonsmokers no. (%)	Smokers no. (%)	Odds ratio (95% CI)
IgA	50 (51)	87 (74)	2.8 (1.60 – 5.04)
IgM	55 (56)	81 (69)	1.8 (1.03 – 3.15)
IgG1	99 (100)	116 (99)	–
IgG2	76 (77)	99 (85)	1.7 (0.84 – 3.30)
IgG3	50 (51)	70 (60)	1.5 (0.85 – 2.51)
IgG4	97 (98)	113 (97)	0.6 (0.10 – 3.25)

*Anti-CCP = anti-cyclic citrullinated peptide; 95% CI = 95% confidence interval.

A trend toward longer disease duration at the time of inclusion was observed for the patients classified as smokers compared with those classified as nonsmokers (P=0.08 by Mann-Whitney U test), and therefore additional logistic regression analyses were performed to correct for disease duration. Smoking was still found to be a significant predictor of both the presence of IgM (P=0.022) and the presence of IgA (P=0.001) after correction for disease duration.

To summarize the extensiveness of the isotype usage, the number of different isotypes participating in the anti-CCP response in individual patients was calculated. Although the median number of isotypes used was equal between patients who were smokers and those who were nonsmokers (median 5 isotypes, range 1–6), the number of isotypes detected per patient was higher in smokers compared with nonsmokers (P=0.013 by Mann-Whitney U test) (Figure 1), indicating that tobacco exposure influences the extensiveness of anti-CCP antibody isotype usage in general, and of IgM anti-CCP and IgA anti-CCP in particular.

To determine whether tobacco exposure influences not only the presence or absence of the different isotypes of anti-CCP antibodies, but also the level of each isotype, the different anti-CCP isotypes were measured by ELISA in the serum, and levels were compared according to tobacco exposure in RA patients who were positive for the respective anti-CCP isotypes. Levels of all isotypes of anti-CCP antibodies, except those of IgG3, were significantly higher in the patients classified as smokers than in the patients who had never smoked (Table 2 and Figure 2), which is consistent with previous results with regard to total levels of IgG anti-CCP antibodies [10].

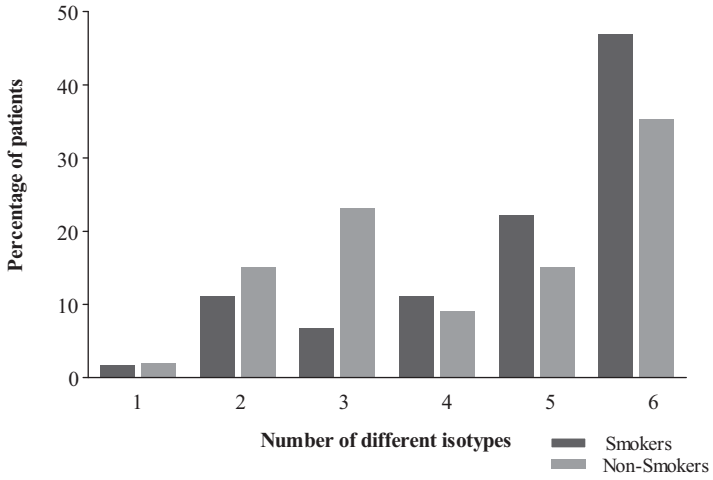

Figure 1. Percentage of patients with a certain total number (per patient) of anti-cyclic citrullinated peptide (anti-CCP) isotypes, among patients with IgG anti-CCP–positive rheumatoid arthritis who were classified as smokers (n=117) or nonsmokers (n=99).

Table 2. Levels of anti-CCP isotypes in smokers versus nonsmokers*

Anti-CCP isotype	Anti-CCP isotype level, units/ml		P†
	Nonsmokers	Smokers	
IgA	67 (42 – 141)	109 (47 – 352)	0.012
IgM	57 (42 – 98)	94 (52 – 166)	0.001
IgG1	145 (79 – 208)	201 (108 – 312)	0.003
IgG2	71 (40 – 167)	125 (57 – 328)	0.016
IgG3	145 (72 – 266)	186 (86 – 870)	0.102
IgG4	17 (5 – 63)	75 (15 – 363)	<0.001

* Values are the median (interquartile range). Anti-CCP=anti–cyclic citrullinated peptide.

† Calculated by Mann-Whitney U test.

We then assessed whether the influence of tobacco exposure on isotype usage can be observed in both SE-positive and SE-negative RA, and whether the influence of smoking is dependent on the presence of SE alleles in RA, as was recently described with respect to the influence of tobacco exposure on the presence of anti-CCP antibodies. In the present analysis, patients were stratified according to tobacco exposure and the presence or absence of SE alleles.

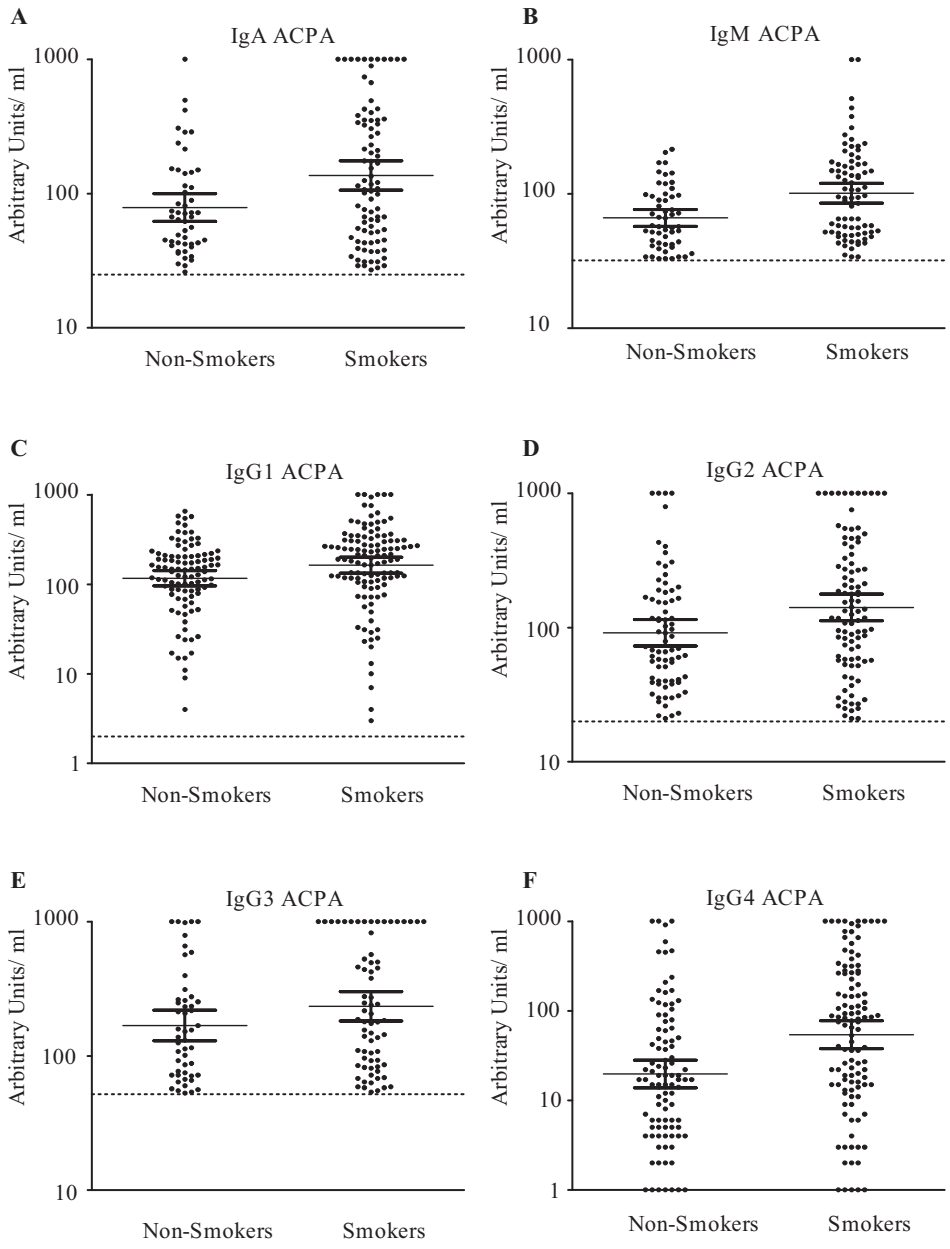

Figure 2. Levels of IgA anti–cyclic citrullinated peptide (anti-CCP) (A), IgM anti-CCP (B), IgG1 anti-CCP (C), IgG2 anti-CCP (D), IgG3 anti-CCP (E), and IgG4 anti-CCP (F) in patients with anti-CCP–positive rheumatoid arthritis who were positive for the respective isotypes and classified as nonsmokers or smokers. Circles indicate individual data points. Broken lines indicate the cutoff level for positivity. Bars show the geometric mean with 95% confidence interval.

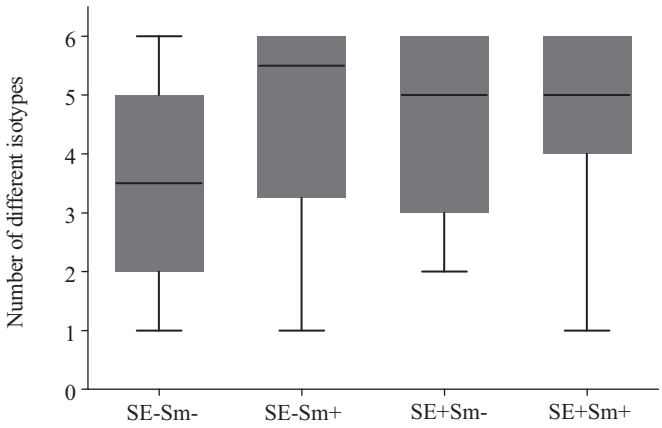

Figure 3. Distribution of the median number of different anti–cyclic citrullinated peptide (anti-CCP) antibody isotypes in patients with anti-CCP–positive rheumatoid arthritis who were classified as nonsmokers (Sm-) or smokers (Sm+) in the presence (SE+) or absence (SE-) of HLA–DRB1 shared epitope alleles. Results are shown as box plots, where the bars indicate the median, boxes indicate the first and third quartiles, and bars outside the boxes indicate the range. P=0.04 for SE-Sm- (n=20) versus SE-Sm+ (n=16); P=0.07 for SE+Sm- (n=70) versus SE+Sm+ (n= 96), by Mann-Whitney U test.

IgA anti-CCP, irrespective of SE status, was significantly more frequent among smokers. Similarly, IgM anti-CCP was more often detected in smokers as compared with nonsmokers regardless of whether these patients had SE-positive or SE-negative disease, although the differences were not statistically significant (data not shown). A trend toward a higher number of different isotypes of anti-CCP antibodies in smokers compared with nonsmokers was observed in those with SE-positive RA (P=0.07).

More intriguingly, however, we observed that in the patients with SE-negative RA, tobacco exposure was associated with a more extensive isotype usage within the anti-CCP response (P=0.04) (Figure 3). No interaction between SE status and smoking status in relation to usage of the anti-CCP antibody isotypes could be detected (data not shown). However, the data obtained indicated that the influence of smoking on isotype usage in patients with anti-CCP–positive RA does not depend on the presence of SE alleles.

Discussion

B–cells activated in the bronchoalveolar tract are prominent producers of IgA antibodies, and the organized BALT that is involved in the generation of IgA producing cells can be detected more frequently in smokers than in nonsmokers [15]. This finding, together with the observation that individuals who are smokers display higher citrullination in cells obtained by bronchoalveolar lavage [9], fueled the hypothesis that IgA anti-CCP would be present more frequently and detected at higher levels in smokers than in nonsmokers.

Indeed, not only were IgA anti-CCP antibodies more frequently present in smokers, but also the levels of IgA anti-CCP antibodies were higher in smokers than in nonsmokers. However, in addition to the findings regarding IgA anti-CCP antibodies, IgM anti-CCP antibodies were also more frequently detected in smokers, and the levels of all isotypes, except IgG3, as well as the number of isotypes used in the anti-CCP response were higher in smokers than in nonsmokers. These data indicate a more diverse anti-CCP response in general in patients with anti-CCP–positive RA who have been exposed to tobacco compared with patients who are nonsmokers.

Smoking not only is associated with anti-CCP–positive RA, but also has been identified as a risk factor for the development of RA among patients with anti-CCP–positive undifferentiated arthritis (UA) [10] and as a factor that influences the extent of joint damage in RA [6]. The differences in isotype usage and/or the differences in levels of anti-CCP antibodies between patients with anti-CCP–positive RA who are smokers and those who are nonsmokers possibly contribute to a more severe progression of RA and a faster fulfillment of the ACR criteria within patients with UA. This is a subject of interest that should be explored further, but was not included in the present study due to insufficient power to detect differences in disease progression.

Tobacco exposure was recently described as a contributor to the risk of anti-CCP–positive RA only among patients with SE-positive disease [8]. In this study, we addressed whether the effect of smoking on the constitution of the anti-CCP response, in terms of isotype usage, was dependent on the presence of the SE as well. We observed a higher number of anti-CCP isotypes in anti-CCP–positive smokers compared with anti-CCP–positive nonsmokers, both in patients with SE-positive RA (P not significant, possibly as a result of a ceiling effect) and in patients with SE-negative RA (P=0.04) (Figure 3). These data indicate that, at least in SE-negative

RA, tobacco exposure influences the extensiveness of isotype usage in the anti-CCP response. Moreover, the results suggest that tobacco exposure is involved in the development of anti-CCP only in patients with SE-positive RA, whereas once the tolerance for citrullinated antigens is broken, the effect of tobacco exposure on the response becomes independent of T cell help via SE-bearing HLA molecules. This could, for example, be mediated by exerting a direct effect on the B cell response and/or a diversification of the underlying T cell response that now recognizes the antigen in the context of other HLA molecules.

