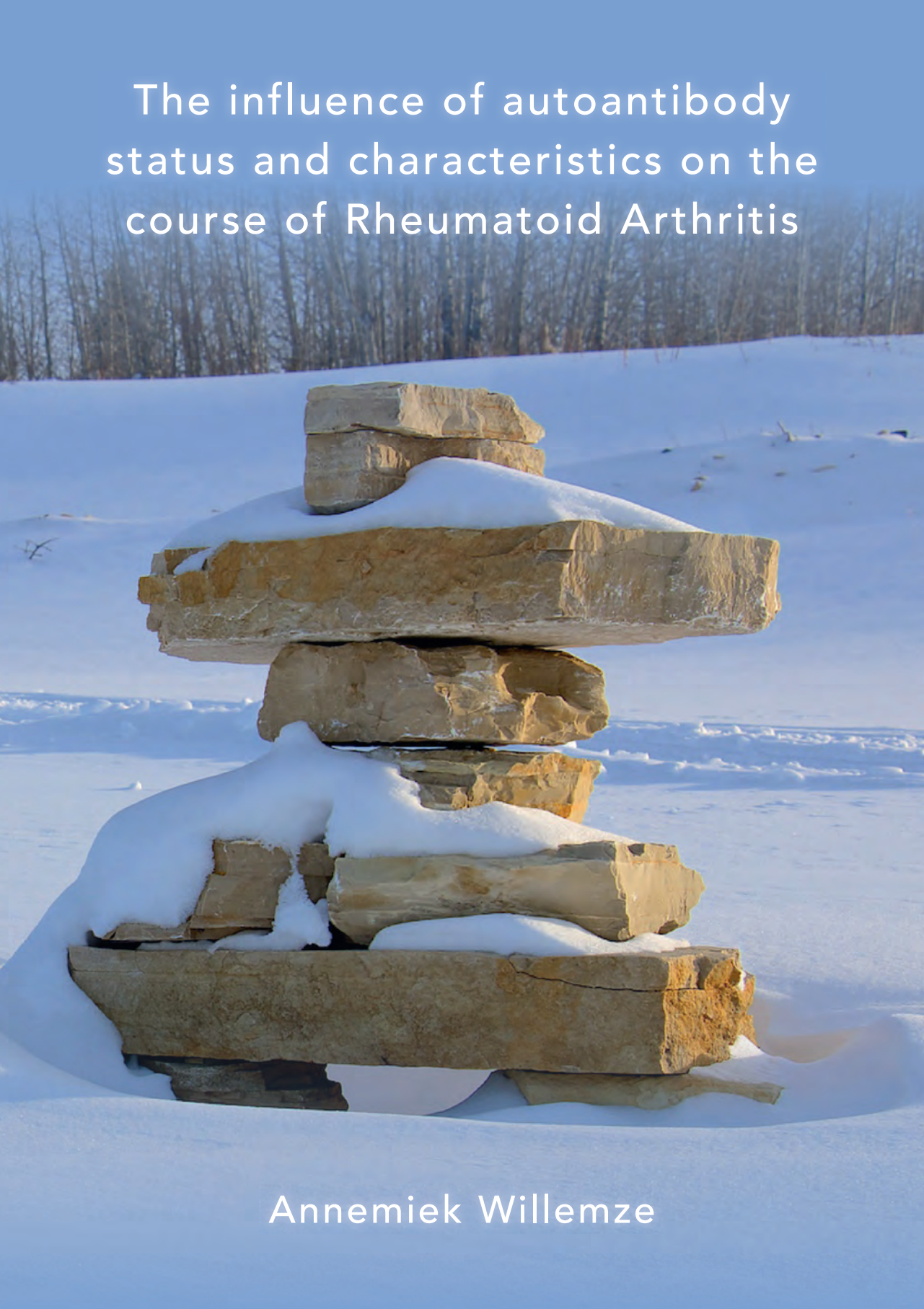
The influence of autoantibody status and characteristics on the course of Rheumatoid Arthritis



Annemiek Willemze

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CHAPTER 1

General introduction

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RHEUMATOID ARHTRITIS

Rheumatoid arthritis (RA), one of the most common autoimmune diseases, is characterized by persistent synovitis, systemic inflammation and expression of autoantibodies.[1] In industrialized countries approximately 0.5-1.0% of the adult population is affected by the disease. There is substantial geographic variation in the occurrence of RA with very high prevalences reported in native American populations[2, 3], and very low prevalences in populations from South-East Asia.[4] The disease is approximately three times more frequent in women than in men, and the prevalence increases with age. As RA is a systemic disease, symptoms as fatique, weight loss and fever as well as disorders of the heart, blood vessels, nerves and kidneys are also relatively common. The disease reveals itself by joint swelling and joint tenderness, in which the small joints of the hands and feet are most commonly affected.[1] The persistency of synovitis can result in the destruction of cartilage and subchondral bone, eventually leading to malformations and disability. If left unattended or not properly treated, RA can lead to increased disability or even invalidity of patients in their normal daily functions, thereby reducing the quality of life. For society this can ultimately lead to enormous costs in healthcare and loss in workforce.[5]

The mainstay of treatment in RA, are the Disease Modifying Anti Rheumatic Drugs (DMARDs), which are a heterogeneous collection of therapeutic agents of which the mechanisms of action are, largely, not well understood. When arthritis stays uncontrolled despite these agents, or when toxic effects arise upon administration of these drugs, biologic agents, such as tumor necrosis factor inhibitors can be used and have proven to be highly effective.[1] RA is considered as having an autoimmune origin because of the presence of self-reactive antibodies, such as anti-citrullinated protein antibodies (ACPA), thereby reflecting the complexity of the disease. Although it poses a considerable health problem, relatively little remains known about the disease pathogenesis and etiology.

The first description of RA was made in 1800 in Paris. Sixty years later, the disease was named 'rheumatoid arthritis' for the first time by an English rheumatologist, Alfred Baring Garrod.[6] As RA seems to be a highly heterogeneous disease, several classification criteria have been developed over the years, such as the 1956 ARA criteria[7], 1961 Rome criteria[8] and 1966 New York criteria[7], with the aim of identifying more homogenous patient groups to facilitate comparison of international studies. Since 1987 the disease has been classified based on the ACR 1987 criteria[9], which were developed using an analytical approach in which RA was defined by a regression analysis of disease characteristics of 'classic cases'. As different disease manifestations are included in these criteria, a heterogeneous set of patients with conceivably different 'pathogenic' backgrounds are identified by them. Therefore, the great variation in disease course and treatment response

among patients with RA can be explained by the classification criteria describing a heterogeneous syndrome. ACPA have an exquisite specificity for RA[10], and ACPA status was included in the new 2010 ACR–European League Against Rheumatism (EULAR) classification criteria for RA[11, 12], alongside RF levels (already included in the 1987 criteria) (figure 1).[9]

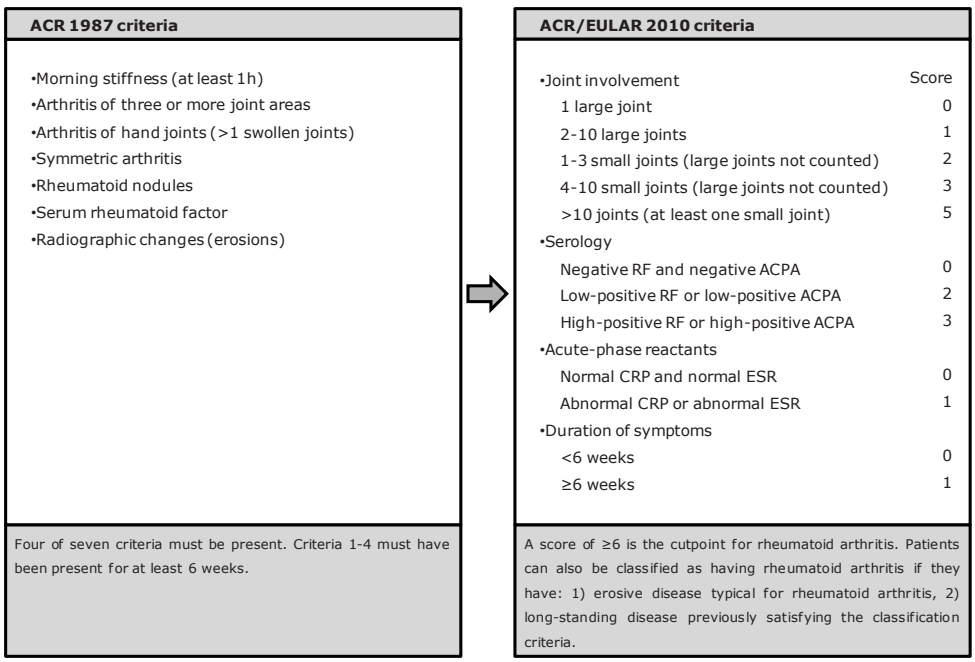

Figure 1. ACR 1987 criteria and ACR/EULAR 2010 criteria. Classification criteria for rheumatoid arthritis. ACR = American College of Rheumatology. EULAR = European League Against Rheumatism. RF = rheumatoid factor. ACPA = Anti Citrullinated Peptide Antibodies. CRP = C-reactive protein. ESR = erythrocyte sedimentation rate. Adapted from Scott et al.[1]

AUTOANTIBODIES IN RA

Various autoantibodies have been described in RA, including antibodies against ribosomal proteins (anti-RA33 antibodies)[13, 14] and against carbamylated proteins (anti-CarP antibodies).[15] The first RA-associated antibody, rheumatoid factor (RF), was known by 1940[16], and was later found to be directed to the Fc region of IgG. More than 20 years later, Nienhuis *et al*.[17] described antibodies labeling perinuclear granules in the superficial cells of the human buccal mucosa epithelium, which were named anti-perinuclear factor antibodies (APF). Although it became clear that APF were highly specific for RA, testing for these antibodies was rather cumbersome; therefore, RF was assessed in daily practice. In 1979, anti-keratin

antibodies (AKA) were described, and found to be present in approximately 50% of patients with RA.[18] Again, although these antibodies were more specific than RF for RA, the presence of RF was much more convenient to visualize. It was not until 1995 that the protein filaggrin was shown to be the common antigen targeted by both APF and AKA.[19] Since then, research into this unique autoantibody system has taken off with unprecedented speed, reaching a climax early in 2011 with the inclusion of anti-citrullinated protein antibodies (ACPA) in the new classification criteria for RA.[11, 12]

Testing for ACPA

After the discovery of filaggrin as the target of APF and AKA, it took only a year to reveal the molecular identity of the antigens that these autoantibodies recognize: the nonclassical amino acid citrulline, embedded on a protein backbone.[20, 21] Citrulline is a non-encoded amino acid, generated by a post-translational modification of arginine mediated by protein-arginine deiminase enzymes (figure 2).[22] This modification takes place during a variety of biological processes, including inflammation. In 1998, the first ELISA using citrullinated peptides (derived from several filaggrin epitopes) was developed[21] and was followed, in 2000, by the first ELISA based on artificial cyclic citrullinated peptides (CCP).[23] The first commercial version of this test, the CCP2 assay, became available in 2002 and enabled routine testing for antibodies directed against citrullinated epitopes as a biomarker for RA.[23-27] As well as the CCP2 assay, a few other assays for ACPA, such as CCP3 and MCV (Mutated Citrullinated Vimentin), have made their way into the clinic. These assays differ slightly in terms of specificity and sensitivity.[28]

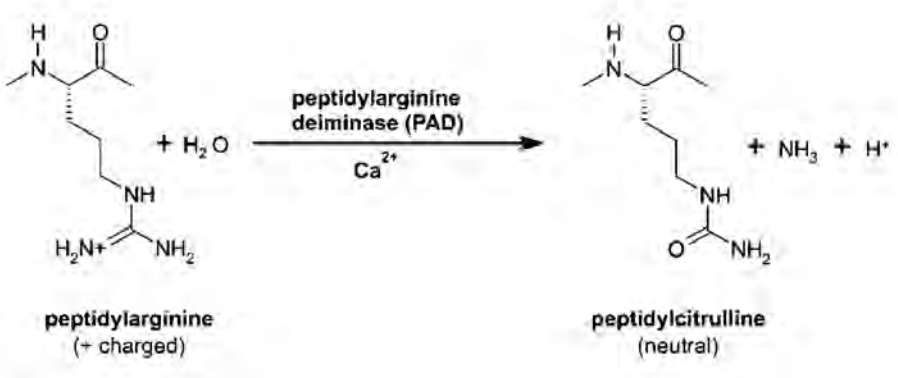

Figure 2. Citrullination of an amino acid. Posttranslational modification of arginine into citrulline, mediated by peptidylarginine deiminase (PAD).

ACPA: cause or consequence?

The association with RA

ACPA are strongly associated with RA, which suggests they have a prominent role in disease pathogenesis. Indeed, it has been suggested that ACPA are the 'spark that lights the RA fire', and are directly involved in a vicious circle that explains the chronicity of RA.[29-31] The efficacy of selective B-cell depletion in the treatment of RA provides evidence for the involvement of B cells and possibly autoantibodies in its pathogenesis.[32, 33] Furthermore, most ACPA-positive patients with RA seem to be ACPA-positive years before the onset of disease[34], although the extent of the ACPA armament seems to be limited at this preclinical stage. Moreover, ACPA levels show an increase around 2 years before the onset of symptoms, after which they seem to stabilize at fairly high levels.

Histological differences in inflamed joints have been found between ACPA-positive and ACPA-negative patients with RA. For example, synovial tissue immune cell infiltrates differ with respect to lymphocyte numbers as well as markers of fibrosis; altered synovial inflammatory architecture might indicate a role for ACPA in synovial inflammation.[35]

Interaction of the ACPA and RF responses

Although the appearance of ACPA in the preclinical phase tends to precede that of RF, RF can also be detected years before clinical disease onset.[34, 36] RF seems to preferentially interact with hypoglycosylated IgG.[37] ACPA are hypoglycosylated as compared with total IgG[38]; thus, one might infer that RF can enhance the pathological effects of ACPA through preferential binding to ACPA and potentiation of the subsequent immune response.

Immunological consequences of ACPA deposition

To be effective, antibodies must, in general, recruit immune effector mechanisms mediated by activation of the complement system[39] or Fc receptor-positive cells.[40] The complement system can be activated via three pathways: the classical pathway, the lectin pathway and the alternative pathway.[41] Each pathway is initiated by a specific recognition molecule. The classical pathway is initiated by complement C1q, the lectin pathway by mannose-binding lectin or ficolins, and the alternative pathway initiated through the spontaneous low level activation of C3. Initiation of complement activation via each of these pathways involves the formation of C3 convertase complexes (composed of different subunits in each pathway), which cleave complement C3 to produce biologically active complement fragments. These fragments attract and activate immune cells through their com-

plement receptors. Intriguingly, ACPA can activate human complement not only via the classical pathway, which is known to be activated by antibodies present in immunecomplexes, but also via the alternative pathway.[42] These observations are reminiscent of findings from animal models of arthritis, which show that the alternative pathway of complement activation is crucially involved in autoantibody-mediated arthritis.[43]

Besides activating complement, ACPA are also readily capable of triggering immune cell responses via Fc receptors (FcR). For example, immune complexes containing ACPA and citrullinated fibrinogen have been shown to trigger TNF secretion through engagement of FcγR on macrophages.[44] Such findings should be interpreted in the perspective that many immune complexed model antigens, such as ovalbumin, elicit similar effects. Thus these data do not necessarily indicate the involvement of ACPA in disease pathogenesis. Nevertheless they are of relevance to understanding the pathogenic role of ACPA, as they show that these autoantibodies do have the potency to recruit powerful immune effector mechanisms.

This notion is further supported by observations made in animal models showing that ACPA, reactive with several citrullinated antigens are able to initiate and enhance arthritis.[45-48] Findings made in these models also indicate that ACPA recognize citrullinated proteins in a hapten-like manner (that is, ACPA binds to citrulline as if it were a small molecule. This molecule can be recognized in the context of different protein backbones. Such a protein backbone is called a carrier which may be one that does not elicit an immune response by itself.).[48] Thus, ACPA and anti-CCP2 antibodies crossreact with a variety of citrullinated proteins that bear little sequence homology; this crossreactivity, however, is not absolute, as indicated by studies using specific citrullinated peptides, some of which did not bind ACPA.[49, 50]

Altogether, these data provide fertile soil to fuel the hypothesis that ACPA play an important part in disease pathogenesis, although further evidence is required to substantiate this putative role in RA. In the following sections, we discuss characteristics of the ACPA response, and summarize emerging evidence for how these antibodies are related to clinical course and treatment outcomes in RA.

ACPA characteristics

The B-cell lineage generates antibody-secreting plasma cells and memory B cells, which have enhanced capability to respond to a specific initiating antigen by producing antibodies. The most important function of antibodies is protection of the host against invading pathogens, primarily through neutralization of molecules essential to the lifecycle of the pathogen and/or the recruitment of powerful immune effector mechanisms capable of killing pathogens or pathogen-infected cells.

To be properly effective, an antibody response needs to develop through avidity

maturation and isotype switching stages. T cells are intimately involved in these processes as they provide the helper activity essential for the maturation of most B cell responses (Figure 3). Similarly, diversification of the antibody response, through recognition of more epitopes, is thought to contribute to its efficacy.

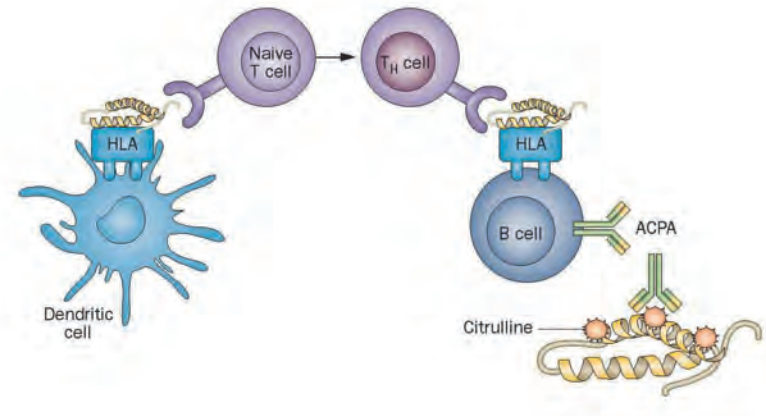

Figure 3. ACPA production by B cells can be stimulated by autoreactive T cells. Dendritic cells present peptides to naive T cells in complex with HLA class II molecules, activating the T cells and leading to TH cell-mediated stimulation of B cells. In the context of APCA production, it has been hypothesized that B cells can recognize citrullinated peptide complexes, for example on apoptotic cells. B cells can internalize these complexes, process them and present peptides from them in complex with HLA molecules encoded by SE alleles (these peptides may or may not be citrullinated), to T cells. When T cells recognize those peptides they can provide help to the B cells, resulting in the production of ACPA. Antibodies to different epitopes (including citrullinated epitopes) of the internalized peptide complex can be produced, leading to the production of ACPA. Therefore, T cell help stimulates the production and maturation of ACPA. Abbreviations: ACPA, anti-citrullinated protein antibodies; SE, shared epitope; TH cell, T helper cell.

Fine specificity and epitope spreading

ACPA can recognize a variety of citrullinated antigens, including citrullinated fibrinogen[51,52], citrullinated vimentin (which is also known as the Sa antigen),[53] citrullinated type II collagen[54], citrullinated α-enolase[55] and many more citrullinated proteins. An increase or shift in the antigen recognition profile (a phenomenon known as epitope spreading) can have important pathophysiological consequences, as has been described in, for example, systemic lupus erythematosus[56] and pemphigus. Autoantibodies such as anti-desmoglein antibodies present in patients with pemphigus vulgaris have been convincingly shown to mediate a pathogenic effect, through transfer into experimental animals.[57] Furthermore, in pemphigus

(of which there are two major types: pemphigus foliaceus and pemphigus vulgaris,) reactivity against different desmoglein epitopes is associated with different outcomes. In patients with pemphigus foliaceus, autoantibodies to desmoglein-1 occur. These autoantibodies mediate blistering of the skin through loss of adhesion in the superficial epidermis, where desmoglein-1, but not desmoglein-3, is expressed. By contrast, in mucosal pemphigus vulgaris, the presence of anti-desmoglein-3 IgG antibodies causes blistering of the mucosae, where desmoglein-3 is expressed.[58] Importantly, the example of pemphigus elegantly demonstrates that intramolecular epitope spreading might modulate remissions and relapses, as autoantibodies that recognize the EC5 domain of desmoglein-1 seem to be nonpathogenic, whereas those directed against the EC1 and EC2 domain of the molecule are associated with disease onset and active disease. Passive transfer experiments have demonstrated that anti-EC1 and anti-EC2 autoantibodies are pathogenic, whereas anti-EC5 autoantibodies are incapable of inducing blisters in mice.[59] Pemphigus is, therefore, a prototype disease that indicates the relevance of epitope spreading in the transition from the preclinical to the clinical stage of autoimmune disease.

The ACPA immune response in RA starts several years prior to diagnosis of the disease, even before the onset of symptoms, but in a restricted manner with low antibody titers and limited peptide reactivity.[60] ACPA titers and peptide-recognition profiles increase as the individual approaches disease onset (figure 4a).[34] Likewise, in patients with arthralgia, the development of arthritis is predicted not only by the presence of ACPA, but also by their levels.[61, 62] Indeed, patients with arthralgia who have an extended ACPA repertoire are at higher risk of developing arthritis.[62] Similarly, ACPA-positive patients with early arthritis that do not fulfill the American College of Rheumatology (ACR) classification criteria for RA are more likely to develop RA if their ACPA response is reactive to more citrullinated epitopes.[63] These findings are consistent with the notion that a 'broader' ACPA recognition profile is associated with the transition towards (persistent) disease, and resemble the observations made in pemphigus with the exception that, thus far, no specific anti-citrullinated epitope or protein reactivity has been identified that would predict disease course in RA. Given the hapten-like recognition of citrullinated antigens and the high crossreactivity towards multiple citrullinated proteins of the ACPA response, this lack of fine specificity is perhaps not surprising.

Isotype profiles

Isotype switching is another event involved in enhancing the efficacy of (auto) antibodies, and leads to an increase in the diversity of antibody structure that enables the activation of more immune effector mechanisms. ACPA can be present in different forms, including IgG, IgA, IgM and IgE (figure 4b).[64, 65]

Multiple ACPA isotypes are present before the onset of RA.[64] Likewise, the ACPA isotype distribution does not seem to significantly expand anymore during disease progression from undifferentiated arthritis (UA) to RA, indicating that most of the expansion of isotype usage by ACPA takes places before the onset of arthritis.

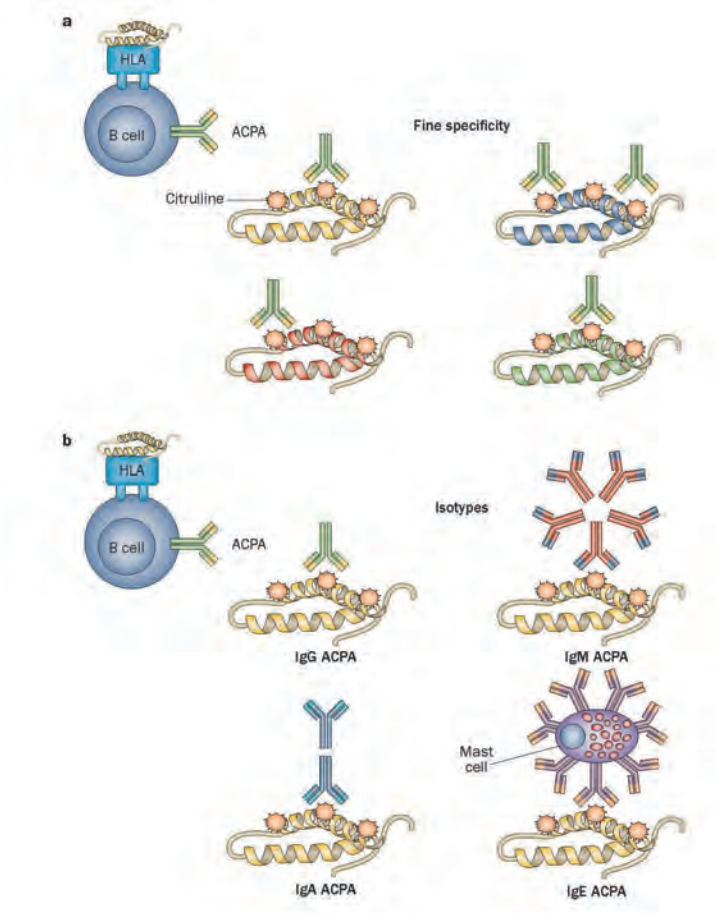

Figure 4. Fine specificity and isotype profiles are important characteristics of an ACPA response. A. ACPA can recognize different citrullinated antigens, including for example citrullinated vimentin, citrullinated fibrinogen and citrullinated α-enolase. Although it is the citrulline moiety that binds to the autoantibody, the context of the amino acids surrounding the citrulline are important for recognition by ACPA with differing fine specificities. The ACPA recognition profile seems to be established in undifferentiated arthritis, with a broader profile being associated with subsequent progression to persistent disease. The peptides presented by the B cells may or may not be citrullinated. **B**. Different antibody isotypes can activate the immune system via different pathways. For example, IgM, IgA and the different IgG subclasses activate the complement system to different extents. Although IgG is the most common isotype of ACPA in RA, the other isotypes also occur

in some patients. Different isotypes recruit different effector functions, for example, of IgE-ACPA it has been hypothesized that it can activate FcεR1-positive cells, such as mast cells, adding participation of these cells to a subsequent inflammatory process. Furthermore, the presence of IgM ACPA indicates an ongoing immune response, with recruitment of new B cells into the ACPA response. Abbreviations: ACPA, anti-citrullinated protein antibodies; RA, rheumatoid arthritis.