In conclusion, patients with anti-CCP–positive RA who are current or former smokers display a more extensive anti-CCP isotype usage and a higher percentage of IgA and IgM anti-CCP antibodies than do patients with anti-CCP–positive RA who are nonsmokers. Additionally, in contrast to the influence of smoking on the presence of anti-CCP antibodies, the influence of smoking on the constitution of the anti-CCP response is not observed exclusively in patients with SE-positive RA, but also in patients with SE-negative RA, possibly reflecting the differential effects of tobacco exposure on the induction as compared with propagation of the anti-CCP response.

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Chapter 8

Isotype distribution of anti-cyclic citrullinated peptide antibodies in undifferentiated arthritis and rheumatoid arthritis reflects an ongoing immune response

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Abstract

Objective

The evolution of the rheumatoid arthritis (RA)-specific anti-cyclic citrullinated peptide (anti-CCP) antibody response, as measured by the isotypes of anti-CCP, has not been described. This study was undertaken to determine anti-CCP isotype usage in patients with undifferentiated arthritis (UA), patients with recent-onset RA, and patients with RA of long duration.

Methods

IgA, IgM, and IgG subclasses of anti-CCP were measured by enzyme-linked immunosorbent assay in serum samples that were obtained from IgG anti-CCP antibody-positive patients with UA (n=110) and IgG anti-CCP antibody-positive patients with RA (n=152) early after the onset of arthritis. Patients with UA in whom RA developed within 1 year (UA→RA) were compared with patients with UA in whom RA did not develop within 1 year (UA→UA). In addition, baseline serum samples obtained from a subset of patients with RA (n=64) were compared with sera obtained from the same patients a median of 7 years later.

Results

IgM anti-CCP was present in early samples from both patients with UA and patients with RA and in followup samples from patients with RA. Several IgG anti-CCP antibody-positive patients who did not have IgM anti-CCP early after disease onset did display IgM anti-CCP later in the course of the arthritis. A diverse pattern of isotype usage was detected in early samples, with a trend toward lower frequencies of all isotypes of anti-CCP in patients with UA compared with patients with RA and in UA→UA patients compared with UA→RA patients. Levels of all isotypes except IgG1 had decreased after 7 years.

Conclusion

These data indicate development of the anti-CCP isotype repertoire into full usage early in the course of arthritis. The sustained presence of IgM anti-CCP indicates ongoing recruitment of new B–cells into the anti-CCP response, reflecting a continuous (re)activation of the RA-specific anti-CCP response during the course of anti-CCP–positive arthritis.

Introduction

Antibodies against cyclic citrullinated peptide (CCP) are highly specific for rheumatoid arthritis (RA) [1], are predictive of the development of RA in patients with undifferentiated arthritis (UA) [2], and are associated with the extent of joint destruction [3]. Furthermore, anti-CCP antibodies have been shown to enhance disease severity in mice with experimental arthritis [4]. Taken together, these findings point to a pivotal role of anti-CCP antibodies in the progression of RA. At present, little information is available regarding isotype usage of the anti-CCP antibody response, because total levels of IgG anti-CCP are commonly measured. Given the possible contribution of these antibodies to the progression of RA, more detailed analyses of the anti-CCP response are valuable, because the results of such analyses could provide insight into the nature of the antibody response.

Naive B lymphocytes express 2 classes of membrane-bound antibodies, IgM and IgD, which function as the receptors for antigens. Activation of mature naive B–cells requires signals delivered through their antigen receptors and, in the case of T cell-dependent antigens, additional signals that are provided after interaction with an antigen-specific helper T–cell.

Activation of naive B–cells upon the first antigen encounter results in proliferation and differentiation into IgM antibody-secreting cells. During their differentiation, upon further contact with T–cells, some B–cells start to produce antibodies of other heavy-chain classes (isotype class switching), and the affinity of the produced antibodies matures. Eventually, this will lead to expanded populations of class-switched high-affinity antibody-secreting plasma cells and to the generation of memory B–cells that will differentiate into plasma cells after antigen reencounter.

Upon repeated antigen exposure, the IgM response is usually absent or relatively low as compared with the primary antibody response [5]. Since immunoglobulin isotype switching is the result of a recombination process in the genetic region that encodes for the different heavy-chain classes of the corresponding isotypes, with deletion of the intervening DNA, switched B–cells are not able to return to IgM production. Furthermore, IgM has a relatively short half-life of ~5 days [6], and long-lived plasma cells producing IgM or IgM memory B–cells against T cell-dependent antigens have not been described. The continuous presence of IgM against T cell-dependent antigens, therefore, points to continuous triggering of newly generated B–cells.

Four subclasses of IgG (IgG1, IgG2, IgG3, and IgG4) can be distinguished in humans. The relative contribution of the different IgG subclasses to an antibody response depends on the nature of the antigen eliciting the response, repeated exposure to the antigen, as well as the cytokines produced in the vicinity of the B–cells. Likewise, the route of entry, the dose of antigen, and the host's genotype can play a decisive role in the isotype usage of B cell responses [7].

IgA, IgM, and IgG subclasses display substantial differences in the ability to mediate effector responses. For example, immunoglobulin isotypes differ considerably in their ability to activate the complement system, with IgM and IgG3 being the most potent complement activators. A recent study demonstrated that the difference in recruiting cellular effector functions is a consequence of differential affinities of IgG subclasses for specific activating IgG Fc receptors compared with their affinities for the inhibitory IgG Fc receptor [8]. Moreover, the different IgG Fc receptors are expressed on different effector cells, adding further to the differential ability of IgG subclasses to mediate effector responses [9].

The concepts described above (with the increase in antibody affinity and the occurrence of isotype switching being crucial for more efficient binding and neutralization of a pathogen and, thus, ultimately for the survival of the host) have been obtained mainly by studying responses against viral or bacterial antigens. It is unknown whether the principles applying to specific humoral immunity to infection also apply to the emergence of autoantibody responses. The dynamics of autoantigen-specific responses in relation to antibody isotype usage and levels have scarcely been studied, and little information is available regarding the evolution of the anti-CCP antibody response in patients with arthritis. For example, it is not known whether one "initial hit" is responsible for the continuous production of these antibodies or whether novel autoantigen-specific antibody-producing cells are continuously activated from newly activated mature B–cells.

Because information on isotype distribution of the anti-CCP response could contribute to an understanding of the effector functions of these antibodies, and because the analysis of isotypes of anti-CCP antibodies early and later in the course of arthritis could provide insight into the underlying immune reaction, we investigated the presence and levels of IgM, IgA, and subclasses of IgG anti-CCP in patients with RA and patients with UA.

Patients and methods

Study population and serum samples

Patients with UA or RA were selected from among patients in the Leiden Early Arthritis Clinic (EAC), which is an inception cohort of patients with arthritis of recent onset (symptom duration <2 years). The EAC was established at the Department of Rheumatology of the Leiden University Medical Center in 1993 and has been described in detail by van Aken et al [10]. For all patients, a diagnosis was registered 2 weeks after the first visit. RA was diagnosed according to the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) 1987 revised criteria for the classification of RA [11]. Patients who could not be properly classified according to one of the ACR criteria were categorized as having UA.

After 1 year of followup, the disease status of all IgG anti-CCP–positive patients with UA was examined in order to determine whether RA (as defined according to the ACR criteria) had developed. Baseline serum samples were drawn at the first visit to the EAC. Additional serum samples obtained after 6-9 years (median 7 years) of followup were available for 64 IgG anti-CCP–positive patients in whom RA was diagnosed within 1 year after their first visit to the EAC. Informed consent was obtained, and the study was approved by the local medical ethics review board.

Anti-CCP autoantibodies

Total IgG anti-CCP was assessed in baseline serum samples from patients with UA and patients with RA, by enzyme-linked immunosorbent assay (ELISA) (Immunoscan RA Mark 2; Euro-Diagnostica, Arnhem, The Netherlands). The cutoff level for IgG anti-CCP positivity was set at 25 units/ml, according to the manufacturer's instructions.

Measurement of anti-CCP antibody isotypes

The levels of IgG1, IgG2, IgG3, IgG4, IgA, and IgM anti-CCP were measured in baseline serum samples obtained from 262 IgG anti-CCP–positive patients (152 with RA and 110 with UA) and in followup samples obtained from 64 patients with RA in whom the diagnosis was made within the first year. Levels of IgA and IgM anti-CCP were also determined in a group of 80 IgG anti-CCP–negative patients with RA.

The levels of IgG1, IgG2, IgG3, IgG4, IgA, and IgM anti-CCP were determined using the sandwich ELISA technique. Microtiter plates coated with CCP (Immunoscan RA Mark 2; Euro-Diagnostica) were incubated for 2 hours with serum

samples, 100 µl/well, at a dilution of 1:50. This and each subsequent incubation step was performed at 37°C in a humidified atmosphere, followed by washing steps with washing buffer for the Immunoscan RA Mark 2 system. All samples and reagents were diluted in dilution buffer for Immunoscan RA Mark 2. For the detection of IgM and (total) IgA, the plates were incubated for 2 hours with either 100 µl/well goat antihuman IgM (1:1,000 dilution) or goat anti-human IgA (1:1,000 dilution) conjugated with alkaline phosphatase (catalog nos. AHI0605 and AHI0105; BioSource International, Camarillo, CA).

For detection of IgG subclasses, the plates were incubated for 2 hours with monoclonal mouse anti-human IgG subclass-specific antibodies, in a 1:10,000 dilution for IgG1 (antibody HP6188; Sanquin, Amsterdam, The Netherlands) and IgG3 (antibody HP6080; Nordic, Tilburg, The Netherlands), in a 1:1,000 dilution for IgG2 (antibody HP6002; SBA, Birmingham, UK), and in a 1:15,000 dilution for IgG4 (antibody HP6206; Nordic). All of the monoclonal antibodies that were used had been evaluated for their specificity in an International Union of Immunological Societies/World Health Organization collaborative study [12,13]. After incubation with the monoclonal antibodies, the plates were incubated overnight at room temperature, with 100 µl/well rabbit anti-mouse immunoglobulin (1:750 dilution) conjugated with alkaline phosphatase (Dako, Glostrup, Denmark). The presence of CCP-specific antibodies was detected using 4-nitrophenyl phosphate disodium salt (Sigma-Aldrich, Steinheim, Germany) as substrate, as previously described [5].

A series of successive dilutions of pooled patient sera that were positive for all isotypes of anti-CCP was used as a reference standard in all plates. Distinct dilutions of this standard (1:25 for IgG2, IgG3, IgA, and IgM; 1:50 for IgG4; 1:200 for IgG1) were defined as containing 1,000 arbitrary units (AU) per milliliter. The number of AUs per milliliter for one isotype is not comparable with the number of AUs per milliliter for other isotypes.

To control for the possibility that IgM rheumatoid factor (RF) interferes with the detection of anti-CCP of the IgM isotype, we depleted RF antibodies in a set of IgM RF–positive, IgM anti-CCP–positive, IgG anti-CCP–positive sera, using IgG-coated CNBr-activated Sepharose beads. This did not result in a reduction of IgM anti-CCP levels. As an additional control, we mixed sera that were highly positive for IgM-RF and negative for IgM anti-CCP and IgG anti-CCP with sera that were IgM RF negative, IgM anti-CCP–negative, and IgG anti-CCP–positive, in order to analyze whether IgM anti-CCP would now be detected. This was not the case (data

not shown). Moreover, several IgM-RF–positive sera were negative for IgM anti-CCP in the presence of IgG anti-CCP, and several IgM-RF–negative sera were positive for IgM anti-CCP, further indicating that IgM-RF did not lead to false-positive results for the detection of IgM anti-CCP.

Cutoff values and specificity control

Cutoff values for the presence of IgG subclasses of anti-CCP antibodies were defined as the mean plus 2 SD for serum samples obtained from a group of 50 IgG anti-CCP–negative control subjects who did not have a diagnosis of RA or UA. This definition resulted in cutoff values for positivity of 2, 20, 52, and 0.1 AU/ml for IgG1, IgG2, IgG3, and IgG4, respectively. Microtiter plates coated with the same amount of the uncitrullinated control peptide were provided by the manufacturer (Euro-Diagnostica) and were used as a control for citrulline specificity of the anti-CCP antibodies. IgG subclass antibodies against the control peptide were not detected, as measured in 101 anti-CCP antibody–positive sera (data not shown).

The cutoff value for anti-CCP reactivity of IgA antibodies was set at 25 AU/ml. In a high proportion of sera (63%) with less than 25 AU/ml, the reactivity against the citrullinated peptide could not be distinguished from that against the uncitrullinated control peptide. Only 4 (2%) of the 162 IgA anti-CCP–positive sera reacted against the control peptide with similar optical density values. These 4 patients were considered negative for the presence of IgA anti-CCP antibodies (Table 1).

For IgM, a cutoff value of 32 AU/ml units was determined by using the definition of the mean value plus 2 SD for the IgG anti-CCP–negative control population. Using this cutoff value, sera from 8 (5%) of the 153 IgM anti-CCP–positive patients reacted against the uncitrullinated control peptide as well. These 8 patients were considered to be negative for IgM anti-CCP, because no specific response was detectable (Table 1).

Statistical analysis

Chi-square tests were performed to compare the proportions of individuals in the different groups who were positive for the various anti-CCP antibody isotypes. If one of the cells in the cross-table contained fewer than 6 subjects, P values were calculated using Fisher's exact test. Odds ratios were calculated in a case-control setting, in which the number of diagnoses of RA and the number of diagnoses of UA in patients with and those without the different isotypes of anti-CCP were compared.

Relative risks for the development of RA were calculated based on the presence of the different anti-CCP isotypes in patients with UA. A *t*-test was used to compare the differences in levels of anti-CCP isotypes between the groups of patients studied. Differences in the frequencies and levels of anti-CCP isotypes between baseline and followup were tested using McNemar's test and a paired-samples *t*-test, respectively. Because the levels of all isotypes were not normally distributed, log transformation was performed first to normalize the data.

Table 1. Anti-CCP isotypes in IgG anti-CCP–positive patients with UA and IgG anti-CCP–positive patients with RA*

Anti-CCP isotype	UA (n=110)	RA (n=152)	OR (95% CI)	P†
IgA				
Positive	64 (58)	94 (62)	1.2 (0.7 – 2.0)	0.55
Negative	46 (42)	58 (38)		
IgM				
Positive	52 (47)	93 (61)	1.8 (1.04 – 3.0)	0.03
Negative	58 (53)	59 (39)		
IgG1				
Positive	107 (97)	150 (99)	2.10 (0.28 – 25)	0.65
Negative	3 (3)	2 (1)		
IgG2				
Positive	74 (67)	125 (82)	2.3 (1.2 – 4.2)	0.005
Negative	36 (33)	27 (18)		
IgG3				
Positive	43 (39)	91 (60)	2.3 (1.4 – 4.0)	<0.001
Negative	67 (61)	61 (40)		
IgG4				
Positive	104 (95)	149 (98)	2.9 (0.62 – 18)	0.17
Negative	6 (5)	3 (2)		

* Values are the number (%). Anti-CCP=anti–cyclic citrullinated peptide; UA=undifferentiated arthritis; RA=rheumatoid arthritis; OR=odds ratio; 95% CI= 95% confidence interval.
† By chi-square test.

Results

Isotypes of anti-CCP at baseline in patients with UA and patients with RA

Different classes and IgG subclasses of anti-CCP isotypes were measured in baseline serum samples obtained from IgG anti-CCP–positive patients with UA (n=110) and IgG anti-CCP–positive patients with RA (n=152) (Table 1). No IgA anti-CCP or IgM anti-CCP was detected in 80 IgG anti-CCP–negative patients with RA, indicating that the occurrence of IgA and IgM anti-CCP is confined to IgG anti-CCP–positive patients. Among IgG anti-CCP–positive patients, those with RA more frequently displayed IgM (P=0.03), IgG2 (P=0.005), and IgG3 (P<0.001) anti-CCP antibodies compared with patients with UA (Table 1). Among patients with UA, a median of 4 isotypes were used in the anti-CCP antibody response, compared with a median of 5 among patients with RA (P=0.007).

A trend toward higher levels of anti-CCP antibodies in RA compared with UA was detected for all isotypes, when all samples were considered. The differences in levels were highest for IgM (P=0.03), IgG2 (P=0.002), and IgG3 (P<0.001) (Figure 1A). However, the exclusion of samples that were negative for the respective isotypes revealed no differences in levels of anti-CCP isotypes between patients with RA and those with UA (P=0.27–0.75), indicating that the level of these isotypes did not differ between patients with RA and patients with UA, but rather, that the number of antibody-positive patients was higher in the RA group than in the UA group.

Thus, among IgG anti-CCP–positive patients, those with RA displayed a more diverse pattern of anti-CCP antibodies, as determined by the presence of different isotypes. No differences in mean levels were detected between the groups of patients who tested positive for the respective isotypes.

Anti-CCP isotypes at baseline in patients with UA in whom RA developed within 1 year (UA→RA) and in patients with UA in whom RA did not develop within 1 year (UA→UA)

Of the 110 anti-CCP–positive patients who had a diagnosis of UA at baseline, 74 had fulfilled the ACR criteria for RA after 1 year of followup, whereas 29 still had a diagnosis of UA. In 7 patients, other diseases had developed by this point in time. Inspired by our observation that patients with RA display a more extensive usage of anti-CCP antibody isotypes compared with patients with UA, we sought to determine whether the anti-CCP response in UA→RA patients differed from that in UA→UA patients.

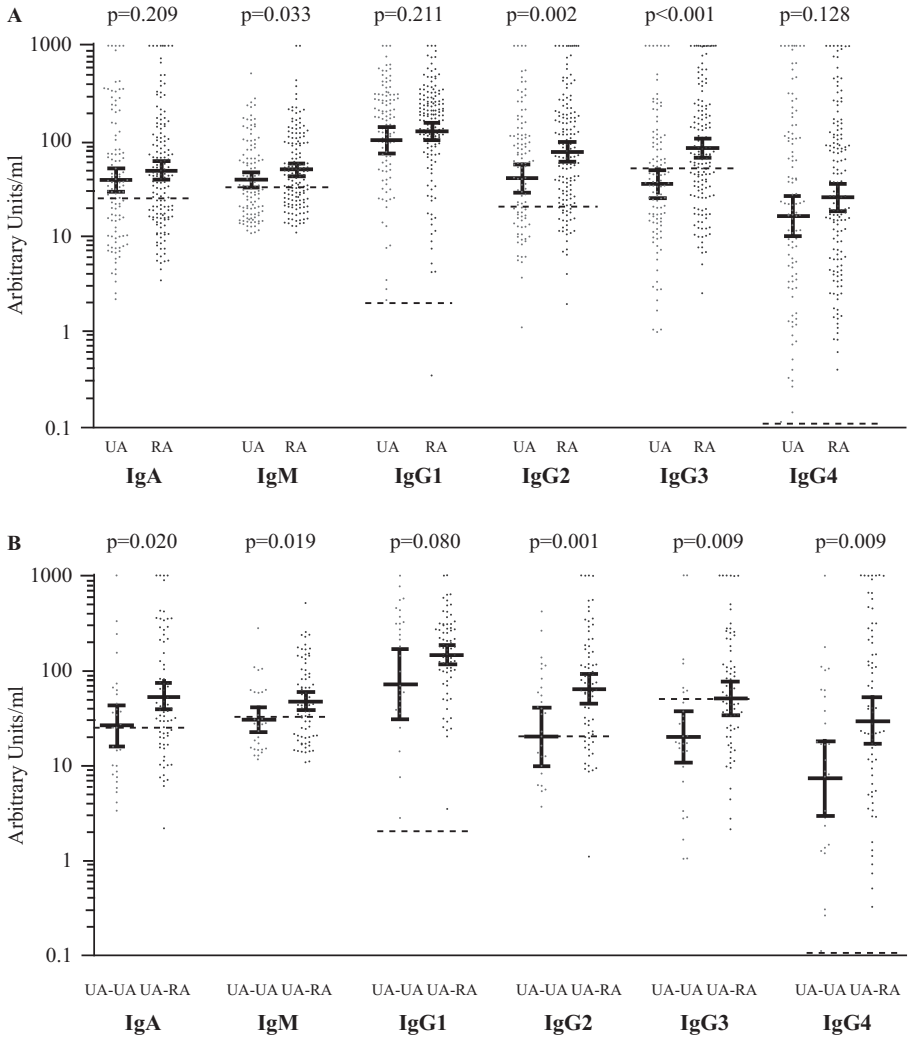

Figure 1. Dot plots showing baseline levels of anti–cyclic citrullinated peptide (anti-CCP) isotypes in **A**, 110 IgG anti-CCP–positive patients with undifferentiated arthritis (UA) and 152 IgG anti-CCP–positive patients with rheumatoid arthritis (RA) and **B**, 29 IgG anti-CCP–positive patients with UA who still had a diagnosis of UA after 1 year (UA–UA) and 74 IgG anti-CCP–positive patients with UA in whom RA developed within 1 year (UA–RA). P values for the group differences in mean log-transformed values (arbitrary units/ml) were calculated. When only patients who were positive for the respective isotypes of anti-CCP were analyzed, the P values for RA versus UA were 0.75 for IgA, 0.67 for IgM, 0.39 for IgG1, 0.27 for IgG2, 0.36 for IgG3, and 0.31 for IgG4; for UA–UA versus UA–RA, the respective P values

IgA, IgM, IgG2, and IgG3 anti-CCP were present at higher frequencies in the UA→RA patients than in the UA→UA patients (P=0.03, P=0.01, P=0.03, and P=0.01, respectively) (Table 2). Among UA→UA patients, a median of 3 isotypes were used in the anti-CCP response, compared with a median of 5 among UA→RA patients (P=0.004); this result served as another indication of more extensive anti-CCP isotype usage in UA→RA patients. A higher risk for the development of RA within 1 year of followup was observed in patients with UA who were positive for IgA anti-CCP (RR 1.3, 95% confidence interval [95% CI] 1.00–1.7), IgM anti-CCP (RR 1.4, 95% CI 1.1–1.8), or IgG3 anti-CCP (RR 1.4, 95% CI 1.11–1.8).

A trend toward higher levels of all isotypes of anti-CCP except IgG1 was observed in UA→RA patients compared with UA→UA patients, when all samples were taken into consideration (Figure 1B). When only those patients who were positive for a respective isotype were considered, only the levels of IgG4 anti-CCP were higher in UA→RA patients (P=0.007).

Taken together, these results show that at the population level, the anti-CCP response in anti-CCP–positive patients with UA in whom RA was not diagnosed within 1 year was less diverse with respect to isotype usage compared with the response in patients in whom RA did develop, and that levels of most isotypes of anti-CCP were similar in both patient groups.

Changes in anti-CCP isotypes in patients with RA, after years of followup

It has been shown for several antigens that repeated antigen exposure results in higher levels of antibodies of the IgG4 subclass [14]. Because RA is a chronic disease and the autoantigens are expected to be present throughout the disease process [15], we hypothesized that after years of arthritic episodes, higher levels of IgG4 anti-CCP would have developed in patients with RA. Therefore, we next investigated whether the pattern of isotype usage changed during disease progression or whether the presence and/or levels of different anti-CCP isotypes remained relatively stable over time. To this end, IgA, IgM, and IgG subclasses of anti-CCP in 64 IgG anti-CCP–positive patients with RA were determined at baseline and after a median of 7 years of followup.

were 0.47, 0.63, 0.23, 0.06, 0.90, and 0.01. The number of arbitrary units/ml for one isotype is not comparable with the number of arbitrary units/ml for another isotype. Bars show the geometric means and 95% confidence intervals. Broken lines indicate the cutoff values.

Table 2. Anti-CCP isotypes in IgG anti-CCP–positive patients with UA, according to the development of RA within 1 year*

Anti-CCP isotype	UA→UA (n=29)	UA→RA (n=74)	RR (95% CI)†	P‡
IgA				
Positive	13 (45)	50 (68)	1.3 (1.00 – 1.7)	0.03
Negative	16 (55)	24 (32)		
IgM				
Positive	9 (31)	43 (58)	1.4 (1.1 – 1.8)	0.01
Negative	20 (69)	31 (42)		
IgG1				
Positive	27 (93)	74 (100)	–	0.08
Negative	2 (7)	0 (0)		
IgG2				
Positive	16 (55)	57 (77)	1.4 (0.98 – 1.9)	0.03
Negative	13 (45)	17 (23)		
IgG3				
Positive	6 (21)	37 (50)	1.4 (1.11 – 1.8)	0.01
Negative	23 (79)	37 (50)		
IgG4				
Positive	27 (93)	71 (96)	1.2 (0.58 – 2.5)	0.62
Negative	2 (7)	3 (4)		

* Values are the number (%). Anti-CCP=anti–cyclic citrullinated peptide; UA=undifferentiated arthritis; RA=rheumatoid arthritis; UA→UA=patients with UA in whom RA did not develop within 1 year; UA→RA=patients with UA in whom RA developed within 1 year; RR=relative risk; 95% CI=95% confidence interval.
† Positive versus negative for the different anti-CCP isotypes.
‡ By chi-square test.

At the time of followup, the proportions of patients who were positive for IgA and IgG3 had decreased (P=0.012 and P=0.007, respectively) (Table 3), whereas no relevant changes were detected for IgM, IgG1, IgG2, and IgG4. A median of 5 isotypes were used at baseline, and a median of 4 were used at followup (P=0.003). Among patients who were positive for a specific isotype of anti-CCP, a trend toward a decreased level of that isotype was observed after followup, for all isotypes except IgG1 (Figure 2 and Table 3). Thus, isotype usage in general (and IgG4 anti-CCP in particular) had

not further increased during a median followup period of 7 years. Instead, the levels and extensiveness of isotype usage had declined during this period.

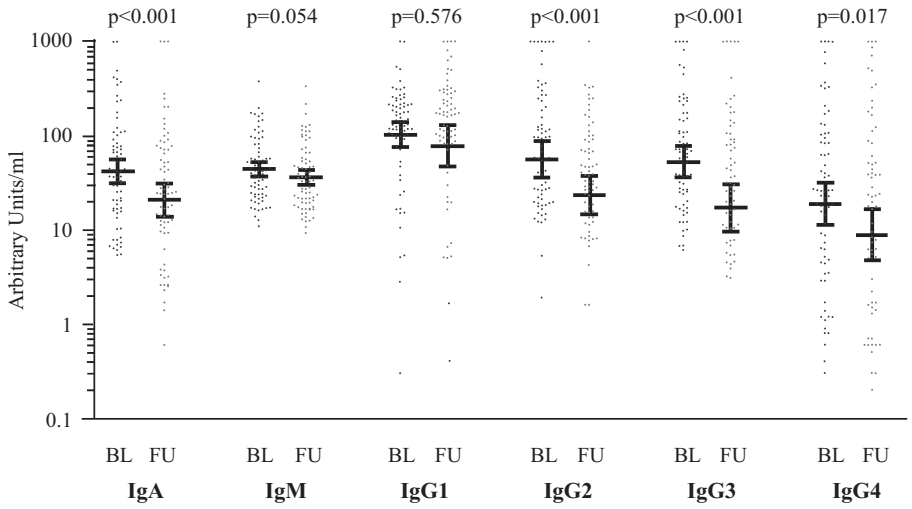

Figure 2. Dot plots showing the levels of anti-CCP isotypes in paired serum samples obtained from 64 IgG anti-CCP–positive patients with RA at baseline (BL) and after a median of 7 years of followup (FU). P values were calculated for paired-sample *t*-tests on log-transformed values (arbitrary units/ml). The number of arbitrary units/ml for one isotype is not comparable with the number of arbitrary units/ml for another isotype. Bars show the geometric means and 95% confidence intervals. See Figure 1 for other definitions.