Maturation of the response

During a B-cell response against recall antigens, isotype switching and affinity maturation typically occur in germinal centers. Following somatic hypermutation, different B-cell clones will compete for antigens presented on follicular dendritic cells. B cells that express immunoglobulins of sufficiently high avidity will acquire the signals necessary for survival and proliferation. As a result, the total avidity of the immune response—defined as the overall binding strength of polyclonal antibodies to a multivalent antigen—increases, because low avidity B cells will not be stimulated and will eventually disappear from the population. The avidity maturation of antibody responses against recall antigens, mostly following vaccination, has been studied extensively, but autoantibody responses seem to behave differently.[66,67,68] For example, the avidity of ACPA is significantly lower than the avidity of antibodies to the recall antigens tetanus toxoid and diphtheria toxoid, pointing to a different regulation of autoantibody responses as compared with recall antigens. In individual patients with RA, ACPA do not show avidity maturation during longitudinal follow up and even in patients who displayed extensive isotype switching, ACPA avidity was relatively low,[69] indicating that these two maturation processes are uncoupled in the ACPA response.

Glycosylation

Although the specificity of antibodies is determined by the variable region, antibody-mediated effector functions are crucially dependent on the interactions of its constant region (Fc part) with the complement system as well as with the Fc receptors. These Fc-mediated effects are influenced by Fc-linked carbohydrate structures known as glycans. By glycosylation, a posttranslational modification process, different kind of glycan structures can be attachted to the Fc-parts of the antibodies, resulting in several different glycoforms in human serum. Glycosylataion of the Fc-tail of antibodies affects the recruitment of effector function, especially the binding to pro-inflammatory respectively anti-inflammatory Fc-receptors.[44] Interestingly, early studies have demonstrated a predominance of IgG-G0 (meaning without galactose) glycoforms in sera of RA patients, which correlated with disease activity and reverted to normal levels in patients who achieved remission.[70, 71] Scherer et al investigated whether the glycan chains carried by ACPA differ from

the glycans carried by other antibodies in the sera of RA patients.[38] A comparison of serum ACPA IgG1 to total serum IgG1 revealed that ACPA were associated with a characteristic glycan profile lacking sialic acid residues. ACPA from synovial fluid of RA patients were highly agalactosylated and due to the attachment of sialic acid to galactose, thereby also lacked sialic acid. Since Fc glycosylation directly affects the recruitment of Fc-mediated effector mechanisms, these data could contribute to the further understanding of the role of ACPA in disease pathogenesis of RA.

The data described above indicate that ACPA-producing B cells behave differently from 'conventional' B cells. In 2010, rituximab was shown in mice to specifically deplete B cells that produce autoantibodies, while sparing the 'conventional' plasma cells that produce protective antibodies.[72] Treatments that target the crucial biological mechanisms underlying 'conventional' B-cell responses might, therefore, prove not to be as effective as anticipated, because ACPA-producing B cells might be following other biological routes.[73]

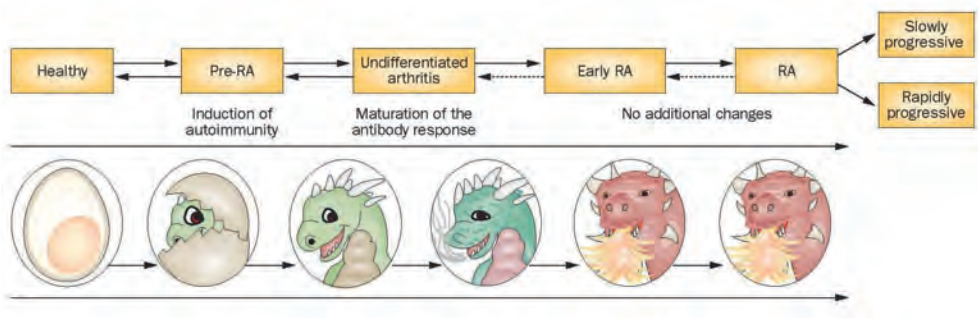

Figure 5. The maturation of the anti-citrullinated antibody response in the development of RA. The maturation of the dragon is depicted as a metaphor for the development of the ACPA response. Breaking of tolerance against citrullinated antigens can occur in healthy individuals, represented by the hatching of a dragon egg. Although immature in terms of isotype profile and breadth of antigen recognition, this early ACPA response might still be harmful, and potentially contributes to the progression of preclinical arthritis to early RA. We hypothesize that maturation of the ACPA response takes place during the preclinical phases, such that by the time early arthritis emerges clinically, the isotype and antigen-recognition profiles of the ACPA response are established. Greater breadth of ACPA isotype usage and/or recognition profiles predispose an individual to develop the full disease, but the ACPA response does not mature any further as the disease becomes more severe. Abbreviations: ACPA, anti-citrullinated protein antibodies; RA, rheumatoid arthritis.

Together, the collection of data described above provides credible support for the notion that the ACPA response shifts from 'infancy' to 'adulthood' before transition to clinical disease (Figure 5). Whether the maturation of the ACPA response is a consequence or a cause of disease initiation is not known, but identifying the

master switches responsible for the expansion of the ACPA response might be instrumental for further elucidation of the disease pathogenesis.

ACPA and clinical features

ACPA-positive and ACPA-negative disease have been shown to be associated with different genetic and environmental risk factors, fuelling the hypothesis that different pathophysiological mechanisms underlie these two separate disease subsets.[74-76] For example, ACPA-negative RA associates with *HLA-DR3*[77 78], whereas the *HLA* shared epitope (SE) alleles predispose to ACPA-positive disease.[74] Likewise, the contribution of smoking to disease risk is mainly confined to the ACPA-positive *HLA*-SE-positive patient group.[76] Stratifying patients with RA on the basis of ACPA status has resulted in the identification of more homogenous patient groups, with respect to both disease course and response to treatment.

ACPA and treatment outcomes

So what are the therapeutic implications of subgrouping patients with RA according to ACPA status? Logically, diseases with distinct pathogenesis might benefit from different treatment strategies. Methotrexate is the most prominent DMARD. A few years ago, we performed a double-blind placebo controlled randomized trial comparing two treatment strategies in patients with UA. Interestingly, the outcome of this study indicated that ACPA-positive patients with UA treated with methotrexate are less likely to progress to RA, and do so at a later time point as compared with a placebo control group. Unexpectedly, no effect of methotrexate therapy on progression to RA in the ACPA-negative group was observed.[79] Interestingly, among patients with UA, those with low or intermediate ACPA levels respond better to methotrexate than patients with high ACPA levels.[80] The data from this randomized trial not only indicate that the two ACPA subgroups respond differently to methotrexate treatment, but also that in patients with high APCA levels methotrexate monotherapy might be insufficient. Indeed, the presence of ACPA and IgM RF together with elevated levels of C-reactive protein is predictive of more rapid radiographic progression in patients with RA. Patients with ACPA and IgM RF are also more likely to respond insufficiently to methotrexate monotherapy for recent-onset RA.[81]

It is not only in regard to response to DMARDs that ACPA status seems to matter. In a trial published in 2011[82], 208 patients with RA refractory to therapy with TNF blockers were treated with rituximab. Rituximab is a monoclonal antibody directed towards the B-cell marker CD20, and has been shown to be an effective treatment in RA. In these patients, the presence of ACPA predicted a better EULAR response to rituximab at 24 weeks. Thus, rituximab might have a greater role in

ACPA-positive patients with RA than in ACPA-negative individuals.[82] The mechanisms of rituximab efficacy, and of B-cell involvement in RA, are incompletely known; the basis for these differing outcomes remains to be elucidated.

ACPA, remission and long-term monitoring

The absence of ACPA and IgM RF are independent predictors of drug-free remission.[83] As we have outlined, the course of ACPA-positive disease seems to be characterized by more persistent inflammation than its ACPA-negative counterpart. Together, these data indicate that treatment decisions in RA can be guided by ACPA status. Seroconversion is uncommon among ACPA-positive and ACPA negative patients; therefore, it does not seem to be useful to repeat ACPA measurements in daily practice.[84, 85] Thus, these data support the hypothesis that RA can be classified into two different disease subsets, and suggest that developing different classification criteria for ACPA-positive and ACPA-negative RA might help to optimize treatment strategies.

ACPA and disease outcome

The emerging relevance of ACPA status to treatment decisions is not only based on differential treatment efficacies, but is also supported by differences in disease outcome. Typically, 50–70% of the patients with RA are ACPA positive.[86] Although ACPA-positive and ACPA-negative patients with RA show a very similar clinical presentation in the early phase of the disease[87,88], their subsequent disease course is different—extra-articular manifestations are clearly influenced by ACPA status. For example, ACPA positivity is associated with an increased risk of developing ischemic heart disease[89] or lung pathology.[90] Likewise, ACPA-positive patients have more destructive disease than ACPA negative patients88, [91-93]; ACPA-positive patients develop erosions earlier and more abundantly than patients without ACPA.[94] Owing to their more severe disease-course, APCA-positive patients require a more aggressive treatment regimen than ACPA-negative patients.[95] Indeed, in the BeSt study ACPA-positive patients initially treated with DMARD monotherapy displayed greater radiographic joint destruction after 2 years than ACPA-negative patients.[95] In patients initially treated with combination therapy, by contrast, no difference with respect to joint destruction was observed between ACPA-positive and ACPA-negative patients. These observations suggest that effective treatment with combination therapy, together with steering treatment according to disease activity, can prevent radiographic progression, even in patients with risk factors for severe damage, such as ACPA-positive patients.

The ACPA isotype profile has been associated with a higher risk of radiographic progression, leading to a 1.4-fold increase in risk per isotype used in the ACPA response, illustrating that an extended isotype usage is associated with a worse outcome.[96]

Altogether, evidence is emerging that ACPA-positive and ACPA-negative RA represent two different disease entities with different outcomes, and, possibly, different responses to medication. The latter notion is especially important as it indicates that treatment regimes can be optimized by developing them according to ACPA status.

OUTLINE OF THIS THESIS

The general aim of this thesis was to elucidate the immunological properties of ACPA and to investigate the association between ACPA characteristics and the disease course of RA.

ACPA display high association with RA and are implicated in its pathogenesis. The presence of ACPAs is known to precede the onset of RA.[34, 36] North American natives have previously been reported to have a younger age at disease onset and an increased prevalence and severity of RA. Genetic studies have also revealed a higher prevalence of HLA DRB1 SE alleles in North American natives and increased frequency of RF positivity in patients with RA in several North American native populations.[97] In order to identify the features of ACPA that could confer its pathogenicity, we extensively characterized this antibody response in a unique North American native population of patients with RA and their unaffected relatives in **chapter 2**.

Furthermore, the synovium is the primary site of pathology in RA, and ACPA are readily detectable in the synovial fluid of patients with RA.[98] The transition from asymptomatic autoimmunity to clinically detectable synovitis is not well understood. **Chaper 3** describes the serological and synovial features of a young woman from a multi-case RA family from a North American Native population who initially had asymptomatic autoimmunity, then subsequently developed clinical features suggestive of early RA.

HLA SE alleles are known to be associated with RA susceptibility[99], specifically susceptibility to ACPA-positive RA.[100] Furthermore, smoking is known to predispose to the development of ACPA-positive RA. The combined effect of HLA SE alleles and smoking are known to exceed the sum of their single effects: a phenomenon known as biological interaction.76, [101, 102] Recent data suggest that a gene–environment interaction between smoking and the HLA shared epitope alleles plays a role in shaping the autoimmune reaction towards a specific citrullinated antigen.[103] More recently, however, our group has reported a similar interaction for 2 peptides derived from 2 other citrullinated proteins, citrullinated vimentin and citrullinated fibrinogen.[104] These latter data indicate that the observed interaction might not be confined to an autoimmune response against a specific citrullinated antigen. Rather, these findings indicate that these interaction effects extend to several citrullinated autoantigens, which might even be explained by a gener-

al predisposition to ACPA development. In **chapter 4**, we aimed to analyze in greater detail the extent of the interaction between HLA shared epitope alleles and tobacco exposure on the antigen-recognition profile of ACPA, especially in ACPA-positive patients in order to exclude the possibility that the association is caused by the presence of ACPA rather than by the presence of an autoimmune reaction to specific citrullinated epitopes.

Another citrullinated protein in inflamed synovial tissue identified previously is fibronectin (FN).[105] FN, one of the most abundant proteins present in the inflamed joint, is a glycoprotein, which can be a component of the extracellular matrix (insoluble form) or present in body fluids (soluble form). FN is involved in a variety of processes, such as wound healing, haemostasis, thrombosis and embryogenesis.[106] In **chapter 5** we characterized the citrullination of fibronectin in the joints of RA patients and studied the prevalence, epitope specificity and HLA association of autoantibodies against citrullinated fibronectin in RA.

HLA SE alleles are not only associated with the presence of ACPA[74], but also with joint destruction[107] and the ACPA fine specificity repertoire.[108] A large variation in joint destruction is seen within the ACPA-positive patient population. It is conceivable that certain citrullinated antigens are more potent than others in activating T cells in the context of HLA SE alleles. HLA SE alleles were found to associate with antibodies targeting peptides from citrullinated vimentin, but not with the presence of antibodies recognizing citrullinated fibrinogen.[108] This differential modulation of the ACPA response by SE-alleles, and the fact that disease phenotypes vary greatly among ACPA-positive patients, has raised the question whether certain ACPA fine-specificities might associate with a more severe disease phenotype. If so, designing assays that test for these specificities would be of prognostic value and could influence treatment decisions in the clinic. Therefore, in **chapter 6** we investigated whether ACPA fine-specificities, which are formed under the influence of SE-alleles, associate with the extent of radiographic joint damage.

Furthermore, it is conceivable that a certain ACPA recognition profile is associated with the emergence of certain clinical features and possibly pathogenicity in RA, as has been shown in other autoimmune diseases. For example, in pemphigus the reactivity against different desmoglein epitopes is associated with different outcomes.[59] In **chapter 7** we determined the association between the ACPA fine specificity and phenotypic characteristics within ACPA-positive RA and investigated whether specific subsets of RA patients can be distinguished on the basis of their epitope recognition profile.

Very early therapy in RA with disease-modifying antirheumatic drugs is associated with lower levels of joint destruction and a higher chance on achieving remission.[109-113] Having symptoms for >12 weeks at treatment initiation is a strong and independent risk factors for a persistent disease course.[109, 111-113] These observations have led to the hypothesis that the 'window of opportunity' exist early in dis-

ease.[110] This hypothesis presumes that underlying disease processes are not fully matured in the very early stage of arthritis, making modulation more successful. As ACPA precede arthritis development and are associated with a severe disease course[114], we hypothesized that the ACPA response broadens within the very early phase of RA and in doing so limits the 'window of opportunity'. Therefore, it was examined in **chapter 8** whether patients who were assessed within 12 weeks of symptom onset have a less broadened ACPA response than patients with longer symptom duration.

The ACPA response likely represents a T-cell-dependent B-cell response, given the protein nature of the antigen recognized and the strong association with the HLA SE alleles. The evolution of such a response is typically characterized by a first wave of IgM antibodies after the first antigen contact, quickly followed by the presence of IgG. After repeated antigen exposure, the IgG responses are further boosted while the IgM peak declines. Therefore, it is conceivable that the presence of IgM ACPA suggests that activation of recently recruited naïve B cells recognize citrullinated antigens because the half-life of circulating IgM is short. In **chapter 9** we determined whether there is a difference in the fine specificity of IgG and IgM ACPA.

Previously it has been shown that the levels of anti-CCP2-antibodies are higher in synovial-fluid than serum.[115-117] However, very limited information on absolute levels of ACPA in either synovial-fluid or serum is present as the levels are generally expressed as arbitrary units. Nonetheless, it is interesting to obtain information on the absolute concentration of ACPA as this would allow the comparison of the ACPA-response to other antibody responses in quantitative terms. In order to quantitate ACPA-levels it is required to isolate ACPA. In **chapter 10** we present data on the estimation of the minimal ACPA quantities in serum and synovial-fluid.

Recently, a novel family of autoantibodies in RA patients was described: anti-carbamylated protein (Anti-CarP) antibodies, which target carbamylated (homocitrulline-containing) epitopes.[118, 119] Since citrulline and homocitrulline have a similar structure, in **chapter 11**, we wished to determine to what extent human autoantibodies can differentiate between them. Unlike human antibodies, the anti-modified citrulline (AMC) antibody developed by Dr Senshu[120] is able to recognise citrullinated epitopes irrespective of the neighbouring amino acids. Therefore, we also aimed in to verify whether the AMC assay could distinguish between these two amino acids.

Finally, **chapter 12**, provides a summary of the results and a discussion of the implication of the studies described in this thesis.

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CHAPTER 2

Marked differences in fine specificity and isotype usage of the anti–citrullinated protein antibody in health and disease

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ABSTRACT

Objective

Anti–citrullinated protein antibodies (ACPAs) display high association with rheumatoid arthritis (RA) and are implicated in its pathogenesis. The presence of ACPAs is known to precede the onset of RA. In order to identify the features that could confer its pathogenicity, we extensively characterized this antibody response in a unique North American native population of patients with RA and their unaffected relatives.

Methods

The levels of IgA, IgM, and IgG ACPAs, as well as IgM and IgA rheumatoid factor (RF), were measured in serum samples obtained from 81 patients with RA and 195 of their unaffected relatives. The isotype distribution, the fine specificity of the ACPA response, and its association with RF were compared in health and disease.

Results

ACPA positivity was observed in 19% of the healthy relatives and ~91% of the patients with RA. ACPA isotype usage was strikingly lower in unaffected relatives than in patients with RA (1–2 versus 5–6 isotypes). Fine specificity studies showed that reactivity to citrullinated fibrinogen and vimentin was present in sera from patients with RA, while it was virtually absent in their unaffected relatives. Finally, the ACPA and RF responses were associated in patients with RA but were discordant in their healthy relatives. Extended analyses revealed that the presence of ACPAs was associated with RA irrespective of RF status, while the association of RF with disease relied on its interaction with ACPAs.

Conclusion

The fine specificity and isotype usage of the ACPA response are qualitatively different in health and disease. Epitope spreading and expansion of the isotype repertoire might be necessary for development of RA, and this could be facilitated by the presence of RF antibodies.

INTRODUCTION

Autoantibodies are characteristic of a large number of autoimmune diseases. Determining the pathogenicity of autoantibodies by showing their capacity to transfer disease, however, is complicated by ethical and practical difficulties. Therefore, only a small minority of autoantibodies, such as antiplatelet antibodies in idiopathic thrombocytopenia purpura or the antidesmoglein antibodies in pemphigus vulgaris, were convincingly shown to mediate a pathogenetic effect through placental transfer[1] or transfer into experimental animals[2], respectively.

Autoantibodies sometimes are found in healthy individuals as well and often are present years before the disease presentation. A detailed understanding of the characteristics of the autoantibody response that render it pathogenic is, therefore, of utmost importance, because such an understanding would allow targeted intervention in individuals at risk, before disease onset. Several characteristics of autoantibodies have been associated with pathogenicity. In pemphigus vulgaris, a skin disease, reactivity to different desmoglein epitopes is associated with different disease outcomes.[3] In antibody-mediated models of arthritis in mice, a combination of antibodies with different fine specificities and isotypes was found to be more efficient than a single antibody in inducing disease.[4, 5] Moreover, in these models, soluble immune complexes were shown to facilitate the access of pathogenic antibodies to the joint.[6]

Rheumatoid Arthritis (RA) is strongly associated with the presence of autoantibodies. Although the involvement of autoantibodies in RA is poorly understood, studies in several mouse models of arthritis indicate that autoantibodies are crucial to disease induction and progression.[7, 8] Among the different autoantibodies described in patients with RA, the anti-citrullinated peptide antibodies (ACPA) display the strongest RA specificity for RA (95-98%), with 70–80% sensitivity.[9] The presence of ACPA in patients with recent-onset RA is predictive for progression to erosive RA[10, 11] and their presence in patients diagnosed with undifferentiated arthritis is highly predictive of progression to RA.[12] Likewise, ACPAs were shown to contribute to disease progression in a mouse model of arthritis.[13] Taken together, data from human and mouse studies indicate that ACPAs are a possible pathogenetic mechanism in RA.

The prevalence of the ACPA response in healthy Caucasians is estimated to be 1-2% of the population.[14] Information about ACPAs in unaffected individuals is therefore scarce in the literature, and originates from retrospective studies that involve serum samples collected from patients with RA before disease onset.[15, 16] These studies, however, did not investigate the characteristics of the ACPA response in health versus disease. Characterization of the ACPA response, including isotype usage and fine specificity, in healthy individuals and patients with RA would provide valuable information about features of the ACPA response that could be

associated with pathogenicity and could indicate the biological pathways involved in disease development.

North American Natives have previously been reported to have a younger age at disease onset and an increased prevalence and severity of RA. Genetic studies have also revealed a higher prevalence of shared epitope (SE) alleles in North American Natives and increased frequency of rheumatoid factor (RF) positivity in patients with RA in several North American native populations.[17] The high predisposition to RA prompted us to investigate the presence of the ACPA response in a unique population of patients with RA and their unaffected relatives from a North American native population in Central Canada.

Surprisingly, our results show a high prevalence of ACPAs in unaffected relatives of patients with RA in this population. This remarkable feature of this population allowed us, for the first time, to extensively study and compare the main characteristics of the ACPA response in health and disease and to identify the features that could confer its pathogenicity.

PATIENTS AND METHODS

Patients, relatives and control subjects

Patients with RA were selected from a Cree and Ojibway population in Central Canada. Probands visiting rheumatology clinics in urban (Winnipeg, Saskatoon) and rural (Norway House, St Theresa's Point) locations were recruited, and each patient was asked to bring along unaffected relatives who were willing to participate in this study. The unaffected population consisted mainly of first-degree relatives (75.5%), with the remainder being second-degree relatives. Additionally, 91 unrelated healthy controls were recruited from the same populations. Eighty-five families were included, comprising 76 probands and 208 relatives. Serum samples from 9 probands are missing. The largest family consisted of 1 proband and 13 relatives.

The characteristics of the patients with RA and their healthy relatives who were included in this study are shown in Table 1. RA was diagnosed according to the American College of Rheumatology (formerly, the American Rheumatism Association) 1987 revised criteria.[18] Clinical assessment of swollen, stiff and painful joints of all patients and relatives was performed by a rheumatologist at the time of inclusion in the study. The number of swollen joints is available for 62 probands (76.3%). Of these patients, 9 had no swollen joints at the first visit. Among the relatives, 5 were found to have RA, and 3 of these patients were also currently receiving disease modifying antirheumatic drugs. These 5 individuals were analyzed as part of the RA population. Of the remaining group of relatives, 8 presented with >1 swollen joint at the time inclusion but did not meet the criteria for RA or any other rheumatic disease and were classified as having undifferentiated arthritis.

The 91 unrelated controls had no swollen joints at the time of inclusion and no first-degree relative with RA. Their median age was 30 years (range 18-88 years) and 39.6% were women. Written consent was obtained from all participants, and the protocol was approved by the Biomedical Research Ethics Boards of the University of Manitoba and by the Band Councils of each rural community.

ACPA isotypes

ACPA isotypes (IgA, IgG1, IgG2, IgG3, IgG4 and IgM) were measured in baseline serum samples obtained from patients with RA, their healthy relatives and unrelated healthy control subjects, using CCP2 plates (Euro-Diagnostica, Arnhem, The Netherlands) and a previously described enzyme-linked immunosorbent assay (ELISA).[19] A successive dilution of one reference standard, consisting of a pool of 20 ACPA-positive samples, was used in all plates. Distinct dilutions of this standard (for IgA and IgM, 1:12.5; for IgG1, 1:400; for IgG2, 1:6.25; for IgG3 and IgG4, 1:12.5) were defined as containing 1,000 arbitrary units (AU) per milliliter.

Fine-specificity measurements

Antibodies against the Sa antigen were detected by ELISA as anti-citrullinated myelin basic protein and confirmed by Western blot analysis as anti-citrullinated vimentin, as previously described.[20] The screening dilutions ensuring 99% RA specificity were 1:300 in ELISA and 1:100 in Western blot analysis. The anti-Sa ELISA cutoff is based on 2 SD of the value for non-RA Caucasian control subjects with connective tissue diseases. Human fibrinogen (Sigma-Aldrich, Zwijndrecht, The Netherlands) was also citrullinated as previously described[21], under nondenaturing conditions. For detection of antibodies against citrullinated fibrinogen, MaxiSorp microtiter plates (Nunc, Sanbio, The Netherlands) were coated with 1 µg/well citrullinated fibrinogen or native fibrinogen in phosphate buffered saline (PBS)(pH 9.0) and were incubated overnight at 4°C. Plates were washed 4 times with PBS/0.05% Tween-20 after this and all other incubation steps. After blocking with 2% Bovine serum albumin (BSA)/PBS (pH 9.0) for 2 hours at room temperature, plates were incubated with serum samples diluted 1:50 in radioimmunoassay (RIA) buffer/5% normal sheep serum (Sigma-Aldrich), for 1 hour at room temperature. Antibodies to citrullinated fibrinogen/fibrinogen were detected using rabbit anti-human IgG-horseradish peroxidise (DAKO, Glostrup, Denmark), diluted 1:10,000 in RIA buffer, by incubating the plate for 1 hour at room temperature. Bound antibodies were visualized using avidin-biotinylated enzyme complex.

The same reference standard as that used for measurements of ACPA isotypes was used in the citrullinated fibrinogen ELISA. The 1:12.5 dilution was defined as containing 1,000 AU/ml of antibody. For the anti-Sa ELISA, reference standards are based on optical density (OD) values given by low, medium and high serum titers at a 1:300 dilution in 1% BSA.[20]

The following linear citrullinated peptides and their native counterparts were used for fine-specificity studies: C2 (vim) (STCit SVS SSS YCitCit MFG G)[22] and C3 (vim) (VYA TCitS SAV CitLCit SSV P)[23] derived from human vimentin, C4 (fib) (NEE GFF SACit GHR PLD KK)[23] and C5 (fib) (FLA EGG GVCit GPR VVE RH) (unpublished data) derived from human fibrinogen, and C6 (enolase) (KIH ACitE IFD SCitG NPT V)[24] derived from human non-neuronal enolase. All synthetic peptides were coated on streptavidin-coated plates (Fisher, Winnipeg, Manitoba, Canada) via a C-terminal long-chain biotin. Detection of antibodies recognizing these peptides was performed as described.[23]

A successive dilution of one serum sample reactive with all vimentin and fibrinogen peptides was used as reference standard for these peptides. The 1:25 dilution of the reference sera was defined as containing 1,000 AU/ml of antibody.

Determination of cut-off values for ACPA ELISA assays

The level of specific antibodies in each serum sample was determined using the reference standard curve. Cutoff values for the citrulline-specific responses were calculated in each assay and were defined as 2 SDs above the mean concentration of serum samples obtained from 30 healthy Caucasian control subjects. In case the concentrations of several control samples were below the detection limit of the ELISA, the cutoff was calculated as the lowest concentration situated on the ascending region of the standard curve. Borderline samples (with concentrations between 10 AU above and 10 AU below the cut-off value) were tested at least twice for IgA ACPA and all IgG isotypes. All samples that reacted with the citrullinated peptides in fine specificity assays were retested. Only samples that were positive every time tested were considered positive.

In fine-specificity studies, citrulline-specific signals were defined as a positive signal against the citrullinated antigen and a negative signal against the uncitrullinated antigen, with a minimum difference of 0.1 in OD values. For uncitrullinated control antigens, a signal higher than 2 SDs above the average OD value for at least 4 Caucasian control subjects tested on the same plate was considered positive. Samples were tested on the same plate for citrullinated and control antigens to minimize experimental variations in OD measurements.

Rheumatoid Factor measurements

IgM RF values reported throughout the study were determined by nephelometry. IgA-RF and IgM-RF were also measured by ELISA, using human IgG1 as the capture antigen and F(ab')2 fragments of peroxidise-conjugated anti-human IgA or IgM, as previously described.[25] The cutoff values used to assign positivity were based on 60 Caucasian controls. The IgM-RF values as determined by ELISA were comparable to those obtained by nephelometry.

Statistical analysis

Statistical analyses were performed using SPSS version 14.0 software (SPSS, Chicago, IL). Differences in the distribution of the ACPA isotypes between patients with RA and their healthy relatives were calculated using the chi-square test. The chi square test was also used for calculating odds ratios (ORs) with 95% confidence intervals (95% CIs), and P values were used for the association of RF and ACPA with RA. When a cell contained fewer than 5 individuals, the P value was calculated with Fisher's 2-tailed exact test. P values less than 0.05 and 95% CIs which excluded the value of 1 were considered significant. Multivariate logistic regression analyses with IgM-RF and ACPAs or IgA RF and ACPAs as covariates were performed for testing the interaction between RF and ACPAs.

Moreover, the individual variables significantly associated with RA were also tested in a multivariate and conditional logistic regression with age and sex as possible confounders (data not shown). These analyses were performed using the Stata 9.0 software (StataCorp, College Station, TX).

RESULTS

The characteristics of the patients with RA and their healthy relatives included in this study are shown in Table 1. The patients were significantly older than the healthy relatives (median 52 years versus 35 years; $p<10^{-8}$ by Mann-Whitney-test) and were more likely to be females (p=0.001 by Chi-square test). Correction for these variables, as described in Materials and Methods, did not result in significant changes in ORs and P values (data not shown).

We first determined the prevalence of ACPA in the patients with RA and their relatives (Table 2). The prevalence of ACPAs was 91.4% in the patients with RA. Intriguingly, an unusually high prevalence of ACPAs (19.0%) was also found in the healthy relatives. Of the unrelated healthy control subjects, 8.8% were positive for ACPAs; this prevalence was significantly lower than the prevalence in relatives (P=0.03 by chi-square test), although it was still higher than the prevalence reported in healthy Caucasians. These data suggest that there is a high background frequency of ACPAs in this healthy North American native population, which is higher in the relatives of patients with RA compared with unrelated healthy individuals. Of note, the small subset of relatives who presented with undifferentiated arthritis at baseline, the frequency of ACPAs was intermediate (37.5%). This group was excluded from further analyses, due do its small size.

The most prevalent isotypes were IgG1 and IgA, with a higher prevalence in patients with RA than in control subjects (Table 2) (for IgG1, 98.6% versus 62.2% [P<10-6]; for IgA, 82.4% versus 32.4% [P<10-6] for ACPA-positive patients with RA versus their healthy relatives). In contrast, IgM, IgG2, IgG3, and IgG4 were

Characteristic	RA patients (n = 81)	Healthy Relatives (n = 195)
Age, median (range) years	52 (19-76)	35 (18-78)
Female sex	90.1	71.8
Location		
Winnipeg	51.9	51.3
Sasketoon	16	7.7
Norway House	23.5	35.4
St. Theresa point	8.6	5.6
Total HAQ score		
Mean	0.74	0.13
Median	0.75	0
No. of DMARDS, mean (range)	3.1 (0-8)	0 (0-0)
Erosions	40.3	Not tested
No. of swollen joints, median (range)	3.5 (0-36)	0 (0-0)
Diagnosis		
RA	100	0
Undifferentiated arthritis	0	0
Healthy	0	100

Table 1. Clinical characteristics of the study populations. Except where indicated otherwise, values are the percent of patients with rheumatoid arthritis (RA) and their healthy relatives. Health Assessment Questionnaire (HAQ) scores were determined in 70 patients with RA. The use of disease-modifying antirheumatic drugs (DMARDs) was assessed in 71 patients. The presence of erosions was determined in 67 patients with RA. The number of swollen joints was determined in 62 patients with RA and in 187 healthy relatives.

detectable in a significant percentage of patients with RA, but were virtually undetectable in the healthy relatives (Table 2). As a consequence, the total number of isotypes used (Table 3) was also markedly different in patients with RA compared with their healthy relatives. Although many serum samples from patients with RA (n=20) were positive for all 6 ACPA isotypes, sear from healthy ACPA-positive relatives showed usage of mostly 1 isotype (n=30) or a maximum of 2 (n=7). These data indicate the presence of a broader ACPA response in patients with RA compared with their healthy relatives, suggesting that the immune response to citrullinated antigens in the healthy relatives is relatively limited compared with that in the patients.

	Healthy relatives (n = 195)	Relatives with undifferentiated arthritis (n = 8)	RA patients (n = 81)	Unrelated healthy subjects (n = 91)
ACPA positive	19.0	37.5	91.4	8.8
Isotype				
IgM	2.1	0.0	49.4	ND
IgA	6.2	0.0	75.3	3.3
IgG1	11.8	25.0	90.1	6.6
IgG2	0.5	0.0	49.4	ND
IgG3	1.5	12.5	43.2	ND
IgG4	0.5	0.0	38.3	ND
IgM-RF	15.4	37.5	81.5	29.7
IgA-RF	21.6	14.3	48.1	19.8

Table 2. Percentage of individuals positive for ACPAs and for isotypes of ACPAs and RF in the healthy and affected population. IgA rheumatoid factor (IgA-RF) positivity was determined in 185 healthy relatives, 7 relatives with undifferentiated arthritis, and 79 patients with rheumatoid arthritis (RA). ACPA = anti–citrullinated protein antibody; ND = not determined.

No. ACPA isotypes used	Healthy relatives (n = 195)	RA patients (n = 81)
0	158	7
1	30	12
2	7	16
3	0	4
4	0	7
5	0	15
6	0	20

Table 3. ACPA isotype usage in probands and relatives. For differences between the distribution of the number of isotypes used in healthy relatives and patients with rheumatoid arthritis (RA), $P < 10^{-35}$; for differences among patients with RA, P= 0.20, by chi-square test. ACPA = anti–citrullinated protein antibody.

Expansion and maturation of antibody responses include not only extended isotype usage but also intramolecular and intermolecular epitope spreading.[3] We therefore studied the fine specificity of the IgG ACPA response in paitents with RA and their healthy relatives. We specifically examined the recognition of citrullinated

fibrinogen and vimentin (the Sa-antigen), autoantigens expressed in the inflamed joint that have been shown to be recognized by IgG ACPAs from patients with RA.[26] [27] The results depicted in Table 4 show that although citrullinated fibrinogen and Sa were recognized by IgG ACPAs from more than half of the patients with RA, virtually none of the IgG ACPA-positive sera from healthy relatives recognized these antigens. (The results described in Table 4 were obtained with a linear α-enolase peptide containing a small difference in sequence compared with the peptide used by Lundberg et al, whose report appears elsewhere in this issue28). Interestingly, most of the RA sera that reacted with citrullinated antigens recognized both citrullinated fibrinogen and Sa.

	Healthy relatives (n = 27)	**RA (n = 27)**
Proteins		
Anti-Sa	0.0	61.1
Anti-Citrullinated fibrinogen	3.7	59.7
Anti-Sa and anti-citrullinated fibrinogen	0.0	47.2
Peptides		
C2 (vim)	0.0	2.8
C3 (vim)	0.0	20.8
C4 (fib)	0.0	26.4
C5 (fib)	0.0	8.3
C6 (enolase)	3.7	13.9

Table 4. Percentage of IgG ACPA-positive individuals whose sera recognized citrullinated vimentin/SA and/or fibrinogen or citrullinated peptides derived from these antigens. Sera from individuals positive for IgG anti–citrullinated protein antibodies (ACPAs) were tested for recognition of citrullinated vimentin (anti-Sa) or anti–citrullinated fibrinogen or peptides derived from citrullinated vimentin (C2 [vim] and C3 [vim]), citrullinated fibrinogen (C4 [fib] and C5 [fib]), or citrullinated enolase (C6 [enolase]). RA= rheumatoid arthritis.

Furthermore, a spectrum of citrullinated epitopes previously shown to be recognized by sera from patients with RA, were also tested for recognition by the IgG ACPA-positive sera in this study. The vimentin and fibrinogen epitopes were selected from larger sets of peptides that were derived from the amino acid sequences of human vimentin and the α- and β-chains of human fibrinogen. The peptides were selected from these sets based on the fact that they allowed adequate differentiation of ACPA fine specificities. The α-enolase peptide was synthesized

based on a study demonstrating that this is the most frequently recognized epitope of that protein.[24] We observed the same striking difference between reactivities, with a significant proportion of RA sera recognizing 1 or more citrullinated epitopes (41.1% of IgG ACPA-positive patients), while virtually none of the sera from healthy relatives reacted against the citrullinated peptides tested. Taken together, these data indicate a markedly different recognition of citrullinated antigens by ACPAs in health and disease.

Previous retrospective studies have shown that the specificity and positive predictive value of ACPAs for RA are increased when ACPAs are combined with RF antibodies, especially of the IgM and IgA isotypes.[14-16] Therefore, we also studied the association of IgM-RF and IgA-RF with the ACPA response in health and disease. The data shown in Table 5 indicate that having both IgM-RF and ACPAs is a characteristic feature of RA (present in 65 of 81 patients with RA), although positivity for both IgM-RF and ACPAs was rare in healthy relatives (7 of 195 healthy relatives) (OR 209, 95% CI 68–647 [$P< 10^{-35}$] for IgM-RF-negative ACPA-negative versus IgM-RF-positive ACPA-positive). Similar data were obtained for IgA-RF and ACPAs (OR 63, 95% CI 22–179 [$P<10^{-22}$] for IgA-RF-negative ACPA-negative versus IgA-RF-positive ACPA-positive) (Table 6).

	Healthy relatives	RA patients	OR (95% CI)	P-value
IgM RF-				
ACPA-	135	6		
ACPA+	30	9	6.8 (2.23-20.40)	$p < 0.0002$
IgM RF+				
ACPA-	23	1		
ACPA+	7	65	213.6 (24.91-1830.86)	$p < 10^{-14}$
ACPA-				
IgM RF-	135	6		
IgM RF+	23	1	0.98 (0.11-8.51)	$p = 1.000$
ACPA+				
IgM RF-	30	9		
IgM RF+	7	65	30.9 (10.53-90.99)	$p < 10^{-12}$

Table 5. Association of ACPAs and IgM-RF with RA. Values are the number of healthy relatives and patients with rheumatoid arthritis (RA) positive for IgM rheumatoid factor (IgM-RF) and/or anti–citrullinated protein antibodies (ACPAs). P values were calculated by chi-square test or, when a cell contained fewer than 5 events, by Fisher's exact test. OR = odds ratio; 95% CI = 95% confidence interval.

	Healthy relatives	RA patients	OR (95% CI)	p-value
IgA RF-				
ACPA-	122	6		
ACPA+	23	5	30.9 (11.68-81.94)	$p < 10^{-16}$
IgA RF+				
ACPA-	28	1		
ACPA+	12	37	86.3 (10.59-703.77)	$p < 10^{-9}$
ACPA-				
IgA RF-	122	6		
IgA RF+	28	1	0.7 (0.08-6.28)	p = 1.000
ACPA+				
IgA RF-	23	35		
IgA RF+	12	37	2.0 (0.88-4.68)	p = 0.096

Table 6. Association of ACPAs and IgA RF with RA. Values are the number of healthy relatives and patients with rheumatoid arthritis (RA) positive for IgA rheumatoid factor (IgA-RF) and/or anti–citrullinated protein antibodies (ACPAs). P values were calculated by chi-square test or, when a cell contained fewer than 5 events, by Fisher's exact test. OR= odds ratio; 95% CI= 95% confidence interval.

Furthermore, after stratification for the presence of either IgM-RF or IgA-RF and ACPAs, we observed that the presence of ACPAs was significantly associated with RA, irrespective of IgM-RF. However, this association became stronger in the presence of IgM-RF. Intriguingly, IgM-RF was not associated with RA in the absence of ACPAs, but the association became significant when ACPAs were also present. These results suggest that the association of IgM-RF with RA is attributable to an interaction with ACPAs. This was further supported by a multiple logistic regression analysis indicating that the odds for having RA when both antibodies were present was 31.64 times higher than expected based on the ORs for the individual antibodies (P=0.005).

When we stratified for the presence of IgA-RF and ACPAs, we observed that ACPAs were associated with RA irrespective of positivity for IgA-RF, but this association is stronger in the presence of IgA-RF. Furthermore, an association between IgA-RF and RA was present only in the presence of ACPAs, but it did not reach the level of significance. Likewise, a logistic regression analysis indicated a trend toward an interaction between IgA-RF and ACPAs (exp(B)=2.79; P=0.276); however, this trend did not reach the level of significance.

Taken together, these data confirm the previously recognized association of IgM/IgA-RF with RA, but this study is the first to show that this association is attributable to an interaction with ACPAs.

DISCUSSION

In this study, we have characterized the ACPA response in a unique population of patients with RA and their unaffected relatives. The cohort was derived from the North American native populations in Central Canada, characterized by a high prevalence of RA and frequent multicase families ([29] and Peschken C et al, unpublished data). We detected a surprisingly high frequency of ACPAs in the unaffected relatives. This offered us the opportunity to study and compare the characteristics of the ACPA response in health and disease. Our data indicate the presence of a qualitatively different ACPA response in the 2 populations, characterized by more extensive isotype usage and recognition of citrullinated antigens present in the inflamed joints exclusively by sera from patients with RA. Moreover, by studying ACPAs in relation to IgM-RF and IgA-RF, we found that ACPAs are associated with RA independently of RF, while RF is associated with RA only in the presence of ACPAs. Our data indicate the presence of an interaction between these risk factors, with high odds for having RA when both antibodies are present.

To our knowledge, this is the first report of such a high prevalence of ACPAs in an unaffected population. Although the cause of this phenomenon is unknown, it has previously been shown that the Cree and Ojibway populations have a high frequency of disease predisposing SE alleles (59%).[30] Furthermore, we have shown in a Dutch population that SE alleles are a risk factor for the development of ACPAs rather than an independent risk factor for RA.[31] We are currently investigating whether this is also true for our North American native population of healthy relatives of patients with RA.

Previous retrospective studies have indicated that the presence of ACPA often precede disease onset. Considering that the unaffected relatives are, on average, younger than patients with RA, it is conceivable that disease will develop in the future in at least some of the ACPA-positive relatives. Follow-up studies are currently under way and will provide valuable information about changes in antibody characteristics that may be associated with disease development.

Although we detected a relatively high prevalence of ACPAs in the population of healthy relatives, our data indicate that the mere presence of ACPAs is not enough to induce disease. The limited ACPA isotype usage in the healthy relatives is consistent with a relatively immature autoantibody response. In patients with RA, chronic exposure to citrullinated antigens in the joint conceivably results in continuous (re)activation of antigen-specific B cells and favors isotype switching. This hypothesis is also supported by the low frequency of IgM ACPAs in healthy relatives (4 of 37 ACPA-positive individuals), as IgM is indicative of ongoing immune responses.

The fine-specificity data indicate that ACPAs recognize at least partially different

antigens in patients with RA and their healthy relatives, with responses against citrullinated fibrinogen and citrullinated vimentin/ Sa being present in more than half of the patients while being virtually undetectable in their healthy relatives. These citrullinated antigens are present in the inflamed joint and may serve as an important source of continuous antigen stimulation in RA synovium.[26, 27] In contrast, the antigens that stimulate the ACPA response in healthy individuals and are responsible for the initial loss of immune tolerance are currently not known. Based on observations made in several populations, it has been hypothesized that the association between smoking and ACPAs may be attributable to increased expression of citrullinated antigens in the lungs of smokers.[32-34] Alternatively, it has been proposed that the oral pathogen porphyromonas gingivalis, which is capable of citrullinating endogenous antigens, may be involved in the initial breaking of immune tolerance.[35] To address these hypotheses, a spectrum of environmental stimuli and their association with fine-specificity of ACPAs will need to be systematically examined in multiple populations, particularly genetically susceptible populations, such as North American natives.

An important observation made in this study is that the ACPA and RF responses are closely associated in the patients with RA while being discordant in their healthy relatives. Although IgG-RF was not tested in this study due to technical difficulties, these results are consistent with results from earlier studies indicating that IgM-RF and IgA-RF are risk factors for RA.[15, 16] Additionally, this study is the first to demonstrate that this association is confined to the ACPA-positive individuals, indicating that the contribution of RF antibodies to RA is dependent on their interaction with ACPAs.

The interaction between RF and ACPAs implies that these autoimmune responses may converge to precipitate disease. Although the biological mechanism underlying this observation is unknown, this association could be explained by a model proposed in a recent study, suggesting that that autoantibody-mediated articular inflammation in mice may be facilitated by soluble immune complexes that enable the access of pathogenic antibodies into the joint.6 Because RF antibodies recognize IgG molecules, they can form soluble immune complexes, which could facilitate access of ACPAs joints. An alternative explanation is that RF could amplify the effector mechanisms induced by ACPAs in the joint, thereby exacerbating joint inflammation.

In summary, our data indicate a diversification of the ACPA response in patients with RA when compared with that in healthy individuals who are at risk of disease. We speculate that the relatively limited ACPA response in healthy individuals will change over time, leading to disease manifestations. These changes likely involve broader isotype usage and/or epitope spreading and could be facilitated by RF antibodies that may allow access of ACPAs into the joint or amplify their effects. Un-

derstanding the pathways responsible for the diversification of the ACPA response in RA is important, because such an understanding could provide new treatment possibilities for targeting the pathological autoimmune response before disease becomes manifest.

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CHAPTER 3

Anti-citrullinated protein antibody response associated with synovial immune deposits in a patient with suspected early rheumatoid arthritis

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Anti-citrullinated protein antibodies (ACPA) are highly specific for rheumatoid arthritis (RA) and precede the onset of clinical symptoms by several years, with increasing titers as patients approach disease onset.[1, 2] The synovium is the primary site of pathology in RA, and ACPA are readily detectable in the synovial fluid and tissue of patients with RA.[3] The transition from asymptomatic autoimmunity to clinically detectable synovitis is not well understood. We describe the serological and synovial features of a young woman from a multi-case RA family who initially had asymptomatic autoimmunity, then subsequently developed clinical features suggestive of early RA.

An 18-year-old Native North American (Cree) woman from Central Canada was recruited to a study that aimed to identify high-risk family members of RA probands and follow them longitudinally for disease onset. This study was approved by the Research Ethics Board of the University of Manitoba, and by the Band Councils of the First Nations communities. At baseline she was asymptomatic and had an entirely normal joint examination. Within 6 months of enrolment she developed pain and stiffness in her left knee and left wrist. Examination revealed joint-line tenderness in these 2 joints, along with several small joints of the hands, although no effusion was detectable. She was suspected of having early RA and started taking hydroxychloroquine and naproxen. After informed consent, at that time she also underwent a Parker-Pearson synovial biopsy of the knee, per an established study protocol. With treatment, her symptoms rapidly improved, and within 3 months she spontaneously discontinued the medications, as she had become pregnant for the first time. She continued to be free of synovitis throughout the pregnancy. After delivering a healthy baby she once again developed tenderness and swelling in the left wrist and left knee, and was again given hydroxychloroquine and naproxen, with improvement in the symptoms over the ensuing 2 months. To date, her symptoms continue to be well controlled with this regimen.

The investigations at baseline had revealed that she was negative for IgM and IgA rheumatoid factor (RF), but was positive for anti-CCP2 (Eurodiagnostica) and anti-CCP3 (Inova) antibodies, at a titer of 52 and 38 units respectively. C-reactive protein and erythrocyte sedimentation rate were in the normal range. HLA testing showed her to have the HLA- DRB1*0901 and *1402 alleles. Her serum samples were tested for ACPA isotypes (IgM, IgA, IgG1-4) using ELISA as described.[4] Anti-Sa was tested by ELISA as described.[5] Response to citrullinated fibrinogen (cit-fib), and 5 different linear citrullinated peptides, including C2 (vim), C3 (vim) derived from human vimentin, C4 (fb) and C5 (fb) derived from human fibrinogen, and C6 (en) derived from human enolase, were assessed by ELISA with 2 non-citrullinated peptides as controls. Cutoff level was established on the basis of the mean + 2 standard deviations of the values obtained from testing 30 healthy Caucasian controls. RF and acute-phase reactants remained negative throughout the study period. IgG1-ACPA was positive the first visit (151 AU), with increasing

titers during followup (Figure 1). IgA-ACPA was initially negative, but became positive with increasing titers after symptom onset. IgM-ACPA, IgG2, IgG3, and IgG4 remained negative throughout the study period. After being initially negative, she developed increasing titers of antibodies against citrullinated fibrinogen, but remained negative for anti-Sa and all of the linear citrullinated peptides tested.

These data suggest that onset of clinical synovitis was heralded by an expansion in ACPA isotype usage, along with epitope spreading to nonlinear cit-fibrinogen-associated antigens. Interestingly, as shown in Figure 1, the autoantibody levels continued to rise despite amelioration of the clinical symptoms with pregnancy. This observation implies that the clinical improvement typically seen during RA pregnancy may not necessarily reflect an attenuation of the underlying autoimmune mechanisms, at least at this early stage of the process.

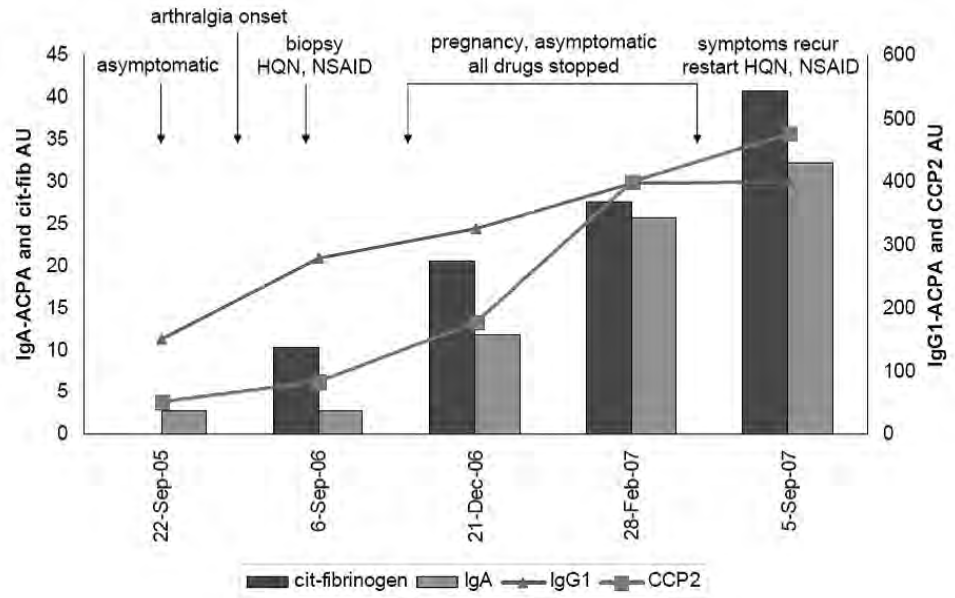

Figure 1. Development of ACPA response over a 2-year period in relation to clinical features in suspected early RA. Levels of all ACPA isotypes and specificity are shown as arbitrary units (AU), with levels of IgA-ACPA and anti-citrullinated-fibrinogen (cit-fib) shown on Y1 axis, and levels of IgG1-ACPA and anti-CCP2 shown on Y2 axis. Approximate cutoff level determined using 30 healthy controls is indicated by broken line. There is an increase in the titers of all ACPA over the study period, with IgA-ACPA and anti-cit-fib both becoming positive after onset of articular symptoms.

In contrast to the findings reported in studies of early RA, the synovium demonstrated normal sublining architecture and minimal microvascular changes. The synovial lining layer was markedly abnormal throughout multiple samples, with the lining cells appearing to float in an amorphous extracellular matrix (Figure 2A). Immunoperoxidase staining revealed the presence of an occasional CD3-positive cell without aggregates, but no CD19 cells. CD68 and CD55 staining were present in the lining layer, and CD86-positive cells were scattered throughout the sublining. Staining using a polyclonal anti-citrulline antibody (AP064, GemacBio) to detect citrullinated proteins demonstrated intense staining of the synovial lining cells (Figure 2B). Immunofluorescence studies showed staining of the lining layer for IgG, IgA, C3, and fibrin (Figure 2C), suggesting that the synovial lining layer may have been the target of an antibody-mediated immune response with complement activation and deposition. Although the specific target of this immune response was not directly tested, the colocalization of citrullinated antigens in lining cells with the immune deposits is consistent with the possibility that the response is directed towards a citrullinated antigen(s). TdT-mediated dUTP-biotin nick-end labeling (TUNEL) demonstrated extensive staining of the synovial lining cells (Figure 2D) compared to that seen in a normal synovial membrane (data not shown), indicating that the lining cells were undergoing wide- spread apoptosis.