Triggering of new B–cells after followup, as indicated by the appearance of IgM anti-CCP

The presence of IgM anti-CCP in the setting of UA, early RA, and established RA suggests a constant recruitment of new anti-CCP–producing B–cells from naive precursors. To substantiate this notion more accurately, we investigated whether IgG anti-CCP–positive patients who were negative for IgM anti-CCP at baseline became IgM positive at followup. Although the presence or absence of IgM anti-CCP seemed to be a relatively stable phenotype in two-thirds of the patients, 8 of the 23 patients who did not have IgM anti-CCP at baseline did display IgM anti-CCP at followup (Table 3 and Figure 3). Because switched B–cells are unable to return to IgM production,

the data showed that new B–cells can be recruited into the anti-CCP response, further indicating that the immune reaction responsible for the production of anti-CCP is still ongoing in patients with established RA. Similar results were observed for IgA, IgG2, IgG3, and IgG4 anti-CCP, for which 4 patients, 7 patients, 5 patients, and 1 patient, respectively, were negative at baseline and positive at followup (Table 3 and Figure 3), again indicating an ongoing immune response in these patients.

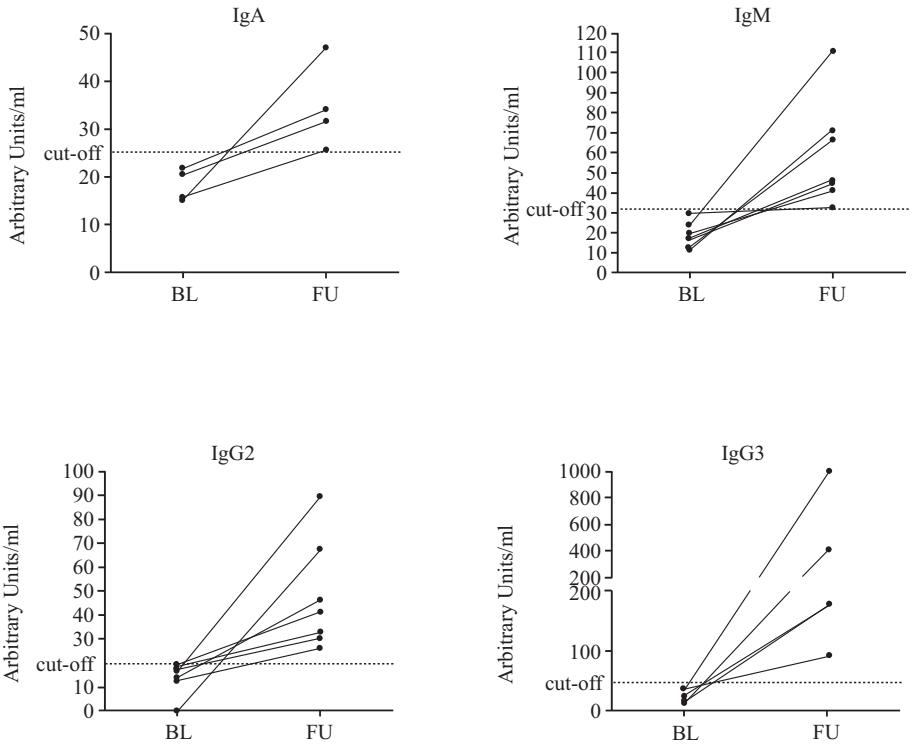

Figure 3. Levels of isotypes in IgG anti–cyclic citrullinated peptide (anti-CCP)–positive patients who were negative at baseline (BL) and positive after a median of 7 years of followup (FU) for IgA, IgM, IgG2, and/or IgG3 anti-CCP. For both IgG1 and IgG4 anti-CCP, only 1 patient changed from negative to positive (results not shown).

Table 3. Changes in the presence and levels of anti-CCP isotypes in paired serum samples obtained from 64 patients with RA at baseline and after a mean followup of 7 years*

	Anti-CCP isotype status at baseline/followup					Median level in anti-CCP–positive patients, AU/ml		
Isotype	**+/+**	**+/–**	**–/–**	**–/+**	**P†**	**Baseline**	**Followup**	**P‡**
IgA	26	16	18	4	0.012	67	47	0.002
IgM	28	13	15	8	0.275	56	46	0.015
IgG1	60	3	0	1	0.625	134	165	0.530
IgG2	36	14	7	7	0.189	98	40	<0.001
IgG3	18	19	22	5	0.007	144	49	<0.001
IgG4	58	4	1	1	0.375	26	18	0.059

* Except where indicated otherwise, values are the number of patients. Anti-CCP=anti–cyclic citrullinated peptide; RA=rheumatoid arthritis; AU=arbitrary units.
† By McNemar's test, baseline versus followup.
‡ By Wilcoxon's signed rank test, baseline versus followup.

Discussion

In this study, we analyzed the presence and levels of IgM, IgA, and subclasses of IgG anti-CCP in patients with RA and patients with UA, in order to determine whether the RA-specific anti-CCP response is stable, early and later in the course of RA. Furthermore, we attempted to determine whether the anti-CCP response reflects an ongoing immune reaction in which newly activated B–cells are recruited.

The reported results indicate a more diverse pattern of isotype usage of anti-CCP antibodies in patients with RA compared with patients with UA (median 5 versus 4 isotypes; P=0.007), with a higher prevalence of IgM, IgG2, and IgG3 anti-CCP in serum samples obtained from patients with RA compared with baseline serum samples obtained from patients with UA. Patients with UA in whom RA developed within 1 year of followup (UA→RA) displayed a more diverse pattern of isotypes of anti-CCP than did patients with UA in whom RA did not develop within 1 year (UA→UA) (median 5 versus 3 isotypes; P=0.004). These results suggest that early during the course of disease progression from UA to RA, isotype switching is occurring. Alternatively, these data suggest that disease that is more severe at the time of onset, as reflected by earlier fulfillment of more of the ACR criteria for RA, is accompanied by a more diverse pattern of anti-CCP antibody isotypes.

IgM responses against T cell-dependent antigens are, in general, not continuously present. In the setting of vaccinations, for example, levels of antigen-specific IgM increased and decreased during the weeks after a primary or a booster immunization against rabies [5]. Similarly, 10 weeks after vaccination, the proportion of measles-specific IgM–positive individuals dropped to <10% [16]. On the basis of the nature of the antigen (i.e., protein) and the association between the presence of anti-CCP antibodies with HLA [17], it is likely that the anti-CCP B cell response is T cell dependent. Intriguingly, even though IgG anti-CCP has been detected years before the first symptoms of arthritis [18,19], our data showed the presence of IgM anti-CCP in a considerably large proportion of early samples from IgG anti-CCP–positive patients with UA or RA as well as in followup samples from IgG anti-CCP–positive patients with RA. Additional regression analyses did not reveal a correlation between the duration of symptoms at baseline or the duration of followup and the presence or absence of IgM anti-CCP at either or both time points (data not shown), indicating that these time intervals did not influence the (change in) presence of IgM anti-CCP. Because IgM antibodies have a half-life of only ~5 days [6], and long-lived plasma cells producing IgM antibodies or IgM memory B–cells against T cell-dependent antigens have not been described, the presence of IgM most likely reflects the presence of recently activated IgM-producing B–cells. We additionally observed that in some IgG anti-CCP–positive patients with RA in whom no IgM anti-CCP was detectable at baseline, IgM anti-CCP was present 7 years later. Taken together, these results are important, because they indicate that novel IgM-producing B–cells are continuously recruited to the anti-CCP response, demonstrating that the anti-CCP response is continuously reactivated during the course of arthritis.

Although the presence of IgG4 anti-CCP has been described previously [20], the observation that IgG4 anti-CCP antibodies were detected at a lower frequency after long-term followup of patients with RA is of interest, because it was hypothesized that patients with RA of long duration would demonstrate a higher frequency of IgG4 anti-CCP antibodies. IgG4 is expressed predominantly under conditions of long-term exposure to protein antigens; this is well illustrated by the longitudinal analysis of the antibody response to bee venom in beekeepers [14] and by the hyposensitization protocols performed in patients with allergies (for review, see ref. 21). Consistent with these observations, the finding that the frequency of IgG4-positive patients with UA or RA was relatively high at the first visit to the EAC could indicate that long-term exposure of autoreactive B–cells to citrullinated antigens already occurred early in the course of symptomatic disease.

After 7 years of followup, not only had the presence and levels of IgG4 anti-CCP decreased, but the levels of the other isotypes (except IgG1) also had decreased. A possible cause of this limited anti-CCP isotype usage at a later time point could be treatment with immunosuppressive medication. However, given the fact that all patients with RA were receiving treatment when the followup serum samples were obtained, the impact of treatment in the present study cohort is difficult to determine and will be the subject of further investigation.

As in other autoimmune diseases, the isotypes of autoantibodies may be of prognostic value. For example, IgG1 and IgG3 isotypes of islet cell autoantibodies in prediabetic children have been associated with progression to type 1 diabetes mellitus (for review, see ref. 22), while IgG4 and IgE autoantibodies have been associated with protection against type 1 diabetes mellitus [23,24]. Similar associations with progression from UA to RA and the presence of 1 or 2 particular isotypes of anti-CCP in patients with UA were not observed in this study (Table 1), although patients with UA who were harboring ≥4 different isotypes displayed a 1.4-fold higher risk for the development of RA within 1 year in comparison with patients who harbored ≤3 isotypes (95% CI 1.01–1.80; data not shown).

Considering the number of hypotheses tested, it can be argued that a correction for multiple testing should be performed. The presence and levels of the different isotypes, however, are not independent phenotypes (data not shown), and the hypotheses tested are far from independent. Determining the correct adjustment strategy is, therefore, not straightforward. Because the main conclusions in this study are drawn from a collection of observations rather than from single hypotheses tested, we chose to mention P values without adding a specific label to the level of significance, and we wish to mention that a P value less than 0.05 may not be statistically significant in the context of a single observation.

In conclusion, the presence of IgM anti-CCP in early serum samples obtained from both patients with UA and patients with RA and in followup samples obtained from patients with RA suggests an ongoing activation of new clones of anti-CCP–producing B–cells. This notion is further supported by the observation that IgG anti-CCP–positive patients who do not display IgM anti-CCP can convert to IgM anti-CCP positivity later in the course of arthritis. Furthermore, relatively extensive isotype usage in the anti-CCP response was detected in patients with recent-onset arthritis. Taken together, these data indicate that full usage of the isotype repertoire occurs early in the course of arthritis, and that a continuous (re)activation of the RA-specific anti-CCP antibody response occurs during the disease course.

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Chapter 9

Fine-specificity of the ACPA response is influenced by shared epitope alleles

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Abstract

Objective

In classic studies on the genetic background of antibody production, the major histocompatibility complex (MHC) has been shown to act as the most prominent immune response gene that controls the magnitude and the specificity of antibody production. The strongest genetic risk factor for rheumatoid arthritis (RA), the human MHC HLA–DRB1 shared epitope (SE) alleles, predisposes for antibodies against citrullinated proteins (ACPAs). ACPA levels are higher in SE-positive patients with RA than in SE-negative patients with RA. The aim of the present study was to determine whether SE influences not only the magnitude but also the specificity of the ACPA response.

Methods

In 2 cohorts of anti–citrullinated peptide 2–positive patients with RA, one from a study of recent-onset arthritis (n=206) and the other from a treatment strategy study (n=141), serum antibodies against a citrullinated peptide derived from vimentin (cVim) and antibodies against a citrullinated fibrinogen peptide (cFibr) were determined by enzyme-linked immunosorbent assay. HLA–DRB1 genotyping was performed.

Results

In the first cohort, SE alleles were significantly associated with the presence of antibodies against cVim (odds ratio [OR] 4.95, 95% confidence interval [95% CI] 1.87–15.3) and were not significantly associated with the presence of antibodies against cFibr (OR 1.71, 95% CI 0.70–4.14). These results were replicated in the second cohort (OR 5.05, 95% CI 1.92–13.6 and OR 1.19, 95% CI 0.30–3.97, respectively).

Conclusion

In 2 cohorts of ACPA-positive patients with RA, SE alleles predisposed for the development of antibodies against cVim but not for the development of antibodies against cFibr. These data indicate that SE alleles act as "classic" immune response genes in the ACPA response, because they influence both the magnitude and the specificity of this RA-specific antibody response.

Introduction

The most prominent genetic risk factors for rheumatoid arthritis (RA), the HLA–DRB1 shared epitope (SE) alleles, encode for a common amino acid sequence in the peptide-presenting part of the HLA class II molecule. These SE alleles have been described recently to be a risk factor for the development of antibodies against citrullinated proteins (ACPAs) rather than the development of RA [1,2].

ACPAs have been postulated to play a pivotal role in the progression of RA, because they are highly specific and predictive for RA [3,4], are associated with the extent of joint destruction [5], and have been shown to enhance disease severity in mice with experimental arthritis [6]. It has been shown that ACPAs recognize a variety of citrullinated antigens, including citrullinated fibrinogen and citrullinated vimentin, which is also known as the Sa antigen [7]. However, not all ACPA-positive sera will recognize all citrullinated antigens, as has been shown by analyzing the reactivity against different citrullinated peptide antigens [8].