We describe a case where there was a transition from asymptomatic autoimmunity to clinically evident synovitis. At this point, it is not possible to determine with certainty whether this patient's synovitis represents the earliest clinical evidence of RA, since she has yet to meet the American College of Rheumatology criteria set. On the other hand, it is possible that the very early use of antimalarials may indeed serve to prevent the full expression of the clinical RA syndrome. This has been shown with the use of methotrexate in anti-cyclic citrullinated peptide-positive patients with undifferentiated arthritis[6]. The serological evolution and synovial features are consistent with the hypothesis that isotype expansion and epitope spreading of ACPA responses to synovial autoantigens are associated with the onset of synovitis in an individual who is genetically susceptible to RA.

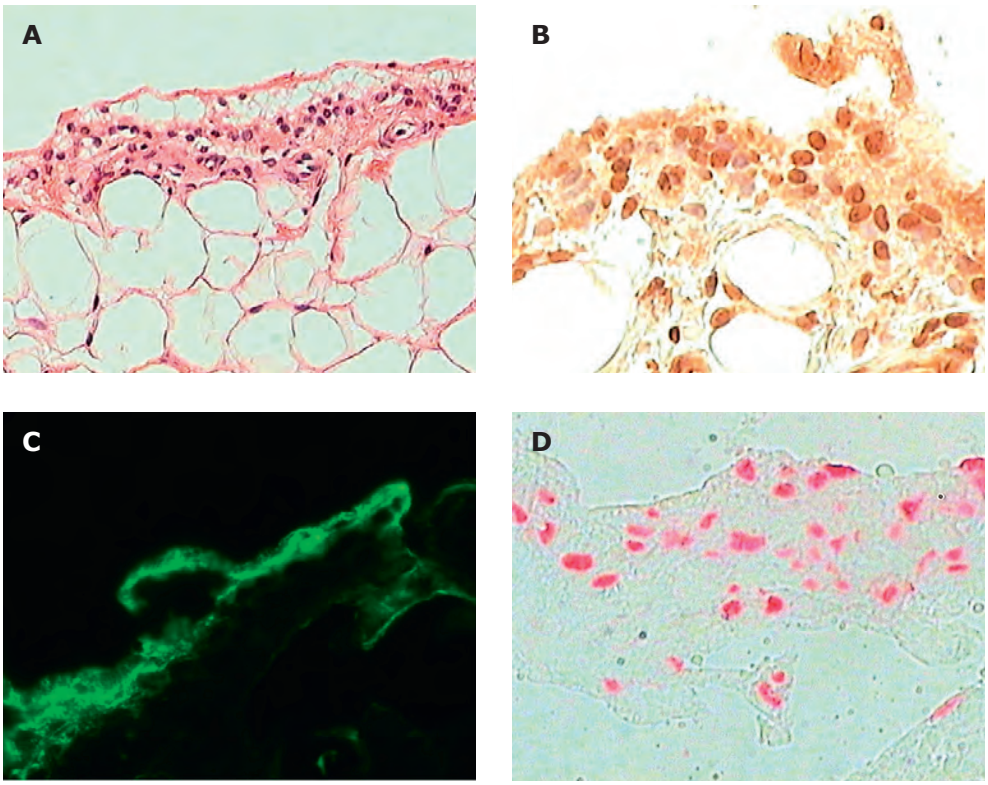

Figure 2. Synovial tissue analysis of biopsy material from a symptomatic knee joint shortly after onset of articular symptoms. A. H&E light microscopy showing marked disruption of the synovial lining cell layer. Lining cells appear to be floating in an amorphous extracellular matrix. Sublining stroma is relatively unremarkable with minimal evidence of inflammatory infiltration. **B.** Intracellular citrullinated antigens are detected by intense staining of the synovial lining cells using a polyclonal anticitrulline antibody. There is less intense staining of the surrounding matrix. **C.** Immunofluoresence staining of synovial tissue for C3 showing positive staining in lining layer. Similar results were seen with IgG, IgA, and fibrin staining. **D.** Synovial lining cells exhibit evidence for widespread apoptosis as detected by TUNEL staining. Original magnification ×200 for **A,** and ×400 for **B, C, D.**

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CHAPTER 4

The interaction between HLA shared epitope alleles and smoking and its contribution to autoimmunity against several citrullinated antigens

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ABSTRACT

Objective

Recent data suggest that a gene–environment interaction between smoking and the HLA shared epitope alleles plays a role in shaping the autoimmune reaction to specific citrullinated antigens. This study was undertaken to determine the effects of HLA shared epitope alleles and tobacco exposure on the immune response against various citrullinated antigens. These associations were analyzed in the anti–citrullinated protein antibody (ACPA)–positive stratum to control for the possibility that the associations found are explained by the known interaction between HLA shared epitope alleles and tobacco exposure on ACPA status.

Methods

In 661 patients with rheumatoid arthritis, reactivity against several citrullinated antigens from vimentin, fibrinogen, enolase, and myelin basic protein was determined by enzyme-linked immunosorbent assay. The effects of the HLA shared epitope alleles and tobacco exposure were assessed by logistic regression analysis. Biologic interaction was analyzed by investigating whether the effects of the risk factors combined exhibited departure from additivity.

Results

A significant interaction between tobacco exposure and HLA shared epitope alleles was found for the presence of ACPA as reported previously. When these interaction effects were studied for several ACPA "fine specificities," significant interactions were noted for several citrullinated peptides. However, these interactions were not present after stratification for ACPA status, indicating that the interaction between tobacco exposure and HLA shared epitope alleles influences autoimmunity not to specific citrullinated antigens, but rather to ACPA development.

Conclusion

Our data indicate that the gene–environment interaction between HLA shared epitope alleles and smoking does not appear to shape the reactivity of the ACPA response. These data suggest that smoking promotes nonspecific citrullination rather than citrullination of specific antigens.

INTRODUCTION

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

ACPA-positive and ACPA-negative disease have been shown to be associated with different genetic and environmental risk factors, fueling the hypothesis that different pathophysiologic mechanisms underlie these 2 separate disease subsets.[11,12] Some types of HLA alleles, particularly HLA–DRB1 alleles encoding an amino acid sequence called the shared epitope, are known to be associated with RA susceptibility[13], specifically susceptibility to ACPA-positive RA.[14]

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

Like the HLA shared epitope alleles, the environmental factor tobacco exposure is also known to predispose mainly to autoantibody-positive disease.[18] The combined effect of HLA shared epitope alleles and tobacco exposure has been shown to exceed the sum of their single effects, a phenomenon known as biologic interaction.[19–21] A gene–environment interaction thus exists between the HLA shared epitope alleles and tobacco exposure with regard to the development of ACPA-positive RA. This interaction displays a dose-dependent effect, as there is an even stronger gene-environment interaction present for heavy cigarette smoking.[22]

Recently, it has been reported that a specific interaction exists between the presence of HLA–DRB1*04, tobacco exposure, and the specific recognition of a peptide derived from citrullinated enolase.[23] Such epidemiologic studies might be of relevance as they link established genetic and environmental risk factors to an autoimmune response against specific citrullinated antigens that are expressed in the

inflamed joints of RA patients. Therefore, such findings might provide relevant clues to aid in the understanding of the pathologic mechanism underlying RA development and progression. More recently, however, we have reported a similar interaction for 2 peptides derived from 2 other citrullinated proteins, citrullinated vimentin and citrullinated fibrinogen.[24] These latter data indicate that the observed interaction might not be confined to an auto-immune response against a specific citrullinated antigen. Rather, these findings indicate that these interaction effects extend to several citrullinated autoantigens, which might even be explained by a general predisposition to ACPA development. In this study, we aimed to analyze in greater detail the extent of the interaction between HLA shared epitope alleles and tobacco exposure on the antigen-recognition profile of ACPA, especially in ACPA-positive patients in order to exclude the possibility that the association is caused by the presence of ACPA rather than by the presence of an autoimmune reaction to specific citrullinated epitopes.

PATIENTS AND METHODS

Patient population

The patients who were analyzed in this study were derived from the Leiden Early Arthritis Clinic (EAC) cohort (n = 661). The Leiden EAC cohort is an inception cohort of patients with recent-onset arthritis (symptom duration of <2 years) that was started at the Department of Rheumatology of the Leiden University Medical Center in 1993 and has been described in detail previously.[25] All patients fulfilled the American College of Rheumatology 1987 revised criteria for the classification of RA26 within the first year of follow-up. At baseline, patients were asked by trained research nurses if they had ever or never smoked. Cumulative smoking data were not included in these analyses, due to insufficient power. The protocols were approved by the relevant local ethics committee, and informed consent was obtained from all participants.

Genotyping

DNA was collected at baseline for genotyping of the HLA–DRB1 region by polymerase chain reaction and hybridization to sequence-specific oligonucleotides as described previously.[27] The HLA–DRB1*01:01, *01:02, *04:01, *04:04, *04:05, *04:08, *10:01, and *14:02 alleles were classified as the shared epitope alleles.

Anti–CCP-2 assays

Total IgG anti–CCP-2 in sera collected at baseline was measured by enzyme-linked immunosorbent assay (ELISA) (Immunoscan RA [Mark 2]; Euro-diagnostica). Samples with a value of >25 units/ml were considered positive according to the recommendations of the manufacturer. Patients with antibodies against CCP-2 were considered ACPA positive.

ACPA fine specificity assays

ELISAs were developed, as previously described[17], against peptides derived from citrullinated vimentin, citrullinated fibrinogen, and citrullinated α-enolase 5–20. Antibody reactivity against both the citrullinated and the noncitrullinated form of 2 linear peptides derived from vimentin (vimentin 1–16 [STCitS VSSS SY-CitCit MFGG] and vimentin 59–74 [VYAT CitSSA VCitLCit SSVP]), 2 linear peptides derived from fibrinogen (fibrinogen α 27–43 [FLAE GGGV Cit GPR VVER H] and fibrinogen β 36–52 [NEEG FFSA CitGHR PLDK K]), and 1 linear peptide derived from α-enolase (enolase 5–20 [KIHA CitEIF DSCitG NPTV]) was determined by ELISA.[28]

Briefly, streptavidin-coated preblocked microtiter plates (Thermo Scientific) were coated with the different fibrinogen, vimentin, and enolase peptides, followed by incubation with the serum samples. After washing, antibodies were detected with a rabbit anti-human IgG horseradish peroxidase–conjugated antibody (P0214; Dako) and ABTS as the coloring substrate, according to the recommendations of the manufacturer (Sigma-Aldrich).

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

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

Cutoff values for the citrulline-specific responses were calculated in each assay as previously described and were defined as 3 SD above the value of the mean concentration of the serum samples obtained from 41 control patients with gout. In fine specificity studies, citrulline-specific signals were defined as a positive signal (above the cutoff value based on findings in patients with gout) against the citrullinated antigen and a minimum difference of 0.1 in delta optical density values compared to the noncitrullinated antigen.[34]

The fine specificity reactivity was confined to the subset of ACPA-positive patients. More than 98% of the patients displaying antibodies against a specific fine specificity epitope were also anti–CCP-2 positive; therefore, we did not analyze all of the anti–CCP-2–negative patients for fine specificity repertoire.

IgM rheumatoid factor was determined by ELISA. Anti-CCP2 autoantibodies (IgA and IgG subforms) and anti-mutated citrullinated vimentin (anti-MCV) Autoantibodies were also measured by ELISA (Quanta Lite CCP version 3.1 for IgG/IgA from Inova Diagnostics and ELISAs from orgentec Diagnostika). The cutoff level for both the anti–CCP-3 test and the anti-MCV test was set at 20 arbitrary units, according to the recommendations of the manufacturers. Sera were tested for an-

tinuclear factor using indirect immuno-fluorescence at a 1:40 dilution on Hep-2000 cells (Biomedical Diagnostics).

Statistical analysis

Logistic regression analysis was performed to determine the odds ratios (ORs) and 95% confidence intervals (95% CIs) of developing autoantibody-positive RA relative to autoantibody-negative RA for the risk factors HLA shared epitope alleles and smoking. The association between the recognition of different citrullinated epitopes and a gene–environment interaction was analyzed using the same method. To take into account the effect of the known association of HLA shared epitope alleles and tobacco exposure with ACPA status, stratified analyses in ACPA-positive patients only were performed.

Biologic interaction, defined as the deviation from additivity, was evaluated by 3 measures: relative excess risk due to interaction (RERI), the attributable proportion due to interaction (AP), and the synergy index. These measures indicate significant biologic interaction if they differ from 0 (RERI and AP) or 1 (synergy index). RERI can be interpreted as the excess risk due to interaction relative to the risk without exposure. AP is interpreted as the attributable proportion of disease that is due to interaction, among persons with both exposures. The synergy index can be interpreted as the excess risk from exposure (to both exposures) when there is interaction, relative to the excess risk from exposure (to both exposures) without interaction.[35] (For a more detailed definition of these measures, see ref. 36). To obtain the parameter estimates needed for calculating these 3 measures, a logistic regression model was fitted.[21] Data were analyzed using SPSS, version 17.0. P values less than 0.05 were considered significant.

RESULTS

Effect of single risk factors on ACPA fine specificity

To investigate whether HLA shared epitope alleles and tobacco exposure are associated with specific reactivities within the total ACPA response, CCP-2 status was determined in 661 RA patients, of whom 348 were found to be anti–CCP-2 positive (Table 1). In baseline sera from 345 of these ACPA-positive RA patients, as well as in sera from 92 ACPA-negative RA patients, we determined reactivity against 2 citrullinated vimentin peptides (citrullinated vimentin 1–16 and citrullinated vimentin 59–74), 2 citrullinated fibrinogen peptides (citrullinated fibrinogen α and citrullinated fibrinogen β), citrullinated a-enolase peptide, and citrullinated MBP. More than 98% of the patients who were positive for a specific citrullinated epitope were also ACPA positive (Table 2). Among ACPA-positive RA patients, 50% displayed antibodies against citrullinated vimentin 59–74 and 12% against citrullinated vimentin 1–16. The highest percentage of reactivity was found for citrullinated fibrinogen

β, for which 70% of the ACPA-positive RA patients displayed reactivity. Reactivity against the citrullinated α-enolase peptide was found in 34% of the ACPA-positive patients, and reactivity against citrullinated MBP was found in 65% of the ACPA-positive patients. Almost all ACPA-positive patients were also positive for anti-MCV and anti–CCP-3, as previously described.[37]

An association between the presence of HLA shared epitope alleles and the recognition of virtually all citrullinated peptides was found when analyzing all RA patients, both ACPA-positive and ACPA-negative (Table 3). The strongest association for HLA shared epitope alleles was found for the recognition of the vimentin 59–74 peptide (OR 8.4 [95% CI 4.8, 14.8]). Similar associations were found for other citrullinated antigen reactivities. To determine whether the presence of the HLA shared epitope alleles influences the recognition of specific citrullinated epitopes, we performed similar analyses in the subset of ACPA-positive patients. In doing so, we controlled for the possibility that the results described above are explained by the known association between HLA shared epitope alleles and ACPA status, rather than by an influence of the HLA shared epitope alleles on specific epitope recognition. Within the subset of ACPA-positive patients, we still found a significant association between HLA shared epitope alleles and some, but not all, citrullinated epitopes (Table 4). This suggests that HLA shared epitope alleles are involved in shaping the immune response against specific citrullinated antigens.

Likewise, we performed a similar analysis to investigate the association between tobacco exposure and the recognition of citrullinated peptides. This analysis revealed smaller but similar associations (Table 3). The strongest association was found between citrullinated α-enolase recognition and tobacco exposure, with an OR of 3.0 (95% CI 1.9, 4.9). To analyze whether the observed association between tobacco exposure and the recognition of citrullinated epitopes was also specific for the recognition of certain epitopes, rather than due to the known association between tobacco exposure and ACPA status, we analyzed the association within the subset of ACPA-positive patients. An association between tobacco exposure and citrullinated α-enolase recognition remained (OR 1.7 [95% CI 1.1, 2.8]), while other citrullinated epitopes were no longer significantly associated with tobacco exposure (Table 4). This may indicate that tobacco exposure plays a modest role in broadening the immune response against some, but not all, citrullinated antigens.

Effect of risk factor interaction on ACPA fine specificity

Next, we investigated the effect of biologic interaction between HLA shared epitope alleles and smoking with regard to ACPA fine specificity. The recognition of most fine specificity peptides indicated biologic interaction between those 2 risk factors when using ACPA-negative patients as reference (Table 5). Significant interaction was ob-

served for the recognition of citrullinated vimentin 59–74, citrullinated fibrinogen β, and citrullinated MBP. The interaction effects were most prominent in the subset of patients with reactivity against citrullinated α-enolase (OR 16 [95% CI 6.1, 42.7], RERI 9.8 [95% CI -0.5, 20.1]) and citrullinated vimentin 59–74 molecules (OR 14 [95% CI 6.3, 31.5], RERI 7.8 [95% CI 0.5, 15.1]) (Figure 1A). Even stronger effects were observed when patients carrying 2 HLA shared epitope alleles were analyzed for citrullinated vimentin 59–74, yielding an OR of 32.0 (95% CI 10.9, 94.1) and an RERI of 19.7 (95% CI-10.3, 49.7) as depicted in figure 2.

Intriguingly, interaction effects were also noted in the subset of ACPA-positive patients who did not show reactivity against, for example, citrullinated vimentin 1–16. This observation indicated that the interactions detected are mainly explained by the known effects between HLA shared epitope alleles and tobacco exposure on ACPA status. To control for this possibility, we next stratified the patients with regard to ACPA status and performed similar analyses in the ACPA-positive patients only. We reasoned that if the interaction only affected specific reactivities, the interaction should be detectable in the total subset of ACPA-positive patients as well. Notably, no significant effects were observed after stratification for ACPA status (Table 6). For example, HLA shared epitope alleles and tobacco exposure no longer showed a significant interaction on the recognition of citrullinated vimentin 59–74, with a combined effect OR of 4.0 (95% CI 1.7, 9.6) and RERI of 0.3 (95% CI -2.0, 2.6), which is comparable to the association found for HLA shared epitope alleles and citrullinated vimentin 59–74 recognition only (OR 3.7 [95% CI 2.0, 6.8]). Similar effects were observed for the other fine specificity reactivities (Figure 1B).

Consistent with the results presented above, the AP due to interaction was comparable among reactivities against all of the citrullinated antigens and ACPA (Figure 3). Since the AP was calculated before stratification for ACPA, these results indicate that the interaction effects between tobacco exposure and HLA shared epitope alleles did not affect the fine specificity, but rather affected the ACPA status. Only citrullinated vimentin 1–16 displayed a slightly higher AP, but this might be a spurious finding due to the small number of RA patients displaying antibodies against citrullinated vimentin 1–16 (6%).

An analysis of the interaction between tobacco exposure and only HLA DRB1*04 shared epitope alleles resulted in similar findings, with strong interactive effects disappearing after stratification for ACPA status (data not shown). Moreover, the interaction effects between HLA shared epitope status and tobacco exposure observed in the unstratified analysis for recognition of >3 citrullinated epitopes (OR 9.7 [95% CI 4.9, 19.1], RERI 5.1 [95% CI 0.7, 9.4]) also became smaller and nonsignificant after stratification for ACPA (OR 3.1 [95% CI 1.3, 7.3], RERI -0.4 [95% CI -3.0, 2.2]).

Similarly, the subset of patients carrying 2 HLA shared epitope alleles displaying antibodies against, for example, citrullinated vimentin 59–74 did not show signs of interaction after stratification (figure 4). Taken together, these data indicate that an

interaction between HLA shared epitope alleles and tobacco exposure contributes to ACPA status, but is not likely to contribute to the recognition of specific citrullinated antigens.

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

Table 1. Baseline characteristics. Except where indicated otherwise, values are the number (%) of patients. Data on rheumatoid factor (RF) status were available for 537 patients with rheumatoid arthritis (RA), 305 patients with anti–cyclic citrullinated peptide 2 (anti–CCP-2)–positive RA, and 232 patients with anti–CCP-2–negative RA.

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

Table 2. Percentage of individuals positive for different fine specificity epitopes. Number of different citrullinated epitopes for which each patient displayed antibodies, including citrullinated vimentin 1–16, citrullinated vimentin 59–74, citrullinated fibrinogen α, citrullinated fibrinogen β, citrullinated enolase 5–20, citrullinated myelin basic protein (MBP), mutated citrullinated vimentin, and anti–cyclic citrullinated peptide 3 (anti–CCP-3). Almost all patients who were negative for anti–CCP-2 were negative for the measured citrullinated epitopes. RA= rheumatoid arthritis; ANF= antinuclear factor.

	SE_pos N	N total	%	OR	95% CI	Smoking N	N Total	%	OR	95% CI
CCP2-	153	293	52.2	1	(ref)	117	300	39.0	1	(ref)
CCP2+	270	335	80.6	3.80*	2.67 – 5.42	170	309	55.0	2.08*	1.51 – 2.88
CCP-	151	290	52.1	1	(ref)	116	297	39.1	1	(ref)
CCP+cVim1-16+	33	41	80.5	3.80*	1.70 – 8.50	23	37	62.2	2.79*	1.38 – 5.65
CCP+cVim1-16-	233	288	80.9	3.90*	2.68 – 5.67	145	268	54.1	2.00*	1.43 – 2.81
CCP-	153	292	52.4	1	(ref)	116	299	38.8	1	(ref)
CCP+cVim59-74+	148	164	90.2	8.40*	4.78 – 14.78	86	150	57.3	2.31*	1.55 – 3.44
CCP+cVim59-74-	120	168	71.4	2.27*	1.51 – 3.41	83	157	52.9	1.93*	1.30 – 2.85
CCP-	153	293	52.2	1	(ref)	117	300	39.0	1	(ref)
CCP+cFib α+	67	87	77.0	3.07*	1.77 – 5.31	39	78	50.0	1.70*	1.03 – 2.81
CCP+cFib α-	201	245	82.0	4.18*	2.81 – 6.23	130	229	56.8	2.24*	1.57 – 3.18
CCP-	150	289	51.2	1	(ref)	116	296	39.2	1	(ref)
CCP+cFib β+	184	227	81.1	3.97*	2.65 – 5.94	115	205	56.1	2.16*	1.50 – 3.11
CCP+cFib β-	75	95	78.9	3.48*	2.02 – 5.99	49	92	53.3	1.93*	1.20 – 3.09
CCP-	152	291	52.2	1	(ref)	116	298	38.9	1	(ref)
CCP+cEno+	98	110	89.1	7.47*	3.93 – 14.19	64	100	64.0	3.04*	1.90 – 4.87
CCP+cEno-	170	222	76.6	2.99*	2.03 – 4.40	105	207	50.7	1.76*	1.23 – 2.52
CCP-	153	292	52.4	1	(ref)	116	299	38.8	1	(ref)
CCP+cMBP+	183	213	85.9	5.54*	3.54 – 8.68	116	199	58.3	2.40*	1.66 – 3.47
CCP+cMBP-	85	119	71.4	2.27*	1.44 – 3.60	53	108	49.1	1.66*	1.06 – 2.58

CCP-	93	186	50.0	1	(ref)	65	179	36.3	1	(ref)
CCP+CCP3+	227	275	82.5	4.73*	3.10 – 7.23	138	245	56.3	2.26*	1.52 – 3.36
CCP+CCP3-	10	17	58.8	1.43	0.52 – 3.91	9	17	52.9	1.97	0.73 – 5.36
CCP-	77	159	48.4	1	(ref)	55	153	35.9	1	(ref)
CCP+MCV+	224	273	82.1	4.87*	3.14 – 7.55	139	244	57.0	2.36*	1.56 – 3.58
CCP+MCV-	12	18	66.7	2.13	0.76 – 5.96	7	17	41.2	1.25	0.45 – 3.46
CCP-	94	175	53.7	1	(ref)	68	172	39.5	1	(ref)
CCP+RF+	210	260	80.8	3.62*	2.36 – 5.55	138	234	59.0	2.20*	1.47 – 3.29
CCP+RF-	32	38	84.2	4.60*	1.83 – 11.55	15	36	41.7	1.09	0.53 – 2.27
CCP-	27	45	60.0	1	(ref)	17	45	37.8	1	(ref)
CCP+ANF+	155	189	82.0	3.04*	1.51– 6.14	97	172	56.4	2.13*	1.09 – 4.18
CCP+ANF-	81	102	79.4	2.57*	1.20 – 5.53	54	94	57.4	2.22*	1.07- 4.61
CCP-	153	293	52.2	1	(ref)	117	300	39.0	1	(ref)
CCP+4-8 reactivities #	167	197	84.8	5.09*	3.24 – 8.00	100	172	58.1	2.17*	1.48 – 3.18
CCP+0-3 reactivities #	58	81	71.6	2.31*	1.35 – 3.94	40	77	51.9	1.69*	1.02 – 2.80

Table 3. Odds ratio for developing antibodies against citrullinated epitopes in the presence of HLA SE alleles or TE. OR=odd ratio, CI=confidence interval, SE-pos=presence of one or two shared epitope alleles. ACPA negative individuals were used as reference group. #Number of different citrullinated epitopes recognized per individual, including cVim1-16, cVim59-74, cFibα, cFibβ, cEno5-20, cMBP, MCV and CCP3. * Statistically significant effect at 0.05 level of significance.

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

Table 4. Odds ratio for developing antibodies against citrullinated epitopes in the presence of HLA SE alleles or TE within the ACPA-positive stratum. OR=odd ratio, CI=confidence interval, SE-pos=presence of one or two shared epitope alleles. **#** Number of different citrullinated epitopes recognized per individual, including cVim1-16, cVim59-74, cFibα, cFibβ, cEno5-20, cMBP, MCV and CCP3. * Statistically significant effect at 0.05 level of significance.

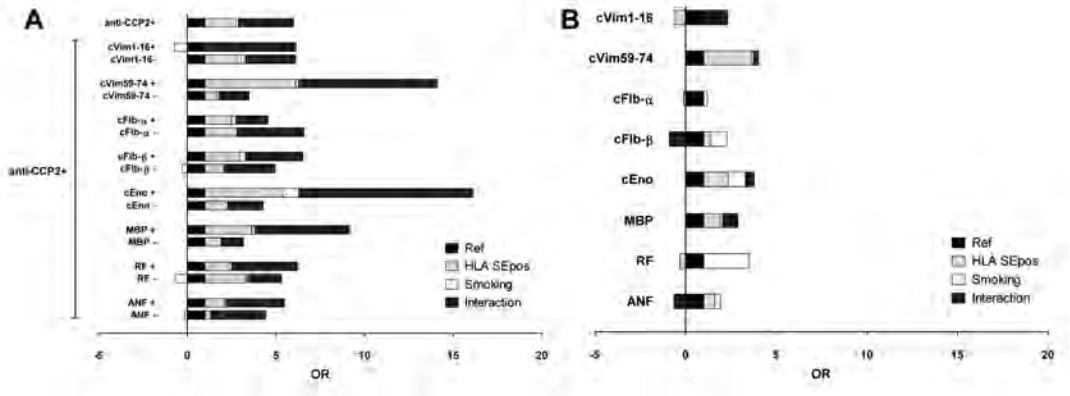

Figure 1. Risk of autoantibody-positive disease conferred by HLA SE positivity, TE and their interaction. Bars are depicted black from OR 0 to 1, light grey to indicate the risk conferred by the presence (1 or 2 alleles) of HLA SE alleles, white for the risk conferred by TE and dark grey for the interaction.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

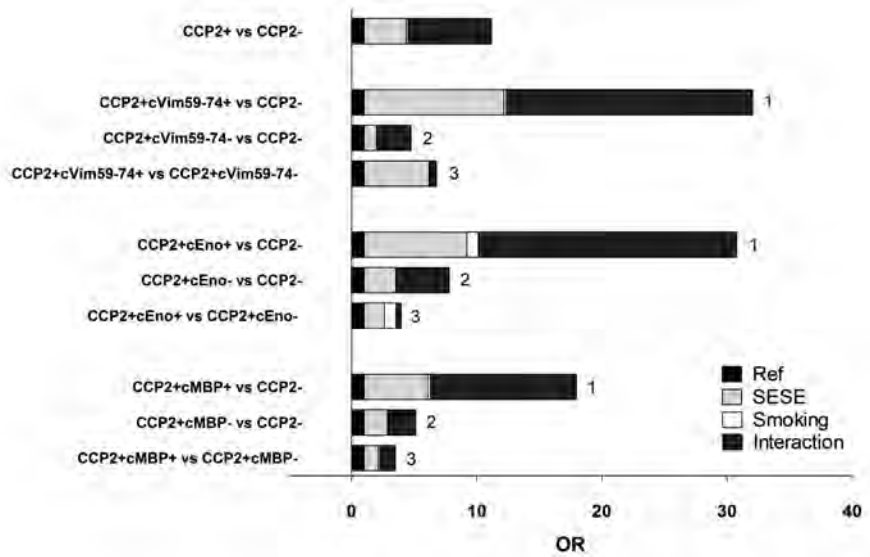

Figure 2. Risk of autoantibody-positive disease conferred by HLA SE homozygosity (SESE), TE and their interaction. Bars are depicted black from OR 0 to 1, light grey to indicate the risk conferred by the presence of two HLA SE alleles, white for the risk conferred by TE and dark grey for the interaction.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

	OR SE	OR smoking	OR SE+smoking	RERI	AP	S
CCP2+	2.74 (1.67 – 4.48)	1.14 (0.61 – 2.11)	5.99 (3.61 – 9.95)	3.11 (0.87 – 5.36)	0.52 (0.29 – 0.75)	2.66 (1.30 – 5.45)*
cVim1-16+	1.07 (0.35 – 3.33)	0.26 (0.03 – 2.18)	5.40 (2.05 – 14.19)	5.07 (0.66 – 9.48)	0.94 (0.70 – 1.18)	-6.55 (N/A)
cVim1-16-	3.03 (1.80 – 5.08)	1.26 (0.66 – 2.40)	6.10 (3.57 – 10.44)	2.82 (0.45 – 5.20)	0.46 (0.21 – 0.72)	2.24 (1.14 – 4.39)*
cVim59-74+	6.10 (2.74 – 13.60)	1.18 (0.39 – 3.60)	14.10 (6.31 – 31.48)	7.81 (0.51 – 15.12)	0.55 (0.32 – 0.78)	2.48 (1.34 – 4.57)*
cVim59-74-	1.67 (0.95 – 2.93)	1.09 (0.55 – 2.18)	3.49 (1.97 – 6.18)	1.73 (0.19 – 3.28)	0.50 (0.17 – 0.82)	3.29 (0.69 – 15.61)
cFib α+	2.49 (1.14 – 5.45)	1.24 (0.46 – 3.34)	4.54 (2.06 – 9.99)	1.82 (-0.68 – 4.31)	0.40 (-0.03 – 0.83)	2.05 (0.65 – 6.47)
cFib α-	2.80 (1.62 – 4.86)	1.03 (0.50 – 2.12)	6.59 (3.77 – 11.51)	3.75 (1.08 – 6.43)	0.57 (0.35 – 0.79)	3.05 (1.37 – 6.78)*
cFib β+	2.97 (1.67 – 5.28)	1.31 (0.64 – 2.68)	6.52 (3.63 – 11.70)	3.24 (0.40 – 6.08)	0.50 (0.21 – 0.79)	2.42 (1.06 – 5.52)*
cFib β-	2.08 (1.01 – 4.27)	0.71 (0.25 – 1.97)	4.65 (2.28 – 9.47)	2.86 (0.44 – 5.29)	0.62 (0.31 – 0.92)	4.66 (0.65 – 33.35)
cEno+	5.40 (2.00 – 14.62)	1.89 (0.55 – 6.52)	16.10 (6.07 – 42.74)	9.81 (-0.53 – 20.14)	0.61 (0.38 – 0.84)	2.85 (1.40 – 5.83)
cEno-	2.28 (1.35 – 3.86)	0.98 (0.50 – 1.94)	4.24 (2.47 – 7.28)	1.98 (0.26 – 3.70)	0.47 (0.18 – 0.76)	2.57 (0.96 – 6.89)
MBP+	3.63 (1.94 – 6.79)	1.18 (0.52 – 2.70)	9.15 (4.88 – 17.17)	5.34 (1.30 – 9.38)	0.59 (0.37 – 0.80)	2.90 (1.45 – 5.79)*
MBP-	1.90 (1.00 – 3.59)	1.05 (0.47 – 2.36)	3.18 (1.65 – 6.10)	1.23 (-0.40 – 2.85)	0.39 (-0.04 – 0.82)	2.30 (0.53 – 9.92)
MCV+	3.40 (1.84 – 6.29)	1.32 (0.62 – 2.79)	10.77 (5.48 – 21.18)	7.05 (1.12 – 12.99)	0.65 (0.44 – 0.87)	3.59 (1.63 – 7.91)*
MCV-	1.47 (0.39 – 5.54)	0.76 (0.13 – 4.40)	2.67 (0.65 – 10.93)	1.44 (-1.67 – 4.55)	0.54 (-0.31 – 1.39)	7.38 (0.0 –>100)

CCP3+	3.20 (1.78 – 5.76)	1.16 (0.55 – 2.44)	9.15 (4.88 – 17.17)	5.80 (1.24 – 10.35)	0.63 (0.42 – 0.84)	3.46 (1.58 – 7.62)*
CCP3-	1.58 (0.36 – 6.93)	2.06 (0.43 – 9.74)	2.48 (0.52 – 11.85)	-0.15 (-4.02 – 3.73)	-0.06 (-1.65 – 1.53)	0.91 (0.08 – 10.35)
RF+	2.34 (1.28 – 4.30)	1.16 (0.56 – 2.42)	6.23 (3.33 – 11.66)	3.73 (0.80 – 6.66)	0.60 (0.36 – 0.84)	3.48 (1.28 – 9.48)*
RF-	3.41 (1.07 – 10.87)	0.33 (0.04 – 3.10)	4.63 (1.40 – 15.33)	1.89 (-1.72 – 5.50)	0.41 (-0.20 – 1.01)	2.08 (0.40 – 10.75)
ANF+	2.13 (0.82 – 5.56)	1.09 (0.34 – 3.54)	5.49 (1.92 – 15.65)	3.26 (-1.19 – 7.70)	0.59 (0.18 –1.01)	3.66 (0.57 – 23.40)
ANF-	1.32 (0.47 – 3.73)	0.83 (0.23 – 3.03)	4.26 (1.42 – 12.82)	3.10 (-0.60 – 6.80)	0.73 (0.34 – 1.11)	20.79 (0 – >100)
4-8 reactivities #	4.10 (2.09 – 8.03)	1.55 (0.67 – 3.59)	9.69 (4.93 – 19.05)	5.05 (0.67 – 9.43)	0.52 (0.28 – 0.77)	2.39 (1.25 – 4.54)*
0-3 reactivities #	1.51 (0.73 – 3.14)	0.88 (0.35 – 2.25)	3.14 (1.53 – 6.45)	1.74 (0.00 – 3.48)	0.55 (0.15 – 0.96)	5.37 (0.15 – 192.71)

Table 5. Risk of autoantibody-positive disease conferred by the presence of HLA SE alleles, TE and their interaction. The interaction was considered statistically significant when the confidence intervals of the RERI and AP exclude 0 and the Synergy index exclude 1. N/A= Not applicable. CCP2 negative individuals were used as reference category. **#**Number of different citrullinated epitopes recognized per individual, including cVim1-16, cVim59-74, cFibα, cFibβ, cEno5-20, cMBP, MCV and CCP3. * Statistically significant effect at 0.05 level of significance.

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

Table 6. Risk of autoantibody-positive disease conferred by the presence of HLA SE alleles, TE and their interaction within the ACPA positive stratum. The interaction was considered statistically significant when the confidence intervals of the RERI and AP exclude 0 and the Synergy index exclude 1. N/A= Not applicable. **#** Number of different citrullinated epitopes recognized per individual, including cVim1-16, cVim59-74, cFibα, cFibβ, cEno5-20, cMBP, MCV and CCP3. ***** Statistically significant effect at 0.05 level of significance.

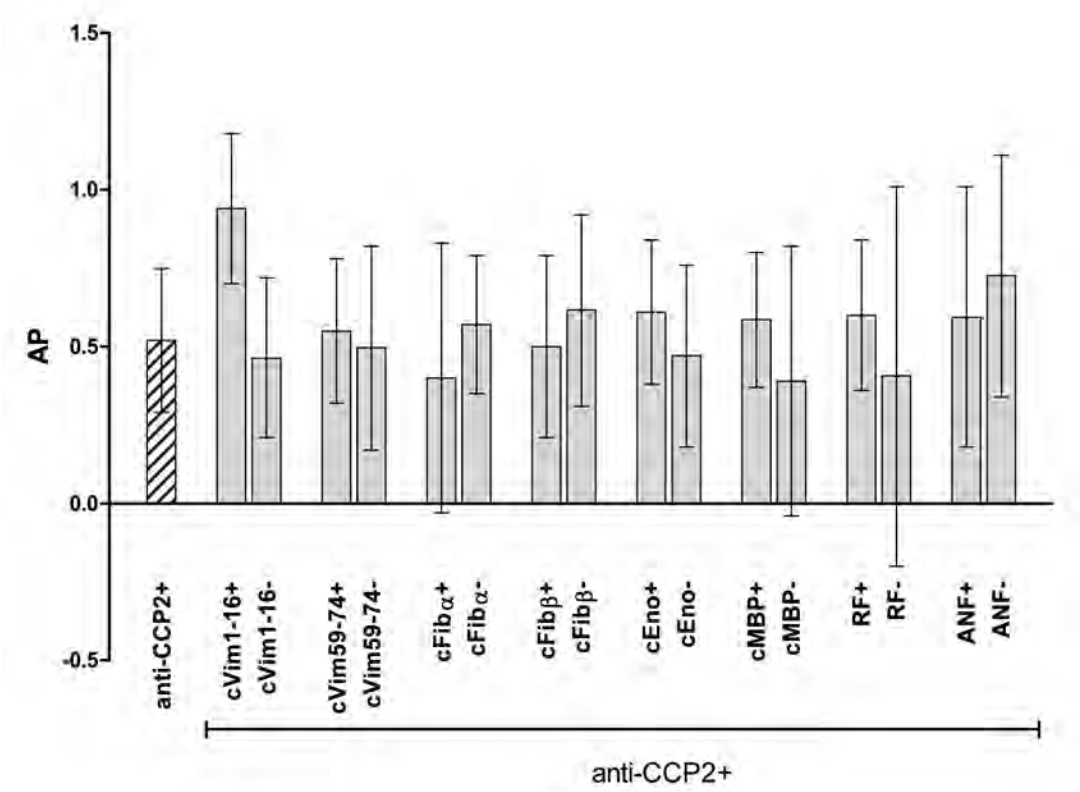

Figure 3. Attributable proportion due to interaction between HLA SE alleles and TE. Attributable proportion due to interaction was calculated by dividing the relative excess risk due to interaction by the total risk of autoantibody positive disease in patients with 1 or 2 HLA SE alleles and TE (AP=RERI/R11)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

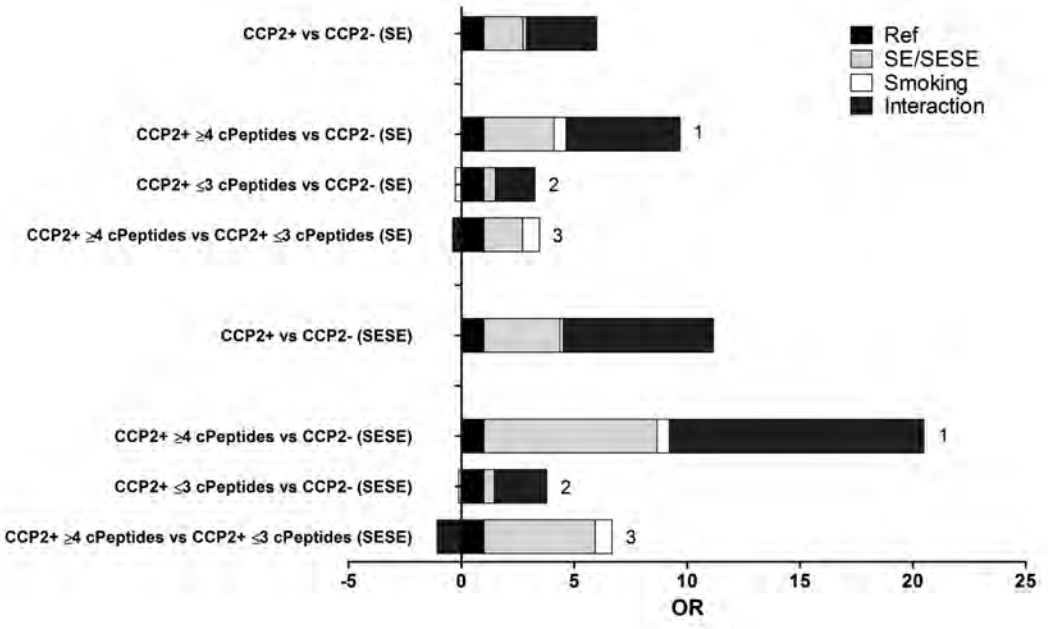

Figure 4. Risk of autoantibody-positive disease with recognition of a limited or extended number of citrullinated epitopes conferred by HLA SE-alleles, TE and their interaction. Bars are depicted black from OR 0 to 1, light grey to indicate the risk conferred by the presence of (1 or 2) HLA SE alleles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

DISCUSSION

Our results show that the apparent interaction between HLA shared epitope alleles and tobacco exposure for ACPA fine specificity disappears after stratification for ACPA status, indicating that this interaction does not greatly affect the fine specificity of the ACPA response. Recently, it has been suggested that a gene–environment interaction between HLA shared epitope alleles and tobacco exposure plays a role in shaping an autoimmune response specifically directed against a peptide derived from enolase.[23] Further experimentation resulted in similar findings for the recognition of other citrullinated epitopes that were derived from vimentin and fibrinogen.[24] These findings could be of relevance, since interactions

between genotype, smoking, and autoimmunity to certain citrullinated antigens could further expand our understanding of RA pathogenesis.

However, no stratification for ACPA status was performed in previous studies, allowing the possibility that the interaction effects were not explained by their influence on the formation of autoimmune reactions against specific citrullinated antigens, but rather by their influence on the formation of ACPA. In the present study, we specifically investigated this possibility. Careful analyses of original data previously published by our group and by Mahdi et al[23,24] suggested that no such interaction effects were present, since, for example, the AP was similar for ACPA-positive disease in the presence and, importantly, also in the absence of a specific ACPA reactivity. Therefore, we reasoned that if such interaction effects only shaped the response to certain specific epitopes and not to other citrullinated epitopes, these effects should still be observed among the subset of ACPA-positive patients. We showed that this is not the case; the ORs observed in the interaction analysis were similar to the ORs observed for the presence of HLA shared epitope alleles only after stratification for ACPA status.

Taken together, these data indicate that the gene–environment interaction between tobacco exposure and HLA shared epitope alleles does not influence the reactivity of the ACPA response. Rather, the presence of HLA shared epitope alleles seems to be the main factor in shaping the antigen recognition of the ACPA response. This is evidenced by the fact that the association between HLA shared epitope alleles and certain fine specificities, such as citrullinated vimentin 59–74 and citrullinated α-enolase, remained after stratification for ACPA status, as has been demonstrated previously.[17] Thus, these results suggest that ACPA fine specificity recognition is mainly dependent on HLA shared epitope status and that tobacco exposure and the interaction between HLA shared epitope alleles and tobacco exposure contribute little. These data also suggest that smoking promotes nonspecific citrullination rather than citrullination of specific antigens.

The findings of this study add to the discussion on the role of tobacco exposure and possibly other environmental triggers, such as infection with certain pathogens, in the pathogenesis of RA. For example, it has been postulated that the fact that immunity to some citrullinated antigens is strongly associated with both tobacco exposure and shared epitope alleles supports the hypothesis that smoking may induce citrullination of certainproteins in the lungs and that immunity to these posttranslationally modified proteins may be determined by certain HLA alleles.[38] This hypothesis is intriguing and warrants further investigation; it would also incriminate certain citrullinated proteins and/or epitopes in the break of tolerance against citrullinated antigens. However, the findings of the present study indicate that the presumed interaction effects previously described by our group and by Mahdi et al[23,24] should not be taken as evidence supporting this hypothesis, but rather as evidence that the main driver of ACPA fine specificity is found in the HLA

shared epitope alleles. In contrast, the interaction effects between HLA shared epitope alleles and smoking are more likely to be confined to the predisposition to ACPA-positive disease.

A potential limitation of our study is that the results were not corrected for multiple testing, allowing the possibility that some associations are false-positives. However, the large ORs found are consistent with previously published data.[17,23] Likewise, we cannot formally exclude the presence of an interaction between HLA shared epitope alleles and tobacco exposure for the peptides analyzed, due to insufficient power. The observed RERI for, e.g., citrullinated vimentin 59–74 (0.29 [95% CI -2.02, 2.59), is not statistically significant.[39] Clearly, a large number of patients would be needed to obtain significant RERI values. The CI surrounding this RERI further indicates that it is unlikely that an RERI of >2.6 will be found in larger studies.

Thus, even if such an interaction existed, it would be modest compared to that found in previous analyses and would not have a very strong influence on the constitution of the fine specificity repertoire. In contrast, the findings of the present study indicate that the HLA shared epitope status is mainly driving the result.

Although our data indicate that there is no clear link between environmental and genetic risk factors in the development of autoimmunity against specific citrullinated antigens, further study of other potential citrullinated autoantigens is needed. Clearly, we cannot exclude the possibility that such an interaction is involved in the development of antibodies against other citrullinated antigens. This would especially be the case in analyses that focus on "heavy smoking," since cumulative smoking exposure might affect such presumed specific interaction. We did not perform such an analysis because of insufficient power.

Taken together, our findings indicate that gene–environment interactions play no major role in shaping the reactivity of the ACPA response by broadening the ACPA recognition profile, but rather that the HLA shared epitope alleles make a significant contribution to the ACPA recognition profile.

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CHAPTER 5

Anti-citrullinated fibronectin antibodies in Rheumatoid Arthritis are associated with human leukocyte antigen-DRB1 shared epitope alleles

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ABSTRACT

Introduction

Fibronectin is one of the most abundant proteins present in the inflamed joint. Here, we characterized the citrullination of fibronectin in the joints of rheumatoid arthritis (RA) patients and studied the prevalence, epitope specificity and human leukocyte antigen (HLA) association of autoantibodies against citrullinated fibronectin in RA.

Methods

Citrullinated residues in fibronectin isolated from RA patient synovial fluid were identified by mass spectrometry. The corresponding citrullinated and non-citrullinated peptides were synthesized and used to analyze the presence of autoantibodies to these peptides in RA sera and sera from other diseases and healthy controls by ELISA. The data were compared with risk factors like shared epitope HLA alleles and smoking, and with clinical features.

Results

Five citrullinated residues were identified in fibronectin from RA synovial fluid. RA sera reacted in a citrulline-dependent manner with two out of four citrullinated fibronectin peptides, one of which contains two adjacent citrulline residues, in contrast to non-RA sera, which were not reactive. The most frequently recognized peptide (FN-Cit$_{1035,1036,}$ LTVGLTXXGQPRQY, in which × represents citrulline) was primarily targeted by anti-CCP (cyclic citrullinated peptide) 2-positive RA patients. Anti-FN-Cit$_{1035,1036}$ autoantibodies were detected in 50% of established anti-CCP2-positive RA patients and in 45% of such patients from a early arthritis clinic. These antibodies appeared to be predominantly of the immunoglobulin G (IgG) isotype and to be associated with HLA shared epitope alleles (odds ratio = 2.11).

Conclusions

Fibronectin in the inflamed synovia of RA patients can be citrullinated at least at five positions. Together with the flanking amino acids, three of these citrullinated residues comprise two epitopes recognized by RA autoantibodies. Anti-citrullinated fibronectin peptide antibodies are associated with HLA shared epitope alleles.

INTRODUCTION

Citrullination or deimination is a post-translational modification, in which a peptidylarginine is converted into a peptidylcitrulline by the enzyme family of peptidylarginine deiminases (PAD). Citrullinated proteins occur at inflamed sites in healthy individuals as well as in patients.[1,2] However, autoantibodies directed against citrullinated proteins (anti-citrullinated protein/ peptide antibodies, ACPA) are very specific for rheumatoid arthritis (RA). More than 70% of RA patients display ACPA, measured via the anti-CCP2 (cyclic citrullinated peptide 2) test, in their sera.[3,4] These antibodies are frequently present prior to disease onset and can predict the development of RA.[5,6]

It is still not fully understood how RA originates and develops, although there is experimental evidence for several steps in this process.[7] Both genetic and environmental factors have been demonstrated to contribute to the development of the disease and ACPA production. The association of several HLA-DRB1 alleles, which all share a highly conserved motif that is known as the shared epitope (SE), has already been reported many years ago.[8,9]. Other genes that have been identified as risk factors for RA include PTPN22, the TRAF1-C5 locus, PADI4, STAT4, IRF5 and CTLA-4.[10-15] Smoking has been demonstrated to be an environmental risk factor for RA and also for ACPA production in RA patients carrying SE alleles.[16,17] Other environmental risk factors that have been suggested to enhance the chance of developing RA include the exposure to mineral oil, diet restrictions and coffee intake.[18-20] However, these data still need confirmation.

Many citrullinated autoantigens (for example, fibrinogen, vimentin, α-enolase) and ACPA directed towards these citrullinated proteins have been identified in RA.[21-26] Currently, the CCP2 test, which is based on a synthetic citrullinated peptide not related to proteins occurring in the inflamed joints of RA patients, is the gold standard[27-29] for ACPA testing. ACPA have recently been included in the new American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) criteria for the classification of RA, because they are present early in the disease and can predict disease development and outcome.[5,30] The ACPA response in established RA patients is very heterogeneous and includes antibodies directed to many citrullinated proteins.[31-33]

Because it has been suggested that ACPA play an important role in the development of the disease, it is important to learn more about the autoantigens that could be involved in the generation of ACPA.[7] Several citrullinated proteins occurring in the inflamed joints of RA patients have been identified previously. In part, their citrulline-containing epitopes have been mapped, particularly using synthetic peptides[24,34] or material from cultured (non-synovial) cells (for example, HL-60 cells).[23] It remains to be established whether these epitopes are relevant from a pathophysiological point of view. ACPA tests based upon citrullinated

autoantigenic proteins may provide information on ACPA fine-specificities[35,36] and may aid the differentiation between clinically distinct RA patient subgroups, although so far no correlations between ACPA fine-specificities and clinical phenotypes have been found.[37-39]

One of the citrullinated proteins in inflamed synovial tissue identified previously is fibronectin (FN).[40,41] FN is a glycoprotein, which can be a component of the extracellular matrix (insoluble form) or present in body fluids (soluble form). FN is involved in a variety of processes, such as wound healing, haemostasis, thrombosis and embryogenesis.[42] Several findings have been published linking (citrullinated) FN with RA. For example, citrullinated FN was found to be present in synovial tissue and synovial fluid (SF) of RA patients.[41,43] It has also been detected in pannus tissue and in immune complexes present in sera of RA patients.[44,45] FN might play a role in articular cartilage destruction, because it has been observed that FN fragments can stimulate the production of multiple mediators of matrix destruction, such as various cytokines and metalloproteinases.[46,47] However, the presence and characteristics of anti-citrullinated fibronectin antibodies in RA patients have not been studied yet.