In classic studies of the genetic determinants that influence antibody production in mice, a region (the immune response [Ir-1] region) that controlled the magnitude and specificity of antibody production was found (for review, see ref. 9). Because the magnitude of the antibody response in first-generation offspring of parents producing high levels of antibodies and first-generation offspring of parents producing low levels of antibodies was comparable with the magnitude of response in the parent producing high levels of antibodies [10], it was concluded that this region influenced antibody production in a dominant manner. Moreover, it was observed that the ability of an animal to generate a proper antibody response against different model antigens strictly depended on the genetic variant located in the Ir-1 region, denoting that the immune response genes control antibody responses to different antigens [10,11]. Subsequently, the Ir-1 region was found to be similar to the H-2 region [12], the major histocompatibility complex region in mice that was originally identified by skin transplantation experiments. The human analog of this region is the HLA region.

In analogy with these classic studies, we recently reported that among ACPA-positive patients with RA, those without HLA–DRB1 SE alleles displayed lower levels of ACPA than did patients with 1 or 2 SE alleles [2]. The number of SE alleles carried by patients did not influence the ACPA levels, indicating a dominant effect of the SE alleles on the level of circulating antibodies. The present study was designed to

determine whether the specificity of the ACPA response, in addition to the magnitude of the response, is influenced by the presence of the HLA–DRB1 SE in patients with RA.

Patients and methods

Study population

The patients who were analyzed in this study were derived from the Leiden Early Arthritis Clinic (EAC) cohort (n=206) and from the BeSt (Behandelstrategieën) study (n=141). The Leiden EAC is an inception cohort of patients with recent-onset arthritis (symptom 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 van Aken et al [13]. The BeSt study is a multicenter, randomized, controlled trial designed to compare the clinical efficacy and radiologic outcomes of 4 different treatment strategies in patients with early-onset RA [14].

All patients fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) 1987 revised criteria for the classification of RA [15] within 1 year of followup (EAC cohort) or at the time of inclusion (BeSt study). In the EAC cohort, 57% of the patients were ACPA positive. In the BeSt study, 61% of the patients were ACPA positive. Only patients who were positive for ACPA and for whom results of HLA–DRB1 genotyping were available were analyzed in this study.

ACPAs

The anti–cyclic citrullinated peptide 2 (anti-CCP-2) test (Immunoscan RA Mark 2; Euro-Diagnostica, Arnhem, The Netherlands) was used to determine whether patients were ACPA positive. A cutoff value of 25 units/ml was used, as described in the manufacturer's instructions.

Antibodies against both the citrullinated and the uncitrullinated form of a linear peptide derived from vimentin (VYATCitSSAVCitLCitSSVP (cVim) and VYATRSSAVRLRSSVP) and of a linear peptide derived from fibrinogen (NEEGFFSACitGHRPLDKK (cFibr) and NEEGFFSARGHRPLDKK) were determined by enzyme-linked immunosorbent assay (ELISA). These peptides were selected from 2 sets of synthetic peptides that were generated; 1 was derived from the amino acid

sequence of human fibrinogen α-chain and β-chain, and 1 was derived from that of human vimentin. The peptides that were synthesized contained at least 1 citrulline at a position of an arginine in the amino acid sequence of the respective proteins. The recognition of these peptides by several sera from patients with RA was determined, after which the peptides that were most frequently reactive with RA sera were selected for our study. For both of these peptides (cVim and cFibr), the corresponding "arginine variant" was synthesized as well and was used in parallel for the analyses. The specificity of the cVim and cFibr ELISAs was established by analysis of their recognition by 30 normal human sera as well as sera from 50 anti-CCP–negative patients with RA. Only 1 of these sera showed very low reactivity against cFibr (data not shown).

A signal higher than the mean optical density (OD) plus 2 SD for serum samples from 5 healthy control subjects that were included on each plate was considered positive. Citrulline-specific signals were defined as a positive signal against the citrullinated peptide and a negative signal against the uncitrullinated peptide, with a minimum difference of an OD value of 0.1. When a sample had a positive signal against both the citrullinated peptide and the uncitrullinated peptide, the sample was excluded from analyses, which was the case for 30 samples against cFibr (12 from the EAC cohort and 18 from the BeSt cohort) and 23 samples against cVim (5 from the EAC cohort and 18 from the BeSt cohort).

Microtiter plates were coated with 10 μg/ml peptide diluted in phosphate buffered saline (PBS)/0.1% bovine serum albumin (BSA) at 4°C overnight. The coated plates were incubated with serum samples (100 μl/well) for 1 hour (diluted 100-fold in PBS/1% BSA/0.05% Tween 20). This and the subsequent incubation step were performed at 37°C in a humidified atmosphere and were followed by washing steps with PBS/0.05% Tween 20. Antibodies were detected after incubation for 1 hour with 100 μl/well rabbit anti-human IgG horseradish peroxidase-conjugated antibody (P0214; Dako, Glostrup, Denmark) (diluted 1:10,000 in PBS/1% BSA/0.05% Tween 20). Bound antibodies were visualized using 100 μl/well 3,3',5,5'-tetramethylbenzidine solution (1:1 ratio) mixed with ureumperoxide as a substrate, followed by 100 μl/well 2M H2SO4 10 minutes later to stop the staining reaction. OD values were measured using an ELISA reader at 450 nm.

HLA genotyping

The HLA–DRB1 (sub)typing was performed using a polymerase chain reaction with specific primers and hybridization with sequence-specific oligonucleotides, as previously described [16]. The SE alleles are DRB1*0101, *0102, *0104, *0401, *0404, *0405, *0408, *1001, and *1402.

Statistical analysis

Odds ratios (ORs) were calculated for the relative proportions of patients with antibodies (anti-cVim or anti-cFibr) among the SE-positive patients compared with the patients without SE alleles. First, the EAC cohort was analyzed. To replicate the data, ORs were calculated for the BeSt study. Subsequently, data from the 2 cohorts were pooled and analyzed with the help of a chi-square test to detect differences in proportions, to provide insight into the robustness of the results. ORs were reported with 95% confidence intervals (95% CIs), which excluded the value of 1 in case of statistical significance.

Results

To investigate whether SE alleles are associated with the specificity of the ACPA response, the presence of antibodies against 2 citrullinated peptides derived from vimentin (cVim) and fibrinogen (cFibr) was determined in serum samples obtained from ACPA-positive patients with RA. These peptides were selected from a panel of vimentin-derived and fibrinogen-derived peptides based on the relatively high frequency of recognition by antibodies from patients with RA.

Among ACPA-positive patients with RA derived from the Leiden EAC cohort, 39% displayed antibodies against both cVim and cFibr, 7% displayed antibodies against only cVim, 36% displayed antibodies against only cFibr, and 19% had no antibodies against either cVim or cFibr. SE alleles were significantly associated with the presence of antibodies against cVim (OR 4.95, 95% CI 1.87–15.3) and not with the presence of antibodies against cFibr (OR 1.71, 95% CI 0.70–4.14) (Table 1). These data indicated a contribution of the HLA–DRB1 SE alleles in determining the fine specificity of the ACPA response.

To confirm and replicate these data, we subsequently performed similar analyses in another patient group consisting of ACPA-positive patients with RA who

were derived from the BeSt study. Among these patients, 55% displayed antibodies against both cVim and cFibr, 2% displayed antibodies against only cVim, 28% displayed antibodies against only cFibr, and 15% had no antibodies against either cVim or cFibr. Comparable with what was observed in patients from the EAC cohort, in patients from the BeSt study, the presence of SE alleles was associated with the presence of antibodies against cVim (OR 5.05, 95% CI 1.92–13.6) and not with the presence of antibodies against cFibr (OR 1.19, 95% CI 0.30–3.97) (Table 1).

An analysis of both cohorts as a single group of patients in order to evaluate the robustness of the results yielded highly significantly more frequent detection of anti-cVim in patients with SE alleles (OR 4.44, 95% CI 2.28–8.75, $P<10^{-6}$) and no significantly higher frequency of anti-cFibr in SE-positive patients (OR 1.39, 95% CI 0.69–2.78, P=0.31). Taken together, these findings indicate that SE alleles influence the specificity of the ACPA response.

Table 1. Presence or absence of antibodies against a citrullinated vimentin peptide (anti-cVim) and against a citrullinated fibrinogen peptide (anti-cFibr) in ACPA-positive patients with RA, according to SE status*

	anti-cVim			anti-cFibr		
Cohort	**positive**	**negative**	**OR (95% CI)**	**positive**	**negative**	**OR (95% CI)**
EAC						
SE positive	86	81		124	38	
			4.95 (1.87 – 15.3)			1.71 (0.70 – 4.14)
SE negative	6	28		21	11	
BeSt						
SE positive	62	30		80	14	
			5.05 (1.92 – 13.6)			1.19 (0.30 – 3.97)
SE negative	9	22		24	5	

* Values are the number of patients. Samples that tested positive against both the citrullinated and uncitrullinated control peptide were excluded from the analyses. ACPA=anti-citrullinated protein antibody; RA=rheumatoid arthritis; SE=shared epitope; OR=odds ratio; 95% CI=95% confidence interval; EAC=Early Arthritis Clinic; BeSt=Behandelstrategieën.

Discussion

Patients displaying antibodies to the citrullinated CCP2 peptide, a peptide that was selected for its ability to identify RA patients with a high sensitivity and specificity, not all recognize other citrullinated peptides to the same degree. In this study we analyzed whether ACPA (anti-CCP2)–positive RA patients with and without SE–alleles, differ in their recognition of two different citrullinated peptides (cVim and cFibr). In two separate cohorts, the presence of SE-alleles was significantly associated with the presence of antibodies against cVim and not with the presence of antibodies against a cFibr, ($P<10^{-6}$ and $P=0.31$, respectively when analyzed together)

SE–alleles have been demonstrated to be a risk factor for the development of ACPA. Within patients displaying ACPA, the level of ACPA has been reported to be higher in patients with SE–alleles [2]. Although only two citrullinated peptides were analyzed in this study, our data are of interest as they show that SE–alleles are not only associated with the magnitude but also with the fine-specificity of the ACPA response. Together these data indicate that SE–alleles act like "classic" immune response genes in the ACPA response.

The observation described above is also of interest as it points to the possibility that a peptide derived from vimentin, or a protein physically linked or structurally related to vimentin is presented to T–cells that are restricted by the HLA–DRB1 SE–alleles (or the HLA–DQ alleles that are genetically linked to the SE–alleles). In this model, citrullinated vimentin/protein complexes presented on, for example, apoptotic cells, could be recognized by citrulline-specific B–cells that would subsequently internalize this complex, process it and present peptides from vimentin(-coupled protein) to T–cells. These T–cells could then provide help to these B–cells, eventually resulting in the production of ACPA. Although this scenario is highly speculative, it is intriguing to note that vimentin is a protein that is known to be citrullinated during apoptosis and is expressed on apoptotic cells [17]. By that means, it may become visible for citrullinated-vimentin-specific B–cells.

In conclusion, in two cohorts of ACPA positive RA patients, SE–alleles predispose for the development of antibodies against a citrullinated vimentin peptide, and not for the development of antibodies against a citrullinated fibrinogen peptide. SE-alleles thereby act as "classic" immune response genes in the ACPA response, since they influence the magnitude as well as the fine-specificity of this RA specific antibody response.

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Chapter 10

Summarizing discussion

Characteristics of the ACPA response in RA and UA and during different stages of disease

RA often is difficult to diagnose at the onset of first symptoms and the current classification criteria were not designed to be used as diagnostic criteria in early disease. Many arthritis patients who eventually will be diagnosed with RA, as a consequence, are defined as having UA at the time they present themselves to the rheumatologist. The prognosis of patients with UA may vary from self-limited to severe destructive disease. The disease outcome of patients who present with UA and develop RA within one year is however the same as that of patients who present with RA. They display similar radiographic progression, disease activity and functional capacity after follow-up [1]. Because early aggressive treatment might offer an effective means to slow disease progression in RA [2;3] and because MTX treatment has been shown to be able to postpone the diagnosis of RA and to retard radiographic joint damage [4], it is important to identify UA patients who will develop RA and treat them as early as possible. At the same time, inappropriate treatment of patients with a more benign disease course could then be avoided.

To give insight in the amount of patients developing RA after having presented with UA, this thesis starts with a review of the literature on this matter of a priori chances. The percentage of patients developing RA is highly determined by the definition of UA at the time a patient first presents to the rheumatologist and the definition of RA. In general, when UA is defined as arthritis with the potential to persist, but without a recognized clinical pattern, 17% to 32% of the patients develop RA according to the 1987 ACR classification criteria (**Chapter 2**).

Antibodies against citrullinated peptides are reported to be predictive for the development of RA in patients with UA. In the Leiden early arthritis clinic, 93% of ACPA–positive recent-onset UA patients had developed RA after 3 years of follow-up, whereas only 25% of ACPA–negative UA patients had developed RA during the first 3 years [5]. This knowledge probably affects interventions. However, it has to be considered that the pre-test probability, and thus the positive predictive value of ACPA–positivity to develop a chronic destructive disease is most likely different in healthy persons, patients with limited disease (e.g. UA patients) and patients that fulfil the ACR criteria for RA, whereas the underlying biology of breaking tolerance to citrullinated antigens has occurred in all three categories of individuals. A much more precise prediction rule that was designed to guide treatment decisions, apart

from ACPA included also known risk factors and clinical characteristics and was published in 2007 [6].

ACPA are thought to play a pivotal role in the development and/or progression of RA because they are highly specific for RA [7], precede the development of RA [8;9] and are associated with the extent of joint destruction [10]. In a mouse model, ACPA have even been described to enhance experimental arthritis [11]. Insight into the ACPA response itself may contribute to our understanding of the possible role of ACPA in the pathogenesis of RA. In **chapter 8,** the occurrence of different isotypes of ACPA is described in different groups of ACPA–positive patients. Early serum samples from both UA and RA patients and follow-up samples from RA patients were analyzed. In general, a more diverse usage of different isotypes of ACPA was detected in serum samples taken early after symptom onset from ACPA–positive RA patients compared to ACPA–positive UA patients. UA patients who developed RA within one year of follow-up displayed more different isotypes of ACPA than UA patients who did not develop RA within that first year. Although it is not formally demonstrated with serial serum samples, these results may suggest that early during the course of disease progression from UA to RA, isotype switching is occurring. Alternatively, a disease that is more severe at the time of onset, as reflected by earlier fulfillment of more of the ACR criteria for RA, is accompanied by a response with a more diverse pattern of ACPA isotypes. This more diverse pattern of isotypes possibly reflects a more active immune response. At the very least it shows a marked difference on the immunological level between UA and RA patients. Furthermore, this difference in ACPA response between UA and RA and the suggestion that isotype-switching occurs during the disease progression from UA to RA may be linked with the fact that patients should be treated early in the disease course.

After 7 years of follow-up, the diversity of isotype-usage had decreased in sera from RA patients. This observation could be a result of treatment with anti-inflammatory drugs. Decreases of ACPA levels after various treatment have been reported and are possibly associated with good clinical response [12-15]. Alternatively, a decreased diversity of isotype-usage could reflect maturation towards one certain pattern of isotype usage in the ACPA response. A single pattern of isotype usage could however not be detected; many combinations were possible. IgG1 and IgG4 were the most frequently detected isotypes of ACPA. IgG1 was present in almost all IgG ACPA–positive patients, which is consistent with the fact that T cell dependent antigens like peptides induce an antibody response that is mainly of the IgG1 isotype

[16;17]. A T–cell dependent antibody response is also indicated by the strong association of the presence of ACPA with HLA.

Another lesson from **chapter 8** is that IgM ACPA could be detected in early serum samples of IgG ACPA–positive UA and RA patients, as well as later in the disease course of IgG ACPA–positive RA patients. In RA samples drawn after 7 years of follow-up, IgM ACPA could even be detected in IgG ACPA–positive patients who did not display IgM ACPA in an earlier sample at baseline. Because IgM cannot be produced by B–cells that first were triggered to produce another isotype of antibody, these results indicate that novel IgM–producing B–cells are recruited to a continuously reactivated ACPA response during the course of ACPA-positive RA.

At present, it is unknown which citrullinated antigens the anti-citrulline response is (initially) directed against, or which specificity of antibody may have pathogenic potential. In the inflamed joint, several citrullinated proteins have been detected, for instance citrullinated fibrin [18] and vimentin [19]. In **chapter 9** the responses against a citrullinated vimentin peptide and a citrullinated fibrinogen peptide were investigated in sera that were known to contain antibodies recognizing the synthetic CCP2 peptide. These serum samples recognized none, one or both antigens. As will be discussed below, the presence of antibodies against the citrullinated vimentin peptide was associated with the presence of SE alleles. From these results it was hypothesized that vimentin, or a protein physically linked to vimentin, is recognized by citrulline specific B–cells that internalize the complex, process it and present the peptides to T–cells that are restricted to SE. These T–cells subsequently provide help to the B–cells, resulting in the production of ACPA. Antibodies against citrullinated fibrinogen may in this model be the result of, for example, cross reactivity or epitope spreading, which was supported by the following observation (data not shown in earlier chapters):

Serum samples were obtained on different time-points after the first visit to the Leiden early arthritis clinic. The presence of antibodies against the citrullinated vimentin peptide and the citrullinated fibrinogen peptide could consequently be analyzed in relation to "time between first visit and serum sampling". It was found that the samples recognizing none of the peptides had the shortest time interval from first visit; those recognizing both displayed the largest time interval. Although a Kruskal-Wallis test could not significantly identify a difference in time interval between the 4 groups (recognizing none, the citrullinated fibrinogen peptide only, the citrullinated

vimentin peptide only or both), a difference was detected between sera recognizing the citrullinated fibrinogen peptide versus those that did not (Mann Whitney U-test, P=0.04). Patients recognizing the citrullinated fibrinogen peptide displayed longer disease duration than those who did not. A similar difference could not be detected between patients who did or did not recognize the citrullinated vimentin peptide. These data may suggest that antibodies against citrullinated vimentin are produced earlier in the disease course of ACPA–positive arthritis, and that later in time an ACPA response against citrullinated fibrinogen develops.

More information from longitudinal serum samples is needed to be able to draw more robust conclusions on the dynamics of isotype changes, avidity maturation and fine-specificity of the ACPA response through time in relation to disease stages, disease activity, treatment and chronicity of the disease. Ideally, for this purpose, serial serum samples collected from the moment before the ACPA-response is initiated and before the first joint complaints express themselves would provide crucial information on the development of the ACPA response and the aspects involved in successive steps in this autoantibody response. A study population that consists of healthy ACPA–positive individuals and diseased ACPA–positive patients became available for research recently and may reveal new information with respect to differences in the ACPA response between health and disease and with respect to changes through time.

ACPA-positive and ACPA-negative disease

In **chapter 3** the aim of the study was to determine whether RA patients with ACPA are different from those who are ACPA–negative, with regard to certain aspects of the disease. It was observed that the clinical presentation of both groups of patients was not different. Neither the reported first symptoms, nor the signs found in physical examination at initial presentation differed between ACPA–positive and ACPA–negative patients. In both groups, symptoms started with pain and swelling, predominantly symmetrical and in the small joints of the hands and feet.

The absence of a distinguishable clinical phenotype in ACPA–positive compared to ACPA–negative disease fits with the hypothesis described in **chapter 3** that one or more common triggers lead to arthritis in similar joints in ACPA–positive and ACPA–negative patients. Antigens may subsequently be citrullinated during

(subclinical) inflammation, promoting autoimmune ACPA formation in genetically predisposed and/or environmentally triggered individuals, which may result in an aggravated inflammation and more severe disease progression in these patients. On the other hand, if ACPA does have a more predominant role in the initiation of the disease process, the distribution of inflamed joints would not necessarily differ, as the mechanisms that determine which joints are affected more regularly than others are still unknown. The similar disease activity at onset can easily be explained by the urge to visit a physician when experiencing complaints being independent of the presence of antibodies, but very much dependent on disease activity. Thus, the clinical similarities between patients with and without ACPA do not exclude an initiating role for ACPA in the pathophysiology of ACPA–positive RA.

Although ACPA–positive and ACPA–negative patients present themselves with the same clinical symptoms, they are different with respect to several other characteristics. To start with, they differ in the severity of progression of the disease. For example, UA patients with ACPA more often develop RA than ACPA-negative patients do [5]. Furthermore, ACPA-positive RA patients display more joint damage on radiological scales [10;20-22]. Similarly, after follow-up in our study cohort ACPA–positive patients displayed a higher number of swollen joints and showed more radiological damage than ACPA–negative patients (**chapter 3**). The localization of joint swelling and radiological abnormalities however remained similar for both ACPA–positive and ACPA–negative RA.

Second, Risk factors for ACPA–positive and ACPA–negative RA differ. It has been shown that the most prominent genetic risk factor for RA, HLA–DRB1 SE, associates only with RA that is characterized by the presence of ACPA, and not with ACPA–negative RA ([23], **chapter 4**). This observation raised the question whether ACPA–negative RA is associated with HLA–DRB1 alleles other than SE alleles. In **chapter 5** the association of HLA–DR3 with ACPA–negative arthritis and not with ACPA–positive arthritis is described. Thus, distinct genetic risk factors are associated with distinct phenotypes of RA. Also with respect to environmental risk factors, ACPA–positive and ACPA–negative RA differ. Smoking is only a risk factor for ACPA–positive disease and, intriguingly, is so only in SE–positive individuals [24;25]. Obesity on the contrary has been reported to be a risk factor only for ACPA-negative RA [26].

Third, histological differences have been observed between synovial infiltrates obtained arthroscopically from patients with ACPA–positive and those from ACPA–

negative RA [27]. Synovial tissue from ACPA–positive patients was characterized by a higher mean number of infiltrating lymphocytes, less extensive fibrosis and a thinner synovial lining layer compared with synovial tissue from ACPA–negative patients.

Finally, the response to treatment may be different between ACPA–positive and ACPA–negative disease. In a trial on the efficacy of methotrexate (MTX) treatment in patients with UA, it was demonstrated that MTX treatment resulted in a postponed diagnosis of RA and in slower radiographic joint damage. Subgroup analysis revealed that these benefits of MTX were most pronounced in patients with ACPA and could not significantly be demonstrated in ACPA–negative patients [4]. A higher degree of efficacy in ACPA–positive RA has been reported in a study on rituximab treatment [28]. On the other hand, pre-treatment positivity for ACPA did not predict clinical response to TNF alpha inhibitors in another study [15]. Information on differences in response to treatment is relatively scarce and is difficult to interpret because of a disparate "natural" course of disease progression. More research on differential treatment for both groups of patients is therefore necessary and outcome measures in clinical trials should preferably be reported separately for ACPA–positive and ACPA–negative patients.

In conclusion, differences in long-term disease outcome and disease progression, differences in genetic risk factors, in environmental risk factors as well as differences in synovial infiltrates all indicate that ACPA–positive and ACPA–negative disease are distinct disease entities. ACPA–positive and ACPA–negative disease therefore possibly result from two different pathophysiological mechanisms, which eventually may indicate a difference in optimal treatment approach for both phenotypes.

HLA-DRB1 SE alleles and their relation with ACPA and smoking in RA

HLA–DRB1 SE alleles have been previously shown to be a risk factor only for ACPA–positive RA and not for RA without ACPA [23]. This observation raised the question whether SE alleles were associated with ACPA, rather than with RA. If SE alleles are indeed a risk factor for ACPA and not for RA, it is predicted that SE alleles do not increase the risk to develop RA in ACPA–positive or in ACPA–negative UA patients.

In **chapter 4**, the percentages of UA patients who did and who did not develop RA according to the ACR criteria during one year of follow-up were therefore compared between ACPA–positive and ACPA–negative patients. It was observed that SE alleles do not independently contribute to the progression from UA to RA, but rather contribute to the chance to be ACPA–positive. Furthermore, in **chapter 6** it is shown that not all SE alleles increase the risk on ACPA to the same degree. As expected because HLA–DRB1*0401 has been reported to display the strongest association with RA susceptibility and severity [29;30], this SE allele confers the highest risk for displaying ACPA (OR 5.8, 95% CI 3.2–10.6). Of note, Hill et al demonstrated that in mice transgenic for DRB1*0401, the citrullination of a vimentin peptide at a position interacting with the shared epitope significantly increased peptide-MHC affinity and resulted in the activation of CD4+ T–cells [31]. Possibly, DRB1*0401 alleles display a relatively high binding affinity for citrullinated peptides, resulting in more effective generation of immunity against citrullinated peptides and a more frequent presence of ACPA.

In classic studies on the genetic determinants that influence antibody production in mice, a genetic region was identified that controlled both the magnitude and the specificity of antibody responses (reviewed in ref [32]). This "Immuneresponse region" (Ir-1), was later recognized to be similar to the H2 region, the murine analogue of HLA [33]. It was found that the ability of a mouse to generate a proper antibody response to certain model peptides strictly depended on the genetic variant in the Ir-1 region. Thus, mice with certain genetic variants could generate antibodies with only certain specificity. Analogous to these classic studies in mice, HLA–DRB1 SE alleles influence the magnitude and the specificity of the ACPA response in human. In **chapter 4** it was observed that the level of ACPA in ACPA–positive patients with SE alleles was significantly higher than the level in SE–negative ACPA–positive patients. Similar to what was found in the classic murine studies, the effect was dominant. With respect to the specificity of ACPA responses, in **chapter 9** it was observed that sera from ACPA–positive patients with SE alleles more often recognized a citrullinated peptide derived from vimentin in comparison to patients without SE alleles. SE alleles did not predispose for the presence of antibodies against a citrullinated peptide derived from fibrinogen. In conclusion, SE alleles act as "classic" immunereponse genes in influencing both the magnitude and the fine-specificity of the ACPA response.

Smoking has been reported to influence the severity of RA in terms of disease expression, disease activity and radiological joint damage [34-36]. Two independent studies have shown that smoking predisposes to ACPA–positive RA only and does so exclusively in the presence of SE alleles [24;25]. Tobacco exposure may therefore influence disease severity via the ACPA response. The gene-environment interaction between smoking and SE alleles on ACPA has been examined in more detail in **chapter 6**. The data suggested that smoking on its own indeed is not significantly associated with an increased risk on ACPA, but smoking did interact with the dose of SE alleles to increase the risk on ACPA. The strength of interaction varied for the different SE alleles. Furthermore it appeared that the stronger the association of the SE alleles was with the presence of ACPA, the weaker was the additional contribution of smoking. To further increase the comprehension of the contribution of smoking to the development of RA, the effect of smoking on the risk for patients with UA to develop RA was determined in relation to the presence of SE and the presence of ACPA. In a group of SE–positive, ACPA–positive patients, the smoking individuals had a significantly increased risk for developing RA, compared to the non-smoking UA patients (OR 8.0 vs OR 3.3 with SE–negative, ACPA–negative, Nonsmokers as a reference, p-value for interaction 0.002).

The effect of tobacco usage might not only be explained by its effect on the presence of ACPA, but also by its effect on the "nature" of the autoimmune ACPA response, e.g. levels and isotypes of ACPA. In **chapter 6** it is shown that tobacco exposure in SE–positive, ACPA–positive patients correlates with a higher level of ACPA, and that both the presence and the level of ACPA are independently associated with the risk of UA patients developing RA (corrected for smoking status, SE status, SE subtypes and interaction between SE and smoking). These data suggested that the observed association between smoking and RA development in SE–positive, ACPA–positive patients could be explained by the correlation between smoking and ACPA levels. **Chapter 7** describes the further exploration whether smoking also affects isotype usage in the ACPA response and whether this effect is dependent on the presence of SE, like the effect on the presence of ACPA has been shown to be. Tobacco usage did influence the extensiveness of ACPA isotype usage and did so in SE–negative patients and non-significantly in SE–positive patients. This observation suggests that once tolerance for citrullinated antigens has been broken and ACPA are generated, the effect of smoking on the ACPA response becomes independent of T cell help via SE-bearing cells.