In the current study, we have mapped citrullinated residues of FN isolated from the SF of RA patients and have used this information to investigate the B-cell response to citrullinated FN in RA.

MATERIALS AND METHODS

Patient material

Synovial fluid (SF) samples from RA patients were a kind gift from Prof. Dr. B. Bozic (Department of Rheumatology, University Medical Center, Ljubljana, Slovenia). After SF samples were obtained by joint punctures (arthrocentesis), using needles with a diameter from 1.6 to 2.2 mm, they were immediately centrifuged at 2,500× g for 10 minutes at 4°C to separate insoluble and soluble components into pellet and supernatant fractions, respectively. Supernatant and pellet fractions were separately stored at -80°C within two hours after taking the samples. The pellets were resuspended in EGTA lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM KCl, 1 mM DTE, 0.1% NP40, 10 mM EGTA, 0.5 mM PMSF and protease inhibitor cocktail). Supernatant fractions were diluted in five volumes of EGTA lysis buffer. After sonification, SDS was added (final concentration 2%) and the fractions were heated and centrifuged at 12,000× g. Supernatants were used for further analysis.

Sera from (established) rheumatoid arthritis (RA; n = 110), systemic lupus erythematosus (SLE; n = 31), and primary Sjögren's syndrome (pSS; n = 31) patients were collected at the Department of Rheumatology of the University Medical Centre Nijmegen and the St. Maartenskliniek Nijmegen (The Netherlands). Sera

from multiple sclerosis (MS; n = 31) patients were collected at the MS Centrum Nijmegen (The Netherlands). Type 1 diabetes (T1D; n = 32) sera were obtained from the Department of Immunohaematology and Blood Transfusion of the Leiden University Medical Center (Leiden, The Netherlands). Early arthritis sera (EAC; n = 301) were collected at the Department of Rheumatology of the Leiden University Medical Center (Leiden, The Netherlands).[48] Tuberculosis (TB; n = 29) sera were collected at Department of Internal Medicine, Tel Aviv Medical Center, Israel. Sera from healthy individuals(NS; n = 32) were collected at the Sanquin Blood Bank in Nijmegen. Sera were stored at -70°C until use.

ACPA levels in RA sera were measured using a commercial CCP2 ELISA kit (Euro-Diagnostica A.B., Malmo, Sweden).

These studies were approved by the local ethics committees; the need for patient consent was waived by the local ethics committees.

Sample preparation and tandem mass spectrometry analysis

Two SF samples from RA patients, the supernatant fraction of one and the pellet of the other, were depleted of albumin as described by Colantonio and coworkers[49], separated by SDS-PAGE and stained with colloidal Coomassie Brilliant Blue. Each lane of the gel, containing material from an individual SF sample, was sliced into 18 pieces and the polypeptides in these gel slices were digested after the addition of 20 µL trypsin solution (15 ng/µL trypsin in 25 mM NH4HCO3 and 5 mM n-octylpyranoglucoside). Peptides were extracted by adding 50% acetonitril, 0.5% trifluoroacetic acid, 5 mM n-octylpyranoglucoside followed by sonication. The protein digests resulting from each of the gel slices were separately analyzed by nano-LC-MS/MS (using a LTQ (linear trap quadrupole) Fourier Transform Ion Cyclotron Resonance mass spectrometer (LTQ FT, Thermo Scientific, Waltham, MA, USA)). Data were converted by BioWorks SEQUEST (Thermo Electron, Waltham, MA, USA) into a peak list, which allowed peptide identification with the Mascot Search database. Additionally, citrullination sites were checked manually. Mass deviations for precursor ions were set to 20 ppm and deviations for the mass of fragment ions were set at 0.8 Da. Fixed modifications, besides citrullination, such as oxidation and methylation, were taken along during the analysis.

Synthesis of citrullinated fibronectin peptides

Peptides (Table 1) were synthesized by a solid-phase procedure using Fmoc chemistry as described previously.[50] The peptides were at least 90% pure as deduced from their elution pattern on reversed phase HPLC.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assays (ELISA) with fibronectin peptides were performed as described previously. Briefly, each well of a microtiter plate (Streptawell, Roche, Basel, Switzerland) was coated with 1 µg biotinylated peptide in 0.1 mL PBS/0.1% BSA overnight at 4°C. After washing three times with PBST0.1 (PBS, 0.1% Tween20), wells were incubated with serum samples 100-fold diluted at in PBST0.05 (PBS, 0.05% Tween20) containing 1% BSA for one hour at 37°C. After incubation, plates were washed three times with PBST0.1, followed by an incubation at 37°C with HRP-conjugated goat anti-human IgG, IgM, IgA, kappa, lambda (DAKO, Glostrup, Denmark) or with either HRP-conjugated rabbit anti-human IgG, rabbit anti-human IgM or rabbit anti-human IgA (DAKO, Glostrup, Denmark). Bound antibodies were detected by the conversion of 3,3',5,5'-tetramethylbenzidine (TMB) and, after terminating the reaction by the addition of sulfuric acid, the absorbance was measured at 450 nm. Cut-off values were determined as the mean value plus two times the standard deviation of normal human control sera.

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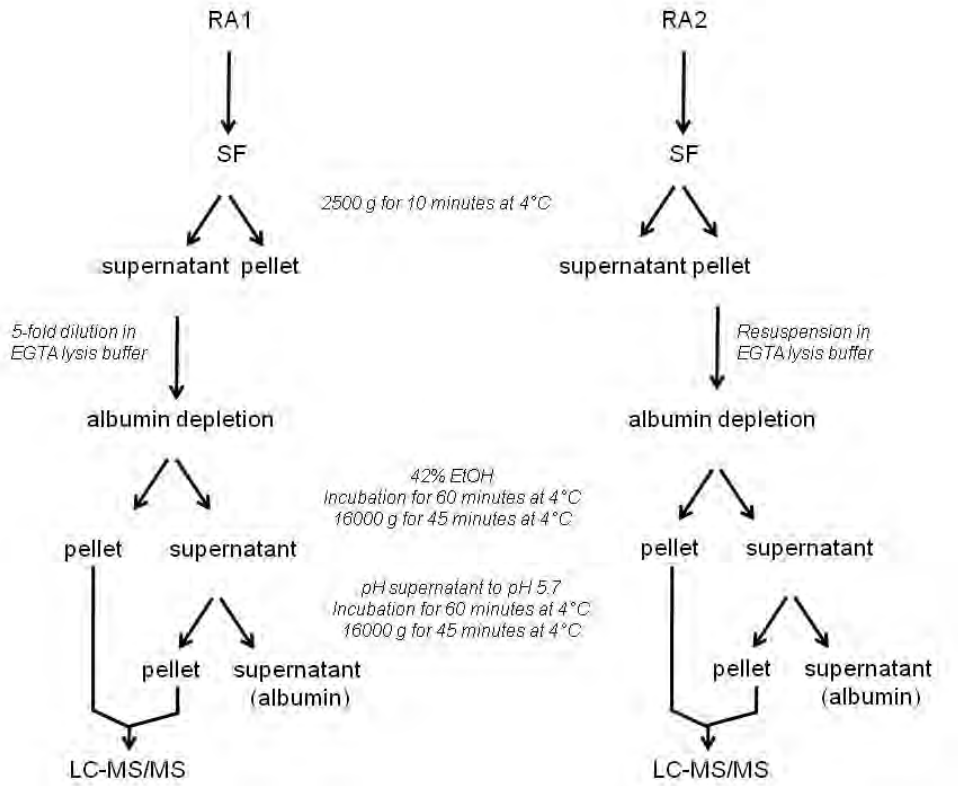
ACPA fine specificity ELISA assays using peptides derived from citrullinated vimentin, fibrinogen and α-enolase were performed as described previously.[39,51]



Supplementary Figure 1. Overview of the handling of synovial fluid samples.

Statistics

A two-tailed unpaired t-test with a CI of 95% was used to observe differences in reactivity between RA sera and non-RA sera with respect to the citrullinated peptides.

Univariate logistic regression analyses were performed for testing the association between several single risk factors (SE alleles as well as smoking) and anti-citrullinated fibronectin peptide antibodies in early arthritis patients. A Mann-Whitney U test was performed to address associations with the clinical phenotype (HAQ (health assessment questionnaire) score, VAS (visual analog scale) score, swollen joint count and Ritchie index) of the early arthritis patients.

RESULTS

Citrullinated fibronectin in synovial fluid samples of RA patients

To study the immune response to citrullinated FN in RA, first the positions of the citrulline residues in FN isolated from the inflamed joints of RA patients were mapped. One supernatant fraction and one pellet fraction from SF samples obtained from two different RA patients (RA1 and RA2, respectively) were depleted of albumin by a differential precipitation procedure as described previously.[49] This resulted in two pellet fractions for each RA SF sample, which were separated by SDS-PAGE and stained with colloidal Coomassie Brilliant Blue (CBB) (Supplementary Figure S1). Subsequently, 18 equal slices, covering the largest polypeptides (slice number 1, molecular weight >94,000) to the smallest polypeptides (slice number 18, molecular weight < 14,000) were excised from the stained gel for both samples (Figure 1A). The polypeptides present in the slices were digested with trypsin and analyzed by LC-MS/MS. The identity of polypeptides and the positions of citrullinated residues were determined by database searches using Mascot (Version 2.1.03, Matrix Science Inc, Boston, MA, USA).

To confirm the presence of citrullinated residues and to distinguish from deamidation of glutamine or asparagine residues, the peptide fragmentation patterns were inspected manually. One of the citrullinated proteins found in the SF of both patients was FN. Figure 1B shows a schematic overview of all the FN peptide sequences obtained. This indicates that (fragments of) FN were present in material from many gel slices, indicative of a large variety of FN polypeptide lengths in SF samples. FN is a protein for which at least 15 different isoforms exist Figure 1C); the canonical isoform (FN1) comprises a polypeptide of 2,386 amino acids, in which three repeats can be discerned.52 FN-derived peptides that were found by these analyses covered most of the FN1 isoform, although for several regions no peptides were detected, which may at least in part be due to poor ionization efficiencies. For one of the alternatively spliced segments, extra domain A (EDA), no

peptides were found in the data obtained, whereas several peptides demonstrating the absence of EDA were present. For IIICS, another alternatively spliced region, some peptide sequences were obtained with material from the high molecular weight fractions. Altogether, the mass spectrometry data covered 53% and 28% of the FN iso- form 1 sequence for RA1 and RA2, respectively (Figure 1B and Supplementary Figure S2).

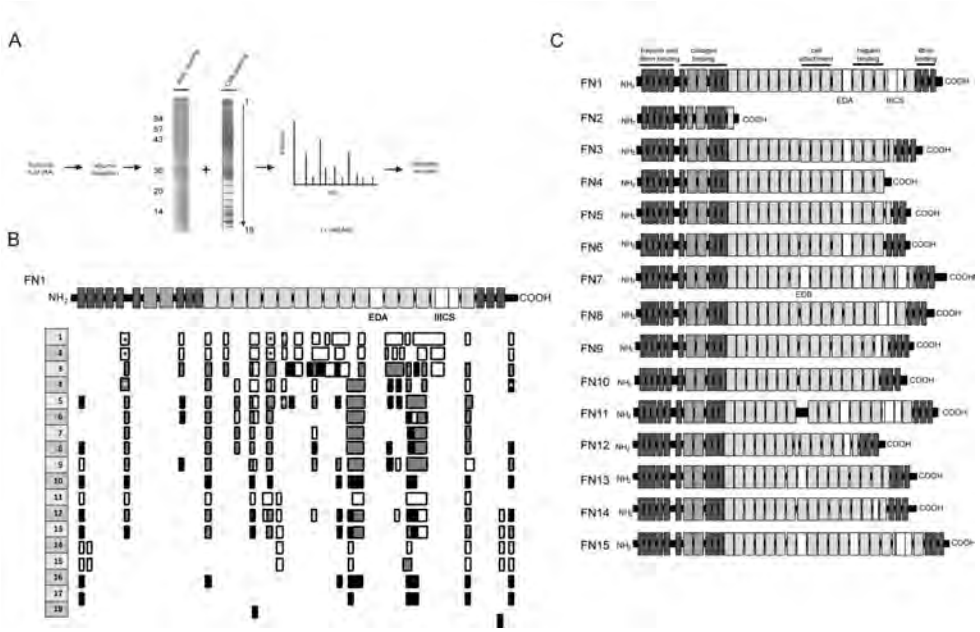
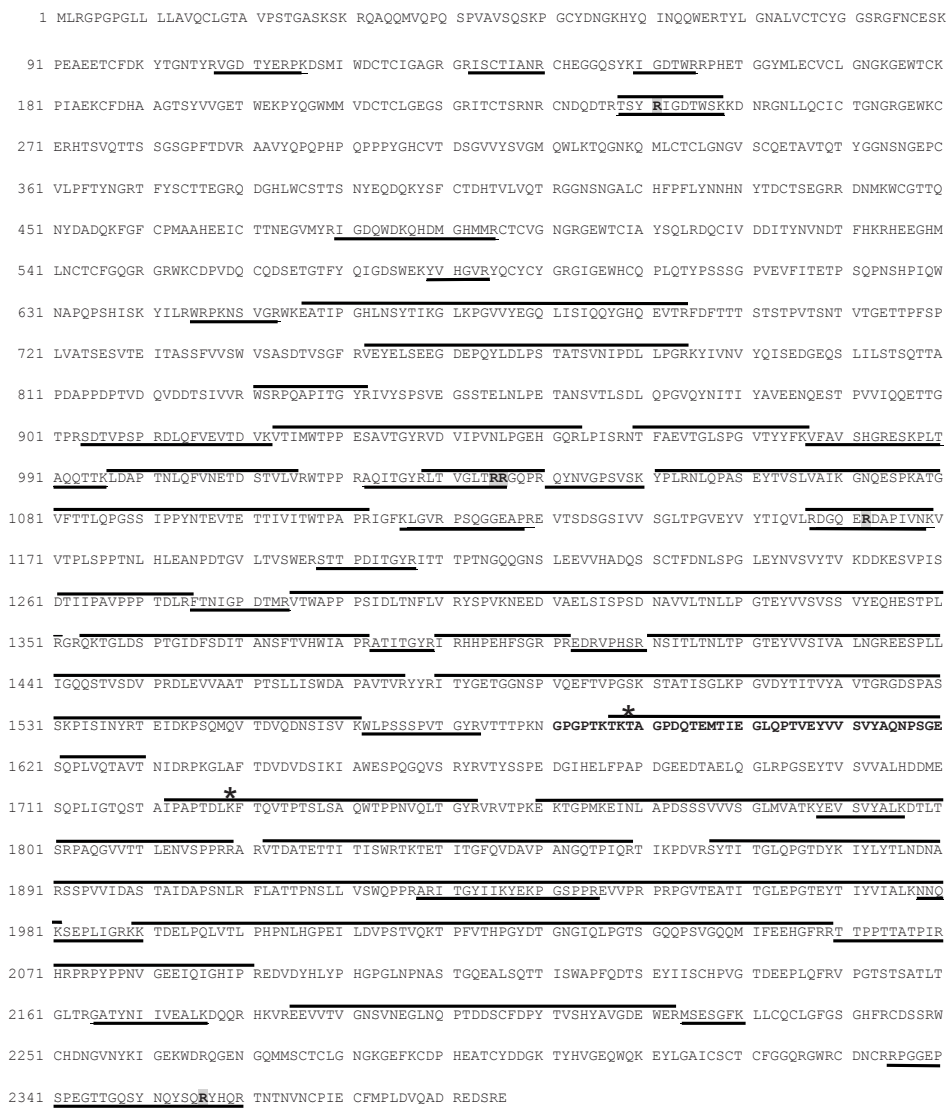


Figure 1. Identification of (citrullinated) FN in RA synovial fluid. A. RA synovial fluid samples were depleted of albumin and separated by SDS-PAGE and stained with colloidal Coomassie Brilliant Blue. Subsequently, 18 equal slices were excised from the stained gel for both samples. The polypeptides present in the slices were digested with trypsin and analyzed by LC-MS/MS. The presence of citrullinated proteins in the gel was visualized by western blotting using anti-modified citrulline (AMC) antibodies after modification of the proteins on the blot. The positions of molecular mass markers are indicated on the left (kDa). **B.** The positions of FN peptides detected in RA SF with LC-MS/MS for each of the 18 gel slices of both patient samples are schematically aligned with isoform 1 of fibronectin (FN1). The white bars represent peptides detected in RA1, the black bars peptides detected in RA2 and the grey bars peptides detected in both patients. The positions of the citrullinated residues detected are marked with asterisks. **C.** Schematic overview of the 15 different FN isoforms, resulting from alternative splicing events, documented in the UniProtKB database. The positions of the main alternatively spliced segments EDA, EDB and IIICS are indicated.



Supplementary Figure 2. Fibronectin-derived peptides identified in the synovial fluid of RA patients. The fibronectin peptides detected in synovial fluid samples of RA patients are indicated by lines above and below the amino acid sequence of human fibronectin (for RA1 and RA2, respectively) and the citrullinated residues present in these peptides are marked with a grey box. The extra domain A (EDA) is represented by the amino acids 1631 till 1720. A tryptic peptide (amino acids 1589-1629), beginning and ending with an asterisk was detected as a single peptide sequence.

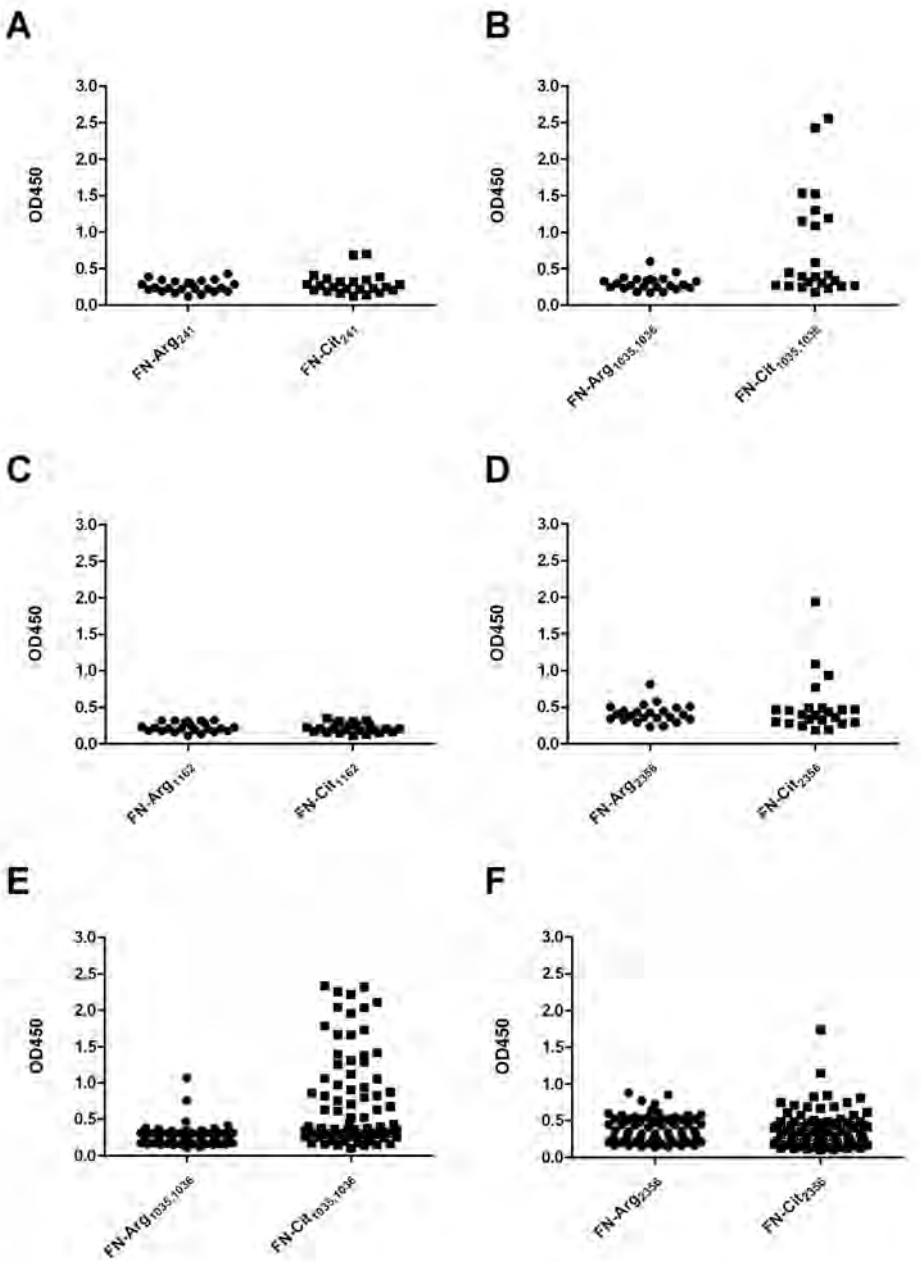


Figure 2. Recognition of citrullinated fibronectin peptides by RA sera. The four fibronectin peptide sets (**A**, FN-Arg/Cit$_{241}$; **B**, FN-Arg/Cit$_{1035,1036}$; **C**, FN-Arg/Cit$_{1162}$; **D**, FN-Arg/Cit$_{2356}$) were analyzed by ELISA with 23 sera from established RA patients. Two peptide sets (**E**, FN-Arg/Cit$_{1035,1036}$; **F**, FN-Arg/Cit$_{2356}$) were analyzed with a larger cohort obtained from established RA sera (n=80). OD450 = optical density at 450 nm.

A search for deiminated arginines identified four citrullinated FN regions containing five citrullinated residues, located at amino acid positions 241, 1035, 1036, 1162 and 2356 (Figure 1B). In RA1, four citrullinated residues were identified (positions 241, 1035, 1036 and 1162), whereas in the second patient (RA2) four citrullinated residues were identified (positions 241, 1035, 1162 and 2356) (Figure 1A and Table 1).

Anti-citrullinated fibronectin peptide antibodies are specific for RA and are associated with the ACPA response

To investigate the antigenicity of citrullinated FN, peptides comprising the citrullination sites identified were synthesized, as well as their arginine-containing counterparts (Table 1). A single peptide was synthesized for the flanking citrullination sites at positions 1035 and 1036, similar to the peptide identified in RA SF. The recognition of these peptides by antibodies in established RA patient sera (n = 23) was analyzed by ELISA. Two of these peptides, which contained either a citrulline at position 241 or at position 1162 (FN-Cit_{241} and FN-Cit_{1162}, respectively), were not recognized by RA sera. In contrast, the other two peptides (FN-$Cit_{1035,1036}$ and FN-Cit_{2356}) were reactive with RA sera, and for both this reactivity appeared to be citrulline-dependent (Figure 2A-D). To substantiate these data and to obtain an indication of the frequency by which these peptides are recognized, sera from a second, larger cohort of 80 established RA patients were analyzed. Also these sera were found to be frequently reactive with the FN-$Cit_{1035,1036}$ and FN-Cit2356 peptides (Figure 2E, F).

Forty-three percent of these sera appeared to recognize FN-$Cit_{1035,1036}$, whereas eight percent was reactive with FN-Cit_{2356}. To analyze the disease-specificity of antibodies to these citrullinated FN peptides, sera from 31 multiple sclerosis (MS), 32 type 1 diabetes (T1D), 31 primary Sjögren's syndrome (pSS), 31 systemic lupus erythematosus (SLE) and 29 tuberculosis (TB) patients, and from 32 healthy individuals, in parallel with 75 established RA sera, were analyzed by ELISA. The results showed that less than two percent of the control sera was reactive with FN-$Cit_{1035,1036}$, whereas one percent displayed reactivity with FN-Cit_{2356} (Figure 3).

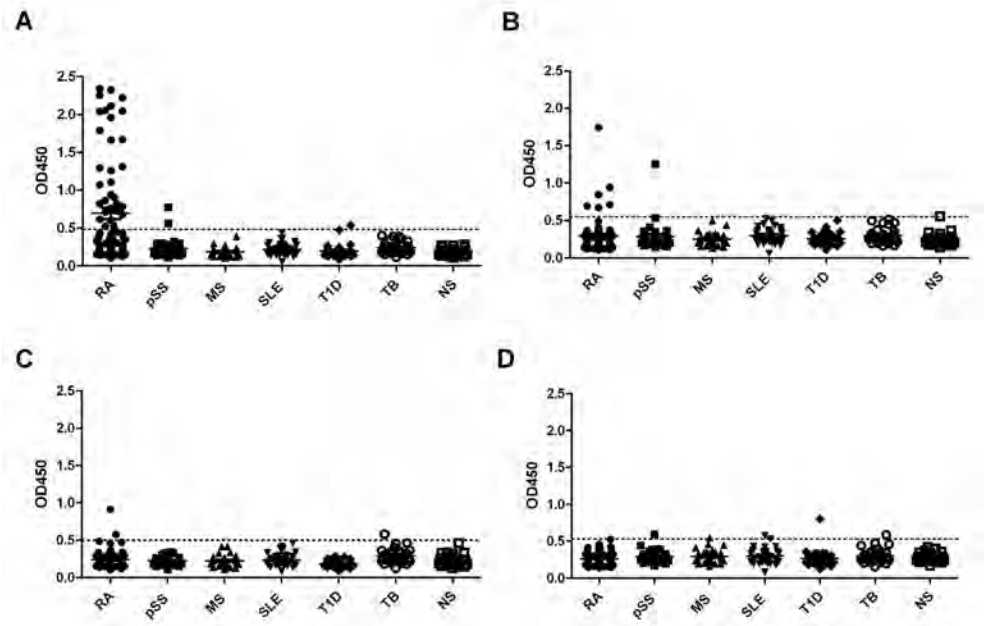


Figure 3. Recognition of citrullinated fibronectin peptides by diseased and healthy control sera. The peptides FN-Arg/Cit$_{1035,1036}$ and FN-Arg/Cit$_{2356}$ were used to study the specificity of the anti-FN antibodies. **A**. Reactivity of RA sera and control sera to FN-Cit$_{1035,1036}$. **B**. Reactivity of RA sera and control sera to FN-Cit$_{2356}$. **C**. Reactivity of RA sera and control sera to FN-Arg$_{1035,1036}$. **D**. Reactivity of RA sera and control sera to FN-Arg$_{2356}$. RA (n=75); pSS = primary Sjőgren's syndrome (n=31); MS = multiple sclerosis (n=31); SLE = systemic lupus erythematosus (n=31); T1D = type 1 diabetes (n=32); TB = tuberculosis (n=29); NS = normal human sera (n = 32). Broken lines indicate cutoff values (mean + 2*SD of NS data); OD450 = optical density at 450 nm.

Recently, it was shown that RA patients can be divided into two subsets based upon the presence or absence of ACPA in their sera and the CCP2 test appeared to be very suitable to differentiate between these subsets.[53] The analysis of anti FN-Cit$_{1035,1036}$ reactivity in 131 anti-CCP2-positive and 28 anti-CCP2-negative RA sera (Figure 4) showed that the anti-citrullinated fibronectin antibodies were hardly present in anti-CCP2-negative RA sera, corroborating the idea that these antibodies are part of the ACPA response in anti-CCP2-positive RA patients. The combined analyses of the established RA sera resulted in a prevalence of 50 percent for the autoantibodies to peptide FN-Cit$_{1035,1036}$ (Table 2), LTVGLTXXGQPRQY (X represents citrulline), in CCP2-positive established RA sera (n = 82).

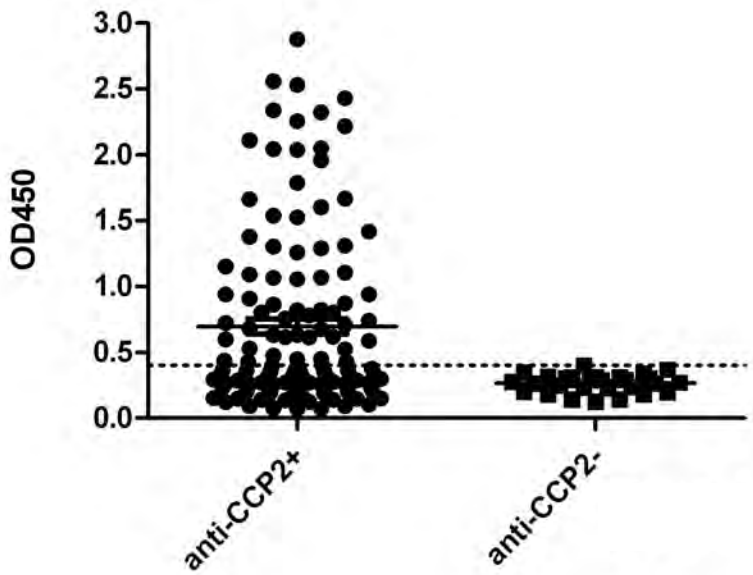


Figure 4. Anti-citrullinated fibronectin peptide antibodies in relation to anti-CCP2 positivity. The peptide set FN-Arg/Cit$_{1035,1036}$ was used to study the presence of anti-FN antibodies in anti-CCP2 positive (n=138) and anti-CCP2 negative (n=28) RA sera. The broken line indicates the cut-off value (mean + 2*SD of data obtained with normal human control sera); OD450 = optical density at 450 nm.

Autoantibodies against citrullinated fibronectin peptide are present early in the disease

Autoantibodies against CCP2 peptides have been demonstrated to be detectable very early in the disease. Moreover, their presence in pre-disease sera predicts the development of RA.[5,6,54] The diversification of the ACPA response was found to occur mainly in the pre-disease stage. To investigate whether autoantibodies against citrullinated FN are also detectable in the early stages of RA development, a number of sera from early arthritis (EAC) patients (CCP2-negative, n = 23; CCP2-positive, n = 24) was analyzed. As observed before for the established RA sera, also the EAC sera reactive with the FN-Cit$_{1035,1036}$ peptide represented a subgroup of the anti-CCP2-positive patients (Figure 5A). To substantiate these data and to obtain an indication of the frequency by which the FN-Cit$_{1035,1036}$ peptide was recognized, additional CCP2-positive early arthritis sera (n = 278) were analyzed. Forty-five percent of these CCP2-positive EAC sera appeared to be reactive with the citrullinated fibronectin peptide containing citrulline residues at positions 1035 and 1036 (Figure 5B and Table 2). Only a small fraction of these early arthritis sera (4%) displayed some reactivity with the corresponding arginine-containing peptide (Figure 5B).

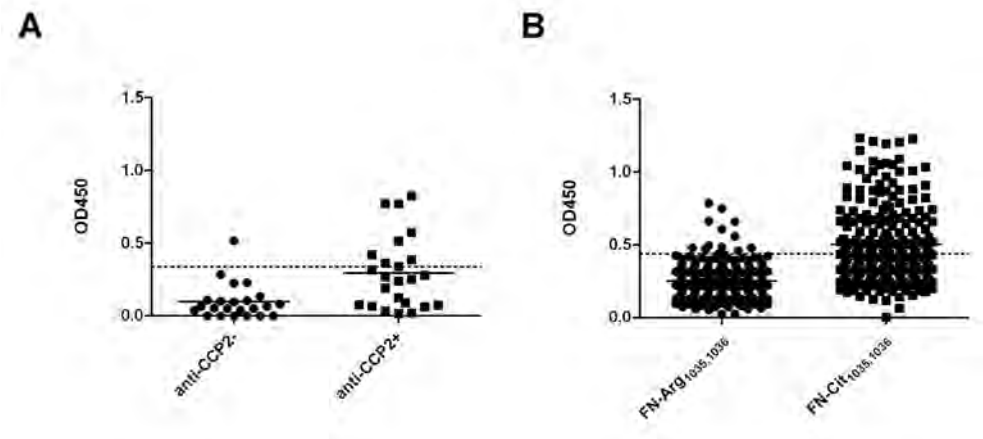


Figure 5. Anti-citrullinated fibronectin peptide antibodies in early arthritis patient sera. **A**. Sera from anti-CCP2-negative (n=23) and anti-CCP2-positive (n=24) early arthritis patients were analyzed in the FN-$Cit_{1035,1036}$ ELISA. **B**. Sera from anti-CCP2-positive early arthritis patients (n=278) were analyzed in the FN-$Cit_{1035,1036}$ ELISA. The broken line indicates the cut-off value (mean + 2*SD of data obtained with normal human control sera); OD450 = optical density at 450 nm.

It is known that after disease onset ACPA isotype switching may occur.[55] To investigate isotype switching of antibodies to citrullinated FN, 23 anti-FN-$Cit_{1035,1036}$-positive sera for which both samples taken at baseline and approximately seven years after disease onset were avail- able, were selected and analyzed in ELISA with isotype-specific (IgG, IgM and IgA) secondary antibody conjugates. At baseline (t = 0), 87% of the reactive sera contained immunoglobulins of the IgG isotype (Figure 6A), whereas 13% contained IgM (Figure 6C) and 4% contained IgA (Figure 6E) type reactivities.

After a median follow-up time of seven years (t = 7) the frequency of IgG type antibodies to FN-$Cit_{1035,1036}$ was slightly decreased to 74% (Figure 6B). In these patients the frequency of IgM type anti-FN-$Cit_{1035,1036}$ antibodies decreased to 4% (Figure 6D) In contrast, at this stage the frequency of IgA type antibodies to this citrullinated FN peptide increased to 13% (Figure 6F). All IgM- and IgA-positive patients were also positive for IgG, whereas the simultaneous presence of IgM and IgA reactivities in the same patients was observed in one patient. Except for two patients, in which the IgG type anti-FN-$Cit_{1035,1036}$ antibodies disappeared, the presence of these IgG antibodies did not markedly change in time (Figure 6G).

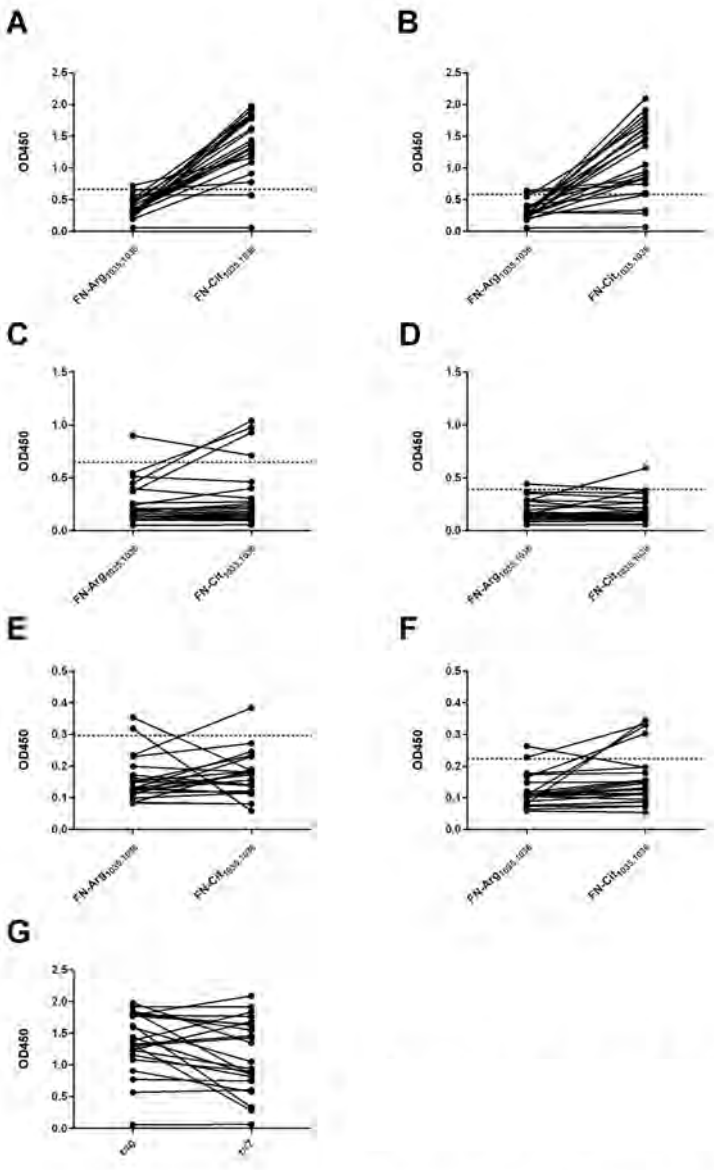


Figure 6. Anti-FN-Cit$_{1035,1036}$ antibodies are predominantly of the IgG isotype.
Sera from a subset of anti-FN-Cit$_{1035,1036}$-positive early arthritis patients (n=23) taken at baseline (t=0) and after a median follow-up of seven years later (t=7) were analyzed for different anti-FN-Cit$_{1035,1036}$ antibody isotypes in ELISA. **A.** IgG isotype reactivity at t=0. **B.** IgG isotype reactivity at t=7. **C.** IgM isotype reactivity at t=0. **D.** IgM isotype reactivity at t=7. **E.** IgA isotype reactivity at t=0. **F.** IgA isotype reactivity at t=7. **G.** Anti-FN-Cit$_{1035,1036}$ IgG reactivity at t=0 and t=7. The broken lines indicate the cut-off values determined by normal human control sera.

Anti-citrullinated fibronectin peptide antibodies are associated with HLA SE alleles

The HLA-DRB1*01, HLA-DRB1*04, HLA-DRB1*10 and HLA-DRB1*14 alleles comprise the group of HLA SE alleles, which are associated with RA.[56] The reactivity to FN-Cit$_{1035,1036}$ was compared with the HLA-DRB1 alleles of the 278 early arthritis patients. The results showed that the presence of HLA SE alleles is associated with the production of anti-citrullinated fibronectin antibodies, because patients carrying HLA SE alleles are more than two times more likely to have autoantibodies against citrullinated fibronectin (OR = 2.11; Table 3 and Supplementary Table S1). When addressing individual HLA-DRB1 alleles, only HLA-DRB1*04 (OR = 1.5), and HLA-DRB1*10 (OR = 1.57) showed a weak to moderate association with the presence of anti-FN-Cit$_{1035,1036}$ antibodies. Although a more pronounced association was observed with HLA-DRB1*08 (OR = 2.45) and HLA-DRB1*16 (OR = 2.43), these data should be interpreted with care because the carriage of these alleles is rare and the data are based on only a low number of patients. Interestingly, a negative association was observed between the presence of anti-citrullinated fibronectin antibodies and two additional HLA-DRB1 alleles, HLA-DRB1*09 and HLA-DRB1*11 (OR = 0.19 and OR = 0.41, respectively) (Supplementary Table S1).

In addition, the potential relationship of smoking with the production of anti-citrullinated fibronectin antibodies was assessed and a weak association was found (OR = 1.42). We did not observe significant associations between the presence of anti-citrullinated fibronectin antibodies and clinical parameters, such as VAS score, HAQ score, Ritchie index or swollen joint count at baseline (data not shown).

Peptide name	Peptides identified in RA SF[a]	Synthetic peptide[a]	Citrulline residues identified in RA1	Citrulline residues identified in RA2
FN-Cit$_{241}$	DTRTSY**X**IGDTWSK, TSY**X**IGDTWSK	DTRTSY**X**IGDTWSZO	X	X
FN-Arg$_{241}$		DTRTSYRIGDTWSZO		
FN-Cit$_{1035,1036}$	LTVGLT**XX**GQPR, AQITGYRLTVGLT**X**R	LTVGLT**XX**GQPRQYZO	X	X[b]
FN-Arg$_{1035,1036}$		LTVGLTRRGQPRQYZO		
FN-Cit$_{1162}$	DGQE**X**DAPIVNK	LRDGQE**X**DAPIVNZO	X	X
FN-Arg$_{1162}$		LRDGQERDAPIVNZO		
FN-Cit$_{2356}$	RPGGEPSPEGTTGQSYN-QYSQ**X**YHQR	YNQYSQ**X**YHQRTNZO		X
FN-Arg$_{2356}$		YNQYSQRYHQRTNZO		

Table 1. Sequences of synthetic fibronectin peptides. [a] X: citrulline; Z: aminohexanoic acid; O: biotinylated lysine**.** [b] Only citrulline at position 1035 was identified

Patient group	n	anti-FN-Cit$_{1035,1036}$ positive	sensitivity (%)	specificity (%)
established RA[a]	82	41	50.0	
early RA[a]	278	124	44.6	
Controls	186	3	1.6	98.4
non-RA	154	3	1.9	98.1
Healthy	32	0	0	100

Table 2. Sensitivity and specificity of anti-citrullinated fibronectin antibodies. [a] Based upon CCP2-positive sera

		Anti-cit FN neg. (%)[d]	Anti-cit FN pos. (%)[d]	OR (95% CI)
Smoking[a]	-	63 (46.7)	40 (38.1)	1.42 (0.85 - 2.39)
	+	72 (53.3)	65 (61.9)	
HLA SE alleles[b]	-	39 (26.5)	57 (14.6)	2.11 (1.13 - 3.92)
	+	108 (73.5)	213 (85.4)	

Table 3. Association of anti-citrullinated fibronectin antibodies with smoking and SE alleles in anti-CCP2-positive early arthritis patients. [a] Based upon information obtained from 240 RA patients. [b] Based upon information obtained from 270 RA patients. OR = odds ratio; 95% CI = 95% confidence interval.

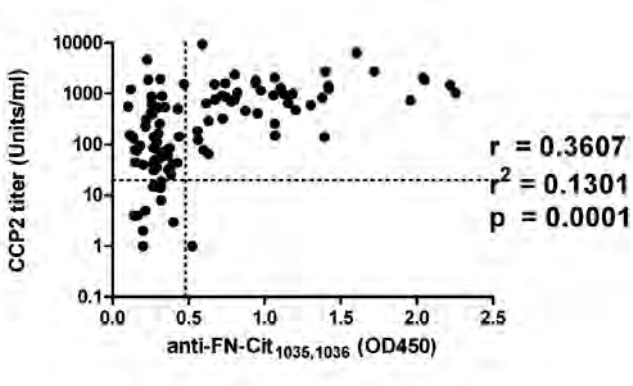
DISCUSSION

The analysis of citrullinated proteins in the synovial fluids of two rheumatoid arthritis patients revealed fibronectin as one of the multiply citrullinated proteins in both patients. Two of the four synthetic peptides that were derived from the citrullinated regions of FN appeared to be reactive with ACPA in the sera of RA patients. The most frequently targeted FN peptide, FN-Cit$_{1035,1036}$, which contains two adjacent citrullines, was recognized by 50% of established and 45% of early ACPA-positive RA patients. Like ACPA in general, anti-FN-Cit$_{1035,1036}$ antibodies appeared to be associated with HLA-DRB1 shared epitope alleles.

FN is a complex protein, characterized by the presence of three types of repeats in its polypeptide sequence, for which many isoforms resulting from alternative splicing events have been described (Figure 1C). The UniProtKB database provides details of 15 isoforms, with isoform 1 (FN1 in Figure 1C) as the canonical sequence. The overall sequence coverage of the mass spectrometry datasets relative to FN1 comprises 53% and 28% for both synovial fluid samples, respectively. It is likely that multiple FN isoforms are expressed in the inflamed synovia of RA

patients and our data do not allow drawing conclusions on their relative abundance. Three regions of FN are especially prone to alternative splicing; at the protein level these are termed extra domain A (EDA), extra domain B (EDB) and type III connecting segment (IIICS). Although peptides were found that match part of the IIICS sequence, no peptides were detected for EDA and EDB. Moreover, a few peptides provide evidence for the absence of EDA and EDB in the isoform(s) they originate from, because their sequences covered the regions immediately N- and C-terminal from EDA or from EDB. The EDA domain, also designated EIIIA, has been implicated in inflammation, because it was shown to be involved in Toll-like receptor (TLR) 4 activation.[57] Although our data do not support the presence of EDA-containing FN isoforms in the synovial fluid of RA patients, it has been demonstrated previously that EDA-containing FN is produced in the RA synovium and is expressed abundantly in RA SF.[58,59] Moreover, recently Lefebvre and colleagues showed that EDA-containing FN stimulated leukotriene synthesis and neutrophil recruitment via TLR activation in a mouse model.[60] Although we could not detect any peptides in the EDA region, our data do not exclude the presence of EDA-containing FN isoforms in RA synovial fluid samples, because we have only analyzed a limited number of patients or because of technical limitations, such as ionization efficiencies. The interpretation of the data is further complicated by the fact that FN-derived peptides were found in material from many gel slices, which indicates a high heterogeneity of FN polypeptide lengths. It is likely that this is at least in part due to the presence of proteolytic enzymes in the synovial fluid from inflamed joints, which cleave the FN polypeptides in different fragments. It is known that during inflammation proteases are active in synovial fluid and can contribute to RA pathogenesis.[61,62] Indeed, the fragmentation of FN in inflammatory SF[63], as well as in cartilage of RA and osteoarthritis patients[64] has been reported previously. Our analyses identified five citrullinated residues in FN from RA patient SF. Except for two adjacent citrullines, these residues are found in distant regions of the FN polypeptide chain. Our data do not provide information on the extent to which these residues are citrullinated in RA SF and it is likely that differences between patients exist. This is substantiated by the observation that in material from RA2 for the region containing the two adjacent citrullines only one of these two residues (1035) was found to be citrullinated, whereas both were citrullinated in RA1. Two other studies have previously reported the presence of citrullinated FN in SF and synovial tissue of RA patients.[41,43] However, these studies did not reveal the positions of the citrullinated residues. Our data do not exclude the possibility that FN is also citrullinated at other positions in RA SF, because material from only two patients was analyzed in detail and the sequence coverage was not more than 53%. Moreover, if citrullination enhances FN's susceptibility to proteolytic cleavage, citrullinated peptides may have escaped detection as a result of cleavages by endogenous proteases that cleave the polypeptide close to the citrullinated residue.

A synthetic peptide approach to investigate the recognition of citrullinated epitopes of FN by RA autoantibodies revealed that the major auto-epitope is located in the region containing the two adjacent citrullines (amino acids 1035 and 1036). Only one of the other three citrullinated peptides was recognized by some RA sera. The fact that RA sera were only reactive with two of these peptides is consistent with the results of other studies showing that the amino acids flanking the citrulline residue contribute to the formation of auto-epitopes.1 Several studies[24,34,65] with synthetic citrullinated peptides (derived from vimentin, fibrinogen and α-enolase) showed that not all peptides containing citrullinated residues are recognized by patient sera, indicating that not only the citrulline is important, but also the amino acids surrounding the citrullinated residue. However, it should be noted that the use of synthetic peptides in general does not allow the identification of reactivities with conformational and/or discontinuous epitopes. Therefore, our data do not exclude the possibility that the citrullinated residues identified are part of conformational epitopes and that autoantibodies to these epitopes may be present in RA patient sera. Although autoantibodies to FN have been detected in RA as well as SLE patients before[66,67], our data are the first to describe antibodies that target FN in a citrulline-dependent manner. The autoantibodies that can be detected with the FN-$Cit_{1035,1036}$ peptide represent a subset of ACPA, which is substantiated by the lack of correlation between the levels of reactivity with CCP2 and with FN-$Cit_{1035,1036}$ (Supplementary Figure S3). In total, 50% of the (anti-CCP2-positive) established RA patients showed reactivity to FN-$Cit_{1035,1036}$, compared to two percent of the controls (non-RA and healthy individuals; Table 2).



Supplementary Figure 3. Correlation between anti-CCP2 and anti-FN-$Cit_{1035,1036}$ reactivities. Cor0lation diagram showing the levels of anti-FN-$Cit_{1035,1036}$ and anti-CCP2 reactivities of (established) RA sera (n=110). Broken lines represent the cut-off values. OD450 = optical density at 450 nm.

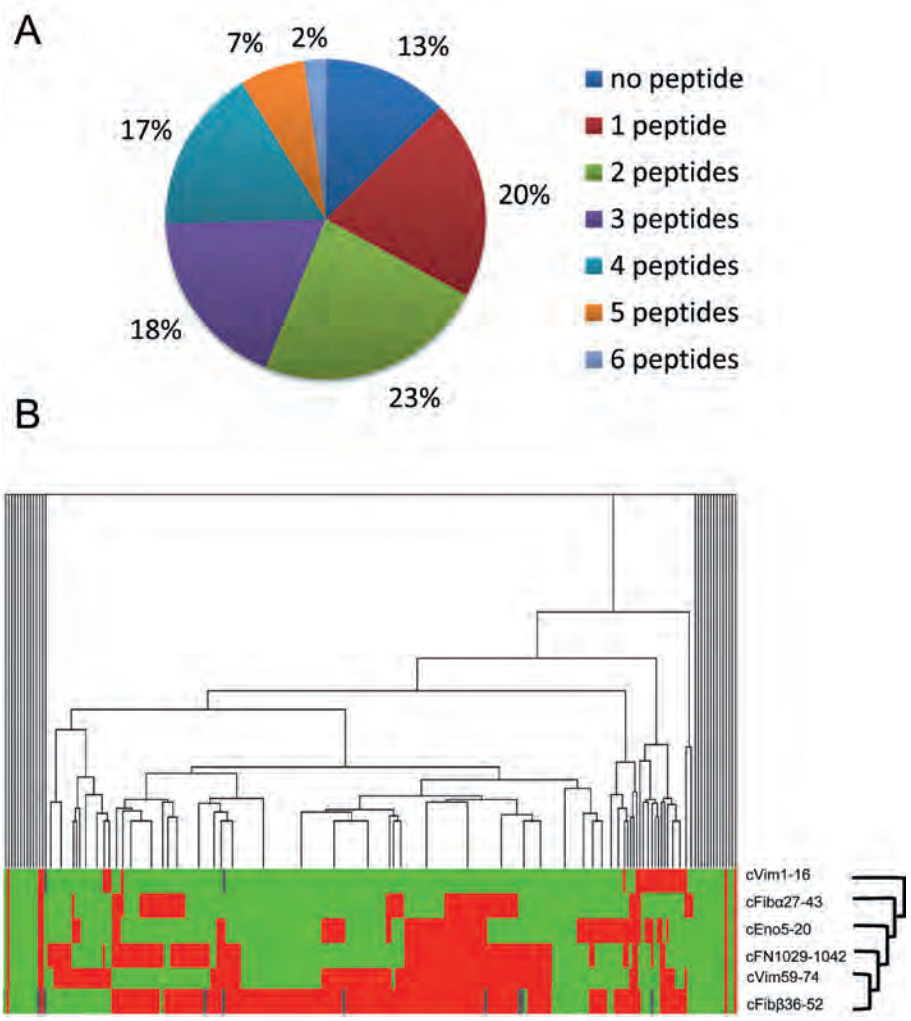


Figure 7. Anti-FN-Cit$_{1035,1036}$ in relation to other ACPA. The reactivity of EAC sera (n=228) with FN-Cit$_{1035,1036}$ was compared with the presence of antibodies to other citrullinated peptides, which are derived from vimentin (1-16: STCitSVSSSSYCitCitMFGG and 59-74: VYATCitSSAVCitLCitSSVP), fibrinogen (α-fibrinogen 27-43: FLAEGGGVCitGPRVVERH; β-fibrinogen 36-52: NEEGFFSACitGHRPLDKK) and α-enolase (5-20: KIHACitEIFDSCitGNPTV) 1. **A.** Fraction of patients recognizing 0 – 6 citrullinated peptides. **B.** Heat map showing the presence of antibodies to the citrullinated peptides obtained by an unsupervised cluster analysis. Red and green mark positive and negative sera, respectively. Missing values are depicted in grey.