Since B–cells in the bronchoalveolar tract are prominent producers of IgA, IgA producing BALT can be detected more frequently in smokers [37], and smokers display higher citrullination in cells obtained by bronchoalveolar lavage [25], the hypothesis in **chapter 7** was that especially the IgA isotype of ACPA would have been more favorably produced in smokers. Indeed IgA ACPA was more frequent among smokers. Similarly however, IgM ACPA were detected more frequently and levels of almost all isotypes were higher in smokers than in nonsmokers, indicating a more diverse ACPA response in general in patients who had been exposed to tobacco compared to those who are nonsmokers. Although a more active ACPA response in smoking individuals may be suggested by these data, a more active clinical disease at baseline was not observed in these patients (data not shown).

After showing that brochoalveolar lavage cells from smoking individuals more often contained citrulline than those from healthy nonsmokers, Klareskog et al. have proposed an etiologic working hypothesis involving genetic, environmental and immunological aspects [25]. Long-term exposure to cigarette smoke, and probably also other environmental stimuli, may induce mechanisms that accelerate deimination of arginine to citrulline in autoantigens present in the lungs, possibly via upregulation of peptidylarginine-deiminase activity in macrophages that are undergoing apoptosis. An immune response to the citrullinated proteins may then be preferentially induced in individuals carrying the HLA–DRB1 SE alleles, possibly by increased binding properties to the DR molecule and thereby enhancing the immunogenecity of the protein. On the basis of the data in **chapter 6**, it is hypothesized that the SE alleles with a relative high binding affinity for citrullinated peptides, anti-citrullinated peptide immunity is generated more easily. In the presence of SE alleles with lower affinity, the effect of smoking may be an increased amount of citrullinated antigens, leading to overcome the threshold to activate T–cells and anti-citrullinated antigen immunity. A shortcoming in this hypothesis so far is that the autoimmune reaction starts against citrullinated antigens in the lung. For ACPA to be able to cause chronic joint inflammation, epitope spreading may occur, and/or access to joint antigens otherwise invisible to the immune system has to be facilitated. As is also indicated by the results in **chapter 7**, that showed an effect of smoking on the isotype-usage independently of SE, smoking probably has a combination of effects on the ACPA response in RA.

Smoking is known to exert many systemic effects (reviewed in [38]). For example it causes systemic inflammation. This is illustrated by an increased number

of circulating leucocytes and a possible selective influence on subsets of T–cells. Smoking is also associated with increased expression of L–selectin, possibly initiating adherence of polymorphonuclear leukocytes to the endothelium and recruitment of these cells in inflamed tissue. Furthermore, it changes levels of all kinds of inflammatory mediators in the lungs and in the circulation of healthy smokers (e.g. a marked increase in CRP and fibrinogen), an effect that can be detected until 10 to 19 years after cessation of smoking. Although limited data exist on circulating concentrations, increased levels of the pro-inflammatory cytokines TNF–alpha and IL–6 have been detected in smokers. Finally, impaired endothelial function as a result of cigarette smoke has been subject of investigation in studies concerning atherosclerosis. Endothelial dysfunction may as well play a part in facilitating joint inflammation. The effect that smoking has on the development of ACPA–positive disease is therefore more likely to result from a combination of triggering the ACPA response in genetically predisposed individuals and facilitating an environment in which ACPA against joint antigens can be formed, or at least in which inflammation is continued by the numerous systemic effects smoking has. The systemic effect may even be of key importance, since the reported duration of CRP elevation after smoking cessation resembles the prolonged risk to develop RA after smoking cessation (up to 20 years [39;40]).

To summarize some of the above discussed results, a model that could explain the observations is proposed in Figure 1. In this model, citrullinated antigens are taken up and presented by professional antigen presenting dendritic cells (DCs) and by B–cells. The DCs, after having matured, will present the antigens to T–cells that will differentiate and proliferate into a group of activated helper T–cells. The helper T–cells will subsequently provide help to the antigen primed B–cells, again via antigen presentation. The B–cells will then be activated and stimulated to differentiate into ACPA secreting cells. As antigen presentation on both levels occurs via HLA class II, HLA–DRB1 SE may have its influence in facilitating presentation of citrullinated antigens because of a strong binding capacity of the SE molecules for certain citrullinated peptides and thereby determines whether the response is initiated (presence and levels of ACPA) and to what exact antigens the response is directed (fine-specificity). Smoking probably affects the antibody response in two ways. If smoking causes high amounts of citrullinated proteins, more citrullinated antigens will be presented to T–cells, which will lead to more frequent breaking of tolerance

and thus more often ACPA production. This effect is dependent on the presence of the antigen presenting molecules and in this model it is thus associated with SE. Once tolerance is broken and ACPA is produced, smoking influences the isotype-usage of the ACPA response in a SE independent fashion, possibly via systemic effects.

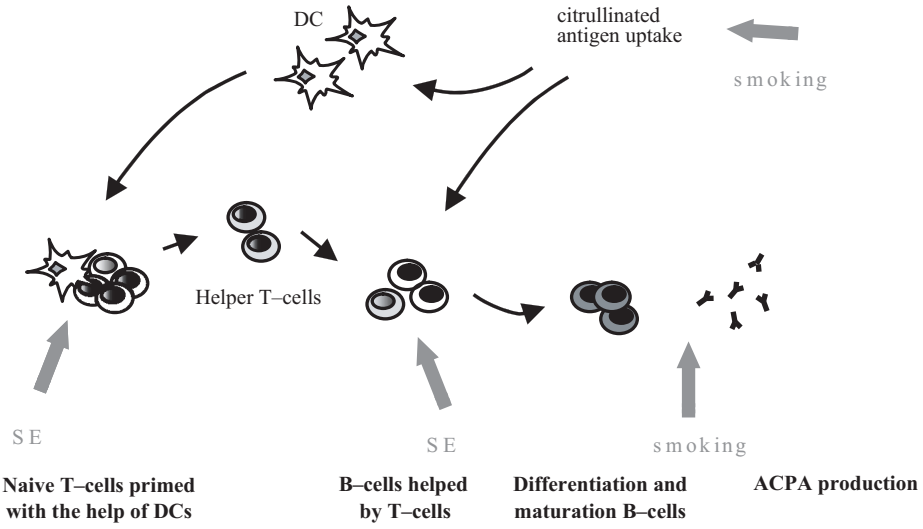

Figure 1. Model for the influence of SE and smoking on the ACPA response. SE influences presence, specificity and levels of ACPA possibly via facilitating antigen presentation of citrullinated antigens between professional antigen presenting DCs and naïve T–cells leading to initiate T–cell help to B–cells. Antigen presentation by B–cells to helper T–cells may also be facilitated by SE, which together initiates B–cell differentiation, maturation and ACPA production. Smoking influences the presence of ACPA in a SE–dependent way, possibly via causing large amounts of citrullinated antigens being presented to the immune system. Once tolerance had been broken, smoking additionally influences ACPA isotype-usage in a SE–independent fashion, possibly via systemic immune-modulatory effects.

A causative role for ACPA in the development of RA is still uncertain. The most valid argument to argue that it does have a pathologic effect is obtained from the results of a study showing enhancement of disease activity in mice with experimental arthritis after injection with ACPA. These results have however, until now, not been

replicated by others. Nonetheless, all circumstantial evidence points to a pivotal role for ACPA in the disease process. The aim of the present thesis was not to investigate whether ACPA cause development or progression of the disease but rather, the results described in this thesis provide information on the influence of known risk factors on ACPA and on RA and may therefore be helpful in unravelling the possible influence of ACPA on the aetiology of RA.

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Nederlandse Samenvatting

Reumatoïde artritis (RA) kenmerkt zich door chronische ontstekingen van meerdere gewrichten (polyartritis). De ontsteking is gericht tegen de binnenbekleding van het gewrichtskapsel (synovium) en komt vooral voor in de kleine gewrichten van handen en voorvoeten, maar kan zich in vrijwel alle gewrichten manifesteren. De belangrijkste symptomen zijn pijn en stijfheid. Uiteindelijk kan de chronische ontsteking leiden tot afbraak van kraakbeen, bot en gewrichtskapsel en daarmee tot gewrichtsvervormingen.

Omdat RA niet één enkel specifiek kenmerk bezit dat in alle gevallen aantoonbaar is en niet voorkomt bij andere ziekten, is de diagnose gebaseerd op een combinatie van bevindingen in de klinische presentatie, in laboratoriumonderzoek en op röntgenfoto's. In 1987 werden criteria ontwikkeld die nu over het algemeen als gouden standaard voor de classificatie van RA gelden. De ziekte RA wordt volgens deze criteria geacht aanwezig te zijn als aan minimaal vier van de volgende zeven criteria is voldaan: ochtendstijfheid, ontsteking van drie of meer gewrichten/ gewrichtsgroepen, gewrichtsontsteking van handen of polsen, symmetrische gewrichtsontsteking, onderhuidse verdikkingen (reumanoduli), reumafactor in het bloed en gewrichtsbeschadigingen zichtbaar op röntgenfoto's.

Aangezien vaak niet al deze kenmerken direct bij het eerste bezoek aan de reumatoloog aanwezig zijn, is de diagnose RA niet altijd snel en makkelijk te stellen. Van de patiënten die met nieuw-ontstane artritis op de polikliniek reumatologie komen heeft ongeveer 20% voldoende kenmerken voor de diagnose RA. Bij ongeveer 40% van de patiënten kan echter geen diagnose gesteld worden en wordt gesproken van het ziektebeeld "ongedifferentieerde artritis". Het beloop van ongedifferentieerde artritis is zeer divers. Bij een deel van de patiënten zullen de symptomen spontaan verdwijnen; bij een ander deel zal zich RA ontwikkelen. Aangezien de ziektelast en radiologische schade van patiënten die in een later stadium voldoende kenmerken voor RA hebben op termijn gelijk is aan die bij patiënten die direct de diagnose RA krijgen en omdat gebleken is dat vroege behandeling van RA gunstig is om de ziekteprogressie te verminderen, is het belangrijk zo vroeg mogelijk te kunnen inschatten of een patiënt met ongedifferentieerde artritis RA zal gaan ontwikkelen of niet. **Hoofdstuk 2** in dit proefschrift behandelt daarom om te beginnen de kans voor een willekeurige patiënt met ongedifferentieerde artritis om RA te ontwikkelen.

Hoe RA precies ontstaat is vooralsnog onduidelijk. Aangenomen wordt dat een combinatie aan omgevingsfactoren en genetische aanleg het ontstaan van de ziekte veroorzaken en het beloop ervan beïnvloeden. In dit proefschrift komt een aantal risicofactoren aan de orde die de kans op het ontstaan van RA vergroten. In de eerste plaats zijn dat reeds langbekende en veel beschreven varianten van het gen dat codeert voor een deel van het antigenpresenterende HLA–DR, namelijk HLA–DRB1 Shared Epitope (SE) allelen. Een andere bekende risicofactor is roken.

Een zeer goede voorspeller voor de diagnose RA die de laatste jaren sterk in de belangstelling staat is de aanwezigheid van autoantistoffen tegen gecitrullineerde peptiden (ACPA). Deze antistoffen zijn gericht tegen bepaalde onderdelen van lichaamseigen eiwitten die niet speciaal voorkomen in alleen RA patiënten. De aanwezigheid van ACPA is echter wel zeer specifiek voor RA. Bovendien zijn deze antistoffen vaak reeds voordat de eerste symptomen zich hebben gemanifesteerd detecteerbaar, voorspelt de aanwezigheid van de antistoffen in sterke mate het ontwikkelen van RA in patiënten met ongedifferentieerde artritis en hebben patiënten met ACPA meer gewrichtsschade in het beloop van hun ziekte. Om die redenen wordt gedacht dat ACPA mogelijk een cruciale rol spelen in de pathogenese van RA.

De **hoofdstukken 3 t/m 9** van dit proefschrift beschrijven hoe deze verschillende bekende risicofactoren met elkaar in verband staan en hoe zij voorkomen in relatie tot ongedifferentieerde artritis en RA in verschillende stadia van de ziekte.

Hoofdstuk 2

Ongedifferentieerde artritis- ziektebeloop beoordeeld in verschillende cohorten met patiënten met nieuw ontstane artritis

Omdat de prognose van ongedifferentieerde artritis kan variëren van spontaan herstel tot ernstig destructieve RA en omdat snelle agressieve medicamenteuze interventie bij RA waarschijnlijk de ziekteprogressie sterk kunnen vertragen, wordt het steeds belangrijker in een vroeg stadium *die* patiënten met ongedifferentieerde artritis te identificeren die RA zullen gaan ontwikkelen. Tegelijkertijd kan in dat geval onnodige behandeling van patiënten met een milder beloop van de ziekte voorkomen worden. In dit hoofdstuk is in verschillende rapportages van vroege artritis klinieken geïnventariseerd welk percentage van de patiënten met ongedifferentieerde artritis

binnen 1 jaar gediagnosticeerd werd met RA. Afhankelijk van welke definitie voor ongedifferentieerde artritis en welke inclusiecriteria voor het beschreven cohort gehanteerd werden, evenals welke definitie voor RA werd gebruikt, varieerden de percentages van 6% tot 55%. In de cohorten die waarneembare artritis vereisten op het moment van inclusie en die RA definieerden aan de hand van de ACR classificatie criteria waren deze percentages 17% tot 32%. Met een dergelijke a-priori kans op het ontwikkelen van RA in een groep patiënten met ongedifferentieerde artritis moet dus rekening gehouden worden bij interventiestudies. Voor een meer individueel, klinisch toepasbare schatting van de kans op RA is inmiddels een voorspelmodel verschenen waarbij rekening gehouden wordt met verschillende klinische en laboratorium parameters.

Hoofdstuk 3

Antistoffen tegen gecitrullineerde eiwitten en verschillen in klinische progressie bij reumatoïde artritis

In dit hoofdstuk stond de vraag centraal of ACPA–positieve en ACPA–negatieve RA gezien zouden kunnen worden als 2 verschillende ziekte-entiteiten. Als eerste werd onderzocht of RA met en RA zonder ACPA dezelfde klinische presentatie hebben. Van 454 patiënten met RA werden de eerste symptomen, het aantal gezwollen en pijnlijke gewrichten en het CRP vergeleken tussen degenen die wel ACPA hadden (N=228) en degenen zonder ACPA (N=226). Er bleken geen aantoonbare verschillen met betrekking tot ochtendstijfheid en type en locatie van de eerste symptomen, CRP niveau of door de patiënt gewaardeerde ziekteactiviteit. Ook het gemiddelde aantal gezwollen en pijnlijke gewrichten was gelijk in beide groepen. Na 4 jaar follow-up hadden de patiënten met ACPA echter wel meer gezwollen gewrichten en was in de ACPA–positieve groep meer radiologische schade waarneembaar.

Hoofdstuk 4

De HLA–DRB1 shared epitope allelen zijn hoofdzakelijk een risicofactor voor antistoffen tegen gecitrullineerde peptiden en zijn geen onafhankelijke risicofactor voor het ontstaan van RA

Nadat in een aantal populaties was gebleken dat de SE allelen, die al meer dan 40 jaar beschreven worden als risicofactor voor RA, alleen geassocieerd zijn met ACPA–positieve RA en niet met RA waarbij geen ACPA detecteerbaar zijn, ontstond de vraag of SE allelen een risicofactor zijn voor RA, of eerder voor het ontwikkelen van ACPA. Om die vraag te beantwoorden werd de invloed van SE allelen en van ACPA op het ontwikkelen van RA in een groep patiënten met ongedifferentieerde artritis onderzocht. In zowel SE–positieve als SE–negatieve patiënten was de aanwezigheid van ACPA significant geassocieerd met de ontwikkeling van RA. In patiënten met en in patiënten zonder ACPA had SE echter geen effect op de kans om RA te ontwikkelen. Bovendien bleek in de groep ACPA–positieve patiënten het hebben van SE allelen geassocieerd te zijn met hogere niveaus ACPA. De SE allelen lijken daarom geen onafhankelijke risicofactor voor het ontwikkelen van RA maar eerder voor het produceren van ACPA.

Hoofdstuk 5

Associatie tussen HLA–DR3 en ACPA–negatieve RA

Wanneer bepaalde HLA–DRB1 varianten het risico op ACPA (positieve RA) verhogen, zou het zo kunnen zijn dat andere varianten geassocieerd zijn met ACPA–negatieve RA. Dit werd onderzocht in **hoofdstuk 5** van het proefschrift, door middel van het vergelijken van HLA–DRB1 allelfrequenties tussen controle personen en RA patiënten in afzonderlijke groepen met en zonder ACPA. HLA–DR3 bleek een variant die alleen geassocieerd is met ACPA–negatieve RA en niet met ACPA–positieve RA. Bepaalde genetische risicofactoren zijn dus geassocieerd met bepaalde verschijningsvormen van RA, wat er mogelijk op wijst dat ACPA–positieve en ACPA–negatieve RA elk een verschillende ontstaanswijze hebben.

Hoofdstuk 6

De HLA–DRB1 shared epitope allelen verschillen in de interactie met roken en de aanleg voor antistoffen tegen gecitrullineerde peptiden

Ook omgevingsrisicofactoren blijken te verschillen tussen ACPA–positieve en ACPA–negatieve RA. Roken vergroot alleen het risico op ACPA–positieve RA en doet dat bovendien alleen in aanwezigheid van genetische predispositie met de HLA–DRB1 SE allelen. In dit hoofdstuk werd onderzocht 1) of verschillende SE varianten een verschillend risico op ACPA geven, 2) of verschillende SE varianten een verschillende interactie vertonen met roken en 3) wat het effect is van roken in relatie tot SE allelen op de ontwikkeling van RA bij patiënten met ongedifferentieerde artritis. De HLA–DR4 SE allelen (HLA DRB1*0401, *0404, *0405 en *0408) bleken een hoger risico op ACPA te geven dan HLA–DR1 en HLA–DR10 SE allelen (HLA–DRB1*0101, *0102 en *1001). De interactie tussen roken en de aanwezigheid van een SE allel was echter juist het minst groot bij de HLA–DR4 SE allelen. In de SE–positieve ACPA–positieve patiënten bleek roken geassocieerd te zijn met hogere spiegels ACPA en met de progressie van ongedifferentieerde artritis naar RA. De aanwezigheid van ACPA en de ACPA spiegel waren bij nadere analyse de enige onafhankelijke voorspellers voor het ontwikkelen van RA in patiënten met ongedifferentieerde artritis. Uit de resultaten van dit onderzoek werd geconcludeerd dat roken mogelijk bijdraagt aan het ontstaan van RA door een interactie met SE allelen en een effect op de ACPA spiegel.

Hoofdstuk 7

Associatie van roken met de samenstelling van de ACPA respons in de afwezigheid van HLA–DRB1 SE allelen

Om na te gaan of roken niet alleen de aanwezigheid en de hoeveelheid ACPA beïnvloedt, maar ook andere karakteristieken van deze antistofreactie, werd in hoofdstuk 7 onderzocht of roken het ACPA isotype-gebruik beïnvloedt. Verschillende isotypes ACPA (IgM, IgA en IgG1–4) werden daartoe vergeleken tussen ACPA–positieve RA patiënten die wel en die niet rookten. Bepaalde isotypes van antistoffen (IgA, IgM) kwamen vaker voor bij rokers dan bij niet-rokers. Het isotype-gebruik van de ACPA respons was bij de rokende patiënten dan ook meer uitgebreid. Opvallend

was dat het effect dat roken had op het voorkomen van verschillende isotypes ACPA waarneembaar was in de patiënten zonder SE allelen. Dit in tegenstelling tot het effect van roken op de aanwezigheid van ACPA op zich, dat alleen gedetecteerd wordt in SE–positieve individuen. Roken blijkt dus op verschillende manieren van invloed op de ACPA respons. Voor de invloed op het initiëren van de antistof reactie (wel of geen productie en ACPA spiegels) is de aanwezigheid van SE allelen, en dus bepaalde varianten van antigeenpresenterende moleculen noodzakelijk. Het effect van roken op het isotype-gebruik is echter niet afhankelijk van de aanwezigheid van deze SE allelen en werkt dus waarschijnlijk via een ander mechanisme.

Hoofdstuk 8

Isotypes van antistoffen tegen gecitrullineerde peptiden in ongedifferentieerde artritis en reumatoïde artritis duiden op een continue immuunrespons

Ook in dit hoofdstuk waren isotypes ACPA het onderwerp van onderzoek. Om meer inzicht te krijgen in de ACPA reactie in het algemeen en in verschillen in de immuunrespons tussen verschillende groepen ACPA–positieve patiënten werd het ACPA isotype-gebruik bepaald in patiënten met recent ontstane ongedifferentieerde artritis, met recent ontstane RA en patiënten met al langere tijd de diagnose RA. In serum dat kort na het ontstaan van de symptomen werd afgenomen kwamen alle verschillende geanalyseerde isotypes voor. Een duidelijke trend werd waargenomen van meer frequent voorkomen van alle isotypes in RA patiënten en meer in patiënten met ongedifferentieerde artritis die binnen jaar aan de criteria voor RA voldeden dan in de patiënten met ongedifferentieerde atritis die dat niet deden. Deze gegevens wijzen mogelijk op een volledig ontwikkelde immuunrespons met aanwezigheid van alle isotypes reeds vroeg in het ziekteproces. Aan de andere kant kunnen ze ook wijzen op een in dat stadium nog in ontwikkeling zijnde (en mogelijk dus nog te beïnvloeden) antistofreactie. IgM, een isotype dat over het algemeen geproduceerd wordt bij een recent ontstane, actieve immuunrespons, bleek ook aantoonbaar in alle groepen patiënten. Tezamen met de observatie dat ook in IgG–ACPA–positieve patiënten zonder IgM–ACPA op een later tijdstip wel IgM–ACPA voorkwam, duidt dit op een continue activatie van de afweerreactie gedurende het ziekteproces van ACPA–positieve artritis.

Hoofdstuk 9

Fijnspecificiteit van de respons tegen gecitrullineerde peptiden wordt beïnvloed door shared epitope allelen

In vroege, klassieke studies naar de genetische achtergrond van antistofproductie is aangetoond dat MHC de meest belangrijke genen bevat die zowel de grootte als de specificiteit van een antistofreactie controleert. In bovenstaande hoofdstukken werd reeds beschreven dat ook de ACPA respons wordt beïnvloed door de aanwezigheid van bepaalde MHC genen, met name HLA–DRB1 SE allelen: SE allelen zijn geassocieerd met predispositie voor de aanwezigheid van ACPA en met de grootte van de respons. In hoofdstuk 9 werd nagegaan of SE allelen zoals in de oude literatuur beschreven is, tevens van invloed zijn op de specificiteit van de ACPA reactie. Tegen welke gecitrullineerde antigenen de ACPA respons initieel gericht is, of welke antistoffen potentieel pathogeen zijn is momenteel niet bekend. In ontstoken gewrichten zijn verschillende soorten gecitrullineerde eiwitten detecteerbaar die van belang zouden kunnen zijn, bijvoorbeeld vimentine en fibrine. In dit hoofdstuk zijn in twee onafhankelijke groepen ACPA–positieve RA patiënten antistofreacties gemeten tegen een gecitrullineerd peptide van vimentine en een gecitrullineerd peptide van fibrinogeen. De aanwezigheid van SE allelen bleek geassocieerd met antistoffen tegen gecitrullineerd vimentine, niet met antistoffen tegen gecitrullineerd fibrinogeen. Deze gegevens zijn indicatief voor een rol als klassiek immuun-respons gen voor HLA–DRB1 in de ACPA respons.

Om de bediscussieerde resultaten samen te vatten is in Figuur 1 een model voorgesteld. In dat model worden gecitrullineerde antigenen opgenomen en gepresenteerd door professionele antigeen presenterende cellen (dendritische cellen, DCs) en B cellen. De DCs presenteren antigenen aan T cellen die vervolgens differentiëren en prolifereren tot een groep helper-T cellen die hulp kunnen bieden aan door antigen gereed gemaakte B cellen. De B cellen zullen hierdoor worden gestimuleerd tot het differentiëren tot ACPA producerende B cellen. Aangezien zowel tussen DC en T cel als tussen T cel en B cel antigen presentatie plaatsvindt via HLA klasse II, kan HLA DRB1 SE het systeem beïnvloeden door presentatie van gecitrullineerde antigenen mogelijk te maken door een sterke bindingscapaciteit van SE moleculen voor bepaalde gecitrullineerde peptiden. Hiermee wordt dan bepaald of een respons wordt geïnitieerd en tegen welke antigenen deze gericht is. Roken beïnvloedt de ACPA

respons waarschijnlijk op ten minste twee manieren. Wanneer roken citrullinering van eiwitten en daarmee veel gecitrullineerde antigenen kan veroorzaken, zullen meer gecitrullineerde antigenen aan T cellen gepresenteerd worden en zal vaker een ACPA reactie ontstaan. Dit effect is afhankelijk van antigen presenterende moleculen, en in dit model daarom geassocieerd met SE. Wanneer eenmaal een ACPA respons is geïnitieerd beïnvloedt roken de respons, gemeten aan de hand van verschillende isotypes, op een manier die niet afhankelijk is van SE; mogelijk door systemische effecten op het immuunsysteem.

Of ACPA oorzakelijk betrokken zijn bij het ontstaan van RA is vooralsnog onduidelijk. Wel wijzen vele gegevens erop dat ACPA in ieder geval een belangrijke rol zouden kunnen spelen in het ziekteproces. Het doel van het onderzoek dat beschreven wordt in dit proefschrift was niet om te onderzoeken of ACPA het ontstaan of de progressie van RA veroorzaken. Wel geven de resultaten informatie over de invloed van bekende risicofactoren op ACPA en op RA. Op die manier kunnen deze gegevens mogelijk wel bijdragen aan het ontrafelen van de eventuele rol van ACPA in de etiologie van RA.

Curriculum Vitae

Kirsten Natascha Verpoort werd geboren op 23 december 1976 te Leiderdorp. In 1995 behaalde zij het diploma Voortgezet Wetenschappelijk Onderwijs aan het Groene Hart Lyceum te Alphen aan den Rijn, waarna zij aanving met de studie Biomedische Wetenschappen aan de Universiteit Leiden. Het jaar daarop startte zij daarnaast de studie Geneeskunde aan dezelfde universiteit. Doctoraal examens voor deze studies werden afgelegd in respectievelijk 2001 en 2000. Het enthousiasme voor het vakgebied reumatologie en de aanpak van onderzoek naar "complexe ziekten" in het algemeen dat werd opgedaan gedurende de hoofdvakstage Biomedische Wetenschappen (Determinants of the development and progression of osteoarthritis; radiology and genetics, begeleid door Prof. dr. P.E. Slagboom, Dr. I. Meulenbelt en Dr. G. Kloppenburg) leidde ertoe dat zij na haar artsexamen in 2003 startte met het promotieonderzoek beschreven in dit proefschrift. Dit onderzoek werd begeleid door Prof. dr. T.W.J. Huizinga en Dr. R.E.M. Toes. Van januari 2007 tot januari 2008 was zij in opleiding tot internist in het HagaZiekenhuis te 's-Gravenhage (opleider: Dr. R.H. Kauffmann). Sinds maart 2008 is zij in vooropleiding tot reumatoloog bij de afdeling Interne Geneeskunde van het Groene Hart Ziekenhuis te Gouda (opleider: Dr. J.T.M. van der Heijden).

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List of Publications

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