The possibility existed that the anti-FN-Cit$_{1035,1036}$ subset of ACPA overlapped with another subset that has been identified previously, as a result of epi-

tope similarities. ACPA fine-specificity data were available for most of the EAC patient sera. The reactivity of these sera with FN-$Cit_{1035,1036}$ was compared with their reactivity with two citrullinated peptides derived from vimentin (vimentin-1-16: STCitSVSSSSYCitCitMFGG; vimentin-59-74: VYATCitSSAVCitLCitSSVP), two peptides derived from fibrinogen (α-fibrinogen-27-43: FLAEGGGVCitGPRVVERH; β-fibrinogen-36-52: NEEGFFSACitGHRPLDKK) and one peptide derived from α-enolase (α-enolase-5-20: KIHACitEIFDS- CitGNPTV).[39] These analyses showed that most of the EAC sera recognized multiple citrullinated epitopes (Figure 7A). A large overlap of anti-FN-$Cit_{1035,1036}$ with reactivities to any of the other peptides was observed, as might be expected from previous data.[26,68] However, some sera appeared to react exclusively with FN- $Cit_{1035,1036}$ and not with the other citrullinated epitopes (Figure 7B). These data are underscoring the large heterogeneity of the ACPA response in RA and indicate that the anti-FN-$Cit_{1035,1036}$ antibodies are one of the most abundant ACPA subclasses that can be detected with synthetic peptides derived from citrullinated synovial proteins. Taken together, these data indicate that this citrullinated FN peptide may be used to investigate the fine-specificity of ACPA in more detail and may be complementary to other citrullinated molecules in ACPA profiling.

The anti-FN-$Cit_{1035,1036}$ antibodies present in early arthritis patients appeared to be predominantly of the IgG isotype. Only a small percentage of sera was found to contain IgM or IgA type anti-FN-$Cit_{1035,1036}$ antibodies. After a median follow-up of seven years, the IgG type reactivities were hardly changed, whereas the prevalence of IgM reactivity against FN-$Cit_{1035,1036}$ was decreased, and that of IgA was somewhat increased. Similar observations have been reported for samples from undifferentiated arthritis patients (who developed RA) taken either at baseline or after one year follow-up.[36] It remains an open question whether the anti-citrullinated FN antibodies play a pathophysiological role. FN-containing immune complexes are likely to be formed in the inflamed joints of RA patients and this may occur already early during disease development. Further studies will be required to elucidate whether citrullinated FN is involved in the inflammatory process.

The presence of anti-citrullinated FN antibodies in the early arthritis patients was associated with HLA SE alleles (OR = 2.11). Of the individual SE alleles, only HLA-DRB1*04 and HLA-DRB1*10 showed a weak association with the presence of anti-citrullinated FN peptide antibodies (Supplementary Table S1). Previously, Snir and co-workers demonstrated that antibodies against multiple citrullinated antigens (for example, vimentin, fibrinogen, α-enolase and the C1-epitope of type II collagen) were associated with SE alleles, particularly with HLA-DRB1*04.[26,69] Our data show a negative association of HLA-DRB1*09 and HLA-DRB1*11 with the presence of anti-citrullinated FN peptide antibodies (OR = 0.19 and OR = 0.41,

		Anti-cit FN neg. (%)[d]	Anti-cit FN pos. (%)[d]	OR (95% CI)
Smoking[a]	-	63 (46.7)	40 (38.1)	**1.42 (0.85-2.39)**
	+	72 (53.3)	65 (61.9)	
HLA SE alleles[b]	-	39 (26.5)	57 (14.6)	**2.11 (1.13-3.92)**
	+	108 (73.5)	213 (85.4)	
HLA-DRB1*01[c]	-	112 (75.2)	93 (75.0)	1.01 (0.58-1.75)
	+	37 (24.8)	31 (25.0)	
HLA-DRB1*03[c]	-	118 (79.2)	105 (84.7)	0.69 (0.37-1.29)
	+	31 (20.8)	19 (15.3)	
HLA-DRB1*04[c]	-	70 (47.0)	46 (37.1)	**1.5 (0.92-2.44)**
	+	79 (53.0)	78 (62.9)	
HLA-DRB1*07[c]	-	131 (87.9)	110 (88.7)	0.93 (0.44-1.95)
	+	18 (12.1)	14 (11.3)	
HLA-DRB1*08[c]	-	147 (98.7)	120 (96.8)	2.45 (0.44-13.61)
	+	2 (1.3)	4 (3.2)	
HLA-DRB1*09[c]	-	137 (91.9)	122 (98.4)	**0.19 (0.04-0.85)**
	+	12 (8.1)	2 (1.6)	
HLA-DRB1*10[c]	-	137 (91.9)	109 (87.9)	**1.57 (0.71-3.5)**
	+	12 (8.1)	15 (12.1)	
HLA-DRB1*11[c]	-	125 (83.9)	115 (92.7)	**0.41 (0.18-0.91)**
	+	24 (16.1)	9 (7.3)	
HLA-DRB1*12[c]	-	145 (97.3)	121 (97.6)	0.9 (0.20-4.09)
	+	4 (2.7)	3 (2.4)	
HLA-DRB1-13[c]	-	129 (86.6)	113 (91.1)	0.63 (0.29-1.37)
	+	20 (13.4)	11 (8.9)	
HLA-DRB1-14[c]	-	144 (96.6)	120 (96.8)	0.96 (0.25-3.66)
	+	5 (3.4)	4 (3.2)	
HLA-DRB1*15[c]	-	113 (75.8)	91 (73.4)	1.14 (0.66-1.97)
	+	36 (24.2)	33 (26.6)	
HLA-DRB1*16[c]	-	148 (99.3)	122 (98.4)	2.43 (0.22-27.08)
	+	1 (0.7)	2 (1.6)	

Supplementary Table 1. Association of anti-citrullinated fibronectin antibodies and HLA-DRB1 alleles in anti-CCP2-positive early arthritis patients. a Based upon information obtained from 240 RA patients. b Based upon information obtained from 270 RA patients. c Based upon information obtained from 278 RA patients. dValues are the number and percentages of early arthritis patients (EAC) negative or positive (cut-off = mean + 2*SD) for anti-FN-$Cit_{1035,1036}$ antibodies. OR = odds ratio; 95% CI = 95% confidence interval.

a Based upon information obtained from 240 RA patients.

b Based upon information obtained from 270 RA patients.

c Based upon information obtained from 278 RA patients.

dValues are the number and percentages of early arthritis patients (EAC) negative or positive (cut-off = mean + 2*SD) for anti-FN-Cit1035,1036 antibodies.

OR = odds ratio; 95% CI = 95% confidence interval.

respectively). This may be in agreement with the previously reported association of the HLA-DRB1*0901 haplotype with reduced levels of anti-CCP antibodies.[70] Paradoxically, HLA-DRB1*09 has been reported previously to be associated with RA in Asian as well as Caucasian individuals.[71,72] In addition, it should be noted that, because ACPA-positive RA patients comprise a strong prevalence for SE-alleles, other HLA-DRB1 alleles are less frequently present than SE-alleles and, therefore, might seem to be protective. As a consequence, the negative association observed with HLA-DRB1*09 and HLA- DRB1*11 might also be the result of skewing.[73]

We did not detect a significant association between clinical phenotype and the presence of anti-citrullinated fibronectin in ACPA-positive RA patients. Recently, Scherer and colleagues also observed no effect on radiographic joint damage in patients that were positive for several citrullinated epitopes.[37] Nevertheless, a weak correlation between the presence of autoantibodies against citrullinated FN and smoking, a risk factor for ACPA generation, particularly in individuals that carry the SE alleles[16,17] was observed.

CONCLUSION

Five citrullinated residues were identified in fibronectin isolated from the inflamed joints of RA patients. An epitope containing two adjacent citrullines at positions corresponding to residues 1035 and 1036 appeared to be most frequently recognized by RA sera. Our data not only show that antibodies against citrullinated FN are present in RA patients, but also demonstrate that the anti-FN antibodies represent a subgroup of anti-CCP2 antibodies and that they can already be detected very early in the disease. Moreover, anti-FN-$Cit_{1035,1036}$ antibodies are associated primarily with HLA SE alleles.

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CHAPTER 6

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

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ABSTRACTS

Objectives

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

Methods

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

Results

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

Conclusions

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

INTRODUCTION

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

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

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

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

METHODS

Patients and Radiographs

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

Replication of the association between SE alleles and radiographic progression in relation to ACPA status was performed using data of Norwegian RA patients with a maximum disease duration of 4 years included in 1992-3 in the Europe-

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

Anti-CCP2 assays

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

ACPA fine specificity assays

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

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

Statistical analysis

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

RESULTS

HLA SE alleles associate with several ACPA fine specificities

SE alleles associate with the presence of certain ACPA fine specificities.[4] We first extended these findings by analyzing reactivity to more citrullinated epitopes, by increasing the number of patients studied, and by analyzing the effect of the SE gene dose on ACPA fine specificity. Three out of six citrullinated epitopes studied associated with the presence of SE alleles (table 1). Reactivity to citrullinated vimentin 59-74, citrullinated α-enolase 5-20 and citrullinated MBP was found in significantly increased frequency in SE positive patients, whereas no such effect was observed for the other three antigens. The effect was independent of the number of SE alleles, as patients with one or two SE alleles displayed a comparable profile of ACPA epitope recognition. These data indicate that one SE allele is sufficient to facilitate the development of certain ACPA fine specificities, and that presence of a second SE allele does not further skew the ACPA profile towards more frequent recognition of a specific epitope.

The ACPA fine specificity repertoire does not predict future radiographic progression

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

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

None of the fine specificities tested was found to associate with radiographic progression. A drawback of this analysis is that lack of association does not exclude that the investigation of other citrullinated peptides would have revealed a positive association with long-term joint damage. As this limitation can persist in case more (non-associating) reactivities would be analyzed, we reasoned that an

additional way to investigate the relationship between the ACPA recognition profile and radiographic outcome is to study the effect of SE alleles on joint damage in ACPA-positive disease only. As SE alleles affect the recognition of certain citrullinated epitopes (table 1), SE alleles can be interpreted as a surrogate marker for the constitution of the ACPA response.

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

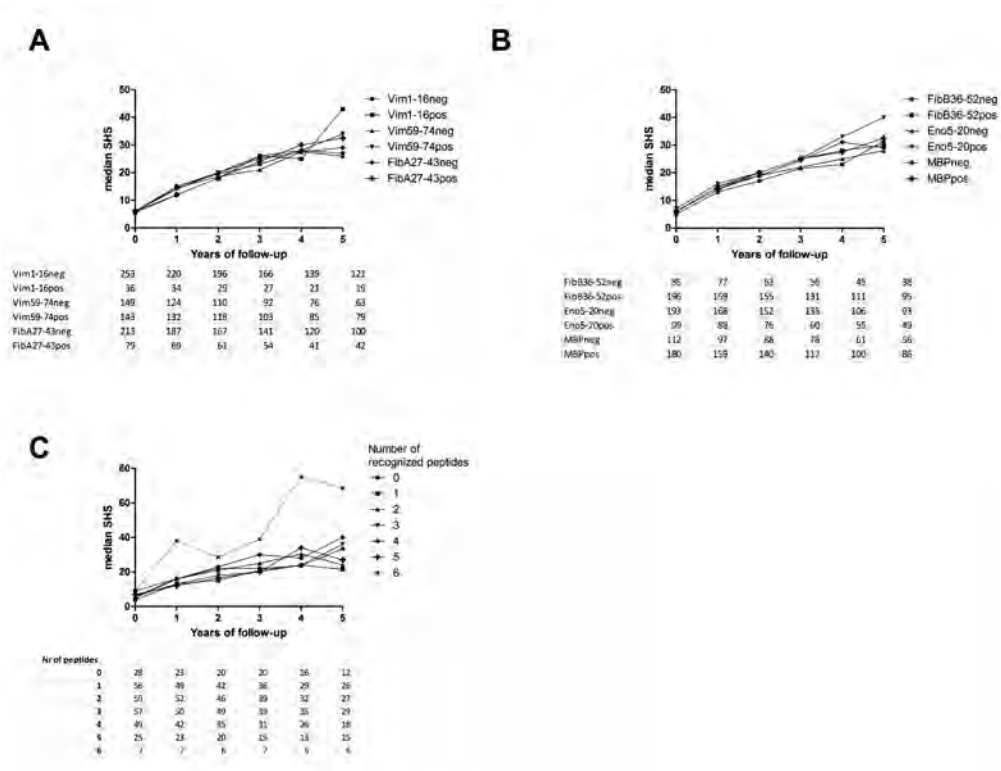

Figure 1. Association between different ACPA fine specificities and radiographic progression. (A,B) Median Sharp-van der Heijde scores (SHS) during 5 year follow-up of ACPA positive patients from the Leiden EAC testing positive or negative for six different ACPA fine specificities at baseline. (C) Median SHS in ACPA-positive patients grouped by the number of ACPA fine specificities recognized. All statistical comparisons (repeated measurement analysis) were non-significant ($p>0.05$). Numbers of patients in each group are given in the table below each figure. The line representing the group of patients recog-

nizing six fine specificities diverts from the rest due to the low number of patients in this group. In order to highlight this, this group is depicted with a dotted line. Radiographic data was available for n=266 ACPA-positive patients at baseline and n=132 at year 5.

Figure 2

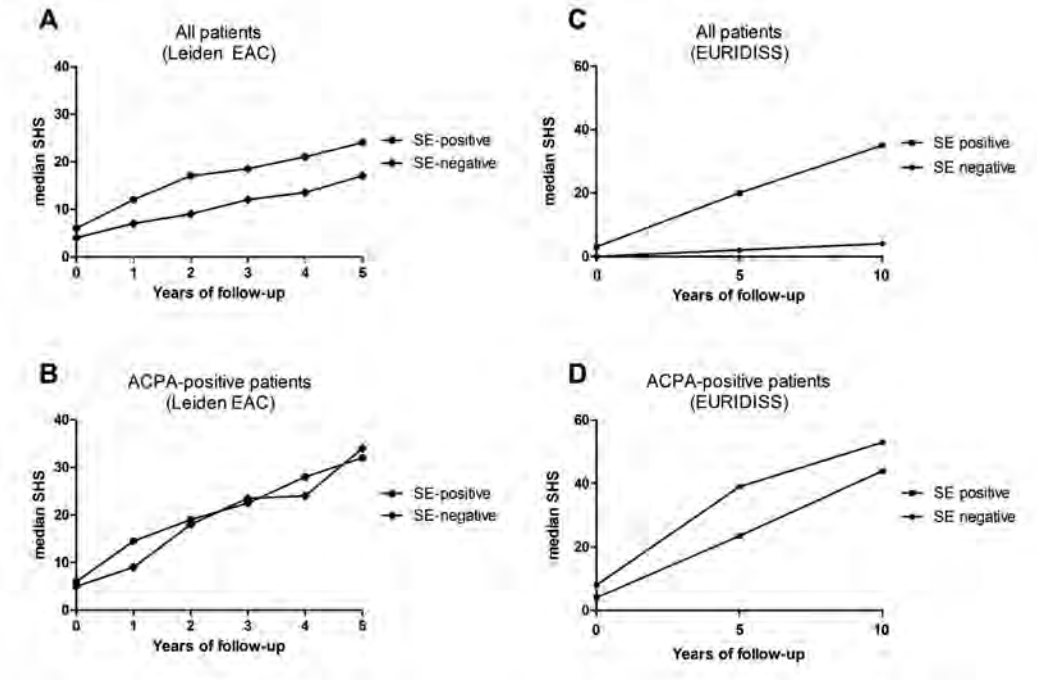

Figure 2. Shared epitope (SE) alleles exert no effect on radiographic progression after stratification for ACPA. Median Sharp-van der Heijde scores (SHS) in relation to SE positivity in the entire RA population and after stratification for ACPA-positive disease in patients of the Leiden Early Arthritis Clinic (A/B) and of the EURIDISS cohort (C/D). For the Leiden EAC: radiographic data was available for n=481 patients at baseline and n=214 at year 5, of which n=266 were ACPA-positive (data for n=132 available at year 5). (A) Repeated measurement analysis: $p = 0.013$; Mann-Whitney U test $p < 0.001$ at year 1, $p < 0.001$ at year 2, $p = 0.007$ at year 3, $p = 0.005$ at year 4, $p = 0.012$ at year 5; (B) repeated measurement analysis: $p > 0.05$; all Mann-Whitney U p-values > 0.05. For the EURIDISS cohort: radiographic data was available for n=154 patients at baseline and n=142 at year 10, of which n=94 were ACPA-positive (data for n=83 available at year 10). (C) Mann-Whitney U test $p = 0.029$ at baseline, $p < 0.001$ at year 5, $p < 0.001$ at year 10; (D) Mann-Whitney U test $p = 0.36$ at baseline, $p = 0.93$ at year 5, $p = 0.97$ at year 10.

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

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

DISCUSSION

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

In this study, we could not detect an association between ACPA fine specificities and radiographic joint damage. An anti-citrulline immune response to 3 out of 6 of the epitopes studied developed preferentially in patients harboring SE alleles, but this did not translate into more severe radiographic outcome. Also, the number of citrullinated epitopes recognized by an individual patient did not influence the degree of joint destruction. Although we accounted for baseline treatment strategy in this analysis, and although no association between year of inclusion and recognition of the separate ACPA fine specificities was found, we cannot fully exclude that treatment effects later in the disease course could have influenced our results. The number of citrullinated epitopes also limits our study, and it cannot be excluded

that other epitopes would have been more useful for this purpose. We addressed this issue by using SE alleles as a surrogate marker for those ACPA fine specificities that develop under the influence of SE alleles. After stratification for ACPA, SE alleles no longer contributed to joint damage. Based on this finding, we consider it unlikely that a SE associated ACPA fine specificity can be identified that predicts disease course in RA. If such a predictive recognition profile exists, antibodies recognizing this epitope are likely to be generated independent of SE alleles.

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

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CHAPTER 7

The ACPA recognition profile and subgrouping of ACPA-positive RA patients

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ABSTRACT

Objective

Anticitrullinated protein antibodies (ACPA) are the most predictive factor for the development of rheumatoid arthritis (RA). Epitope spreading towards more citrullinated epitopes occurs before the onset of RA. Here, the authors investigated whether specific epitope recognition allows the identification of specific RA subgroups and whether it is associated with clinical features of RA.

Methods

The reactivity of 661 patients with RA from the Leiden Early Arthritis Clinic against several citrullinated antigens was determined by ELISA. Cluster analyses were performed to identify subgroups of patients on the basis of their ACPA recognition profile. The association of the specific reactivities with clinical characteristics was studied.

Results

ACPA-positive patients displayed a heterogeneous ACPA recognition profile. After performing cluster analyses, no apparent clustering of patients was found, and on the basis of the reactivities analysed, 64 different subgroups could already be identified. The extent of epitope recognition was associated with anticyclic citrullinated peptide-2 levels. The recognition of specific citrullinated epitopes was not associated with baseline characteristics. Likewise, patients with an extended fine specificity repertoire did not display differences in baseline characteristics or joint damage after 7 years of follow-up using cyclic citrullinated peptide-2 levels as a proxy, compared to ACPA-positive patients recognising fewer peptides.

Conclusion

These data show that the ACPA response is highly diverse with respect to recognition of specific citrullinated epitopes. Furthermore, the authors' data indicate that clinical correlates in established ACPA- positive RA are independent from the specific (group of) citrullinated peptides recognised.

INTRODUCTION

Anticitrullinated protein antibodies (ACPA) have been shown to be able to initiate and enhance arthritis in murine models of arthritis[1,2] and are able to activate Fc Receptor-positive cells[3,4] and the complement system, leading to the argument that they could play a role in disease pathogenesis.[5]

The detection of ACPA, which is most commonly assessed by reactivity against cyclic citrullinated peptide (CCP)-2, is an accepted diagnostic tool for rheumatoid arthritis (RA).[6] ACPA are highly specific for RA, can be detected years before the first clinical manifestation of RA[7,8] and are reported to be a good predictor for the development of RA.[9] Anti-CCP antibodies recognise multiple citrullinated peptides and proteins and are thus a collection of ACPA.[10]

Some types of human leucocyte antigens (HLA) alleles, particularly HLA-DRB1 alleles encoding the shared epitope sequence, are known to be associated with RA susceptibility,[11] more specifically susceptibility to ACPA-positive RA.[12] ACPA-positive and ACPA-negative disease have been shown to be associated with different genetic and environmental risk factors, fuelling the hypothesis that different pathophysiological mechanisms are underlying these two separate disease subsets.[13,14] Next to isotype usage[15] and avidity[16], the fine specificity of an antibody response is also thought to contribute in determining its efficacy.[17] It has been shown that ACPA can recognise a variety of citrullinated antigens, including citrullinated fibrinogen (cFib), α-enolase[18], citrullinated vimentin (cVim) and citrullinated myelin-binding protein (cMBP)—the latter mimicking the Sa antigen.[19,20]

Levels of specific ACPA are elevated in synovial fluid, suggesting local antibody production and/or retention of ACPA at the site of inflammation.[21]

However, not all ACPA-positive sera will recognise all citrullinated antigens, as has been shown by analysing the reactivity against different citrullinated peptide antigens.[10,22]

It has been shown that patients with RA display a much more extended citrullinated epitope recognition pattern compared to ACPA-positive first-degree relatives without symptoms.[23] Indeed, epitope spreading with an increase in the recognition of citrullinated antigens occurs before the patients fulfil the 1987 classification criteria of RA. Differences in ACPA fine specificity between patients having undifferentiated arthritis who do or do not develop RA after 1 year are already present at baseline.[24] Likewise, patients with arthralgia and with an extended ACPA repertoire have a higher risk of developing arthritis.[25]

It is conceivable that a certain ACPA recognition profile is associated with the emergence of certain clinical features and possibly pathogenicity, as has been shown in other autoimmune diseases. For example, in pemphigus the reactivity against different desmoglein epitopes is associated with different outcomes.[26]

Here, we determined the association between the ACPA fine specificity and phe-

notypic characteristics within ACPA-positive RA and investigated whether specific subsets of RA patients can be distinguished on the basis of their epitope recognition profile.

MATERIALS AND METHODS

Patient population

All patients who fulfilled the American College of Rheumatology 1987 revised criteria for the classification of RA[27] within 1 year of follow-up from the Leiden Early Arthritis Clinic were analysed (n=661). The Leiden Early Arthritis Clinic is an inception cohort of patients with recent onset arthritis (symptoms duration, <2 years) that was started at the Department of Rheumatology of the Leiden University Medical Center in 1993 and was described in detail previously.[28] Sustained disease-modifying antirheumatic drug (DMARD)-free remission was defined as having no current use of DMARDs, no swollen joints and classification as DMARD-free remission by the patients' rheumatologist.[29]

Anti-CCP2 assays

Total IgG anti-CCP2 was measured in baseline sera by ELISA (Immunoscan RA Mark 2; Eurodiagnostica, Arnhem, The Netherlands). Samples with a value >25 units/ml were considered positive according to the manufacturer's instructions. Individuals with antibodies against CCP2 were considered ACPA-positive. From patients with antibody levels reaching the plateau of the standard, we further diluted the serum to determine ACPA levels.

ACPA fine specificity assays

ELISA assays were developed, as previously described[30], against peptides derived from cVim, cFib and citrullinated α-enolase (cEno)5-20. Antibody reactivity against the citrullinated (Cit) and the uncitrullinated form of two linear peptides derived from vimentin (Vim 1-16: STCitS VSSS SYCitCit MFGG and Vim 59-74: VYAT CitSSA VCitLCit SSVP), two linear peptides derived from fibrinogen (Fibα 27-43: FLAE GGGV Cit GPR VVER H and Fibβ 36-52: NEEG FFSA CitGHR PLDK K) and one linear peptide derived from α-enolase (Eno 5-20: KIHA CitEIF DSCitG NPTV) were determined by ELISA as described previously.[23,31] Citrullination of myelin basic protein (MBP) (Sigma-Aldrich, Zwijndrecht, Netherlands) and the specific ELISA were performed as previously described.20 Antibodies reactive with citrullinated MBP were determined in 1:100 diluted sera.

Although there are also other targets for ACPA,[32,33] we chose primarily to investigate epitopes from these proteins because they have been most consistently identified as citrullinated autoantigens.[18, 19, 34] We analysed reactivity to citrullinated MBP, as its ELISA has been previously standardised to be the clinical equiva-

lent (positive versus negative) of the original anti-Sa (cit-Vimentin) western blot assay.[20]

Cut-off values for the citrulline-specific responses were calculated as previously described.[24,31] IgM-rheumatoid factor (RF), anti-CCP3 autoantibodies (IgA and IgG subforms) and anti-mutated citrullinated vimentin (MCV) autoantibodies were also measured by ELISA as described previously.[31] Sera were tested for antinuclear factor (ANF) using indirect immunofluorescence at a 1:40 dilution on Hep-2000 cells (biomedical diagnostics).

Statistical analysis

Differences between groups were analysed with the Mann– Whitney test or the t test, or χ^2 test where appropriate. Association between anti-CCP2 antibody levels and number of peptides recognised was studied by linear regression of log-transformed anti-CCP2 antibody levels on number of recognised peptides. Principal component analyses (PCA)[35] were applied to covariance matrices and included the different fine specificity epitopes. In order to normalise distributions, all variables were log-transformed concentration levels plus one. Based on scree plots and eigenvalues, components with an eigenvalue ≥0.4 were selected.

The association of the specific reactivities with baseline characteristics and joint damage over time was studied. Annual radiographs of hands and feet were assessed chronologically for radiographic damage according to the Sharp–van der Heijde score[36] by one experienced reader. For this, a repeated measurement analysis was applied. This method takes advantage of the longitudinal, repetitive character of the data and does not exclude patients with incomplete follow-up data, avoiding selection bias. In a multivariate normal regression model with radiological score as response variable, the effect of time was entered as factor to fit the non-linear slope of joint destruction. The components were entered with an interaction term with time as continuous variable to test the effect of the components over time. Age, gender and inclusion period (a proxy for treatment strategy) were entered in the model to correct for possible confounding effects. Analyses were performed using SPSS version 17.0, and p values below 0.05 were considered to be statistically significant.

Cluster analyses were used to identify subgroups of patients on the basis of their ACPA recognition profile. Clustering illustrates the relationship between different patients and reactivities, as described by Eisen et al.[37] Hierarchical clustering was performed using average linkage clustering where patient correlation was performed centred and the reactivities uncentred. To illustrate the influence on anti-CCP2 level, we performed a supervised cluster analysis where the patients were fixed and the reactivities correlated uncentred.

RESULTS

ACPA recognition profile

Baseline characteristics of the 661 patients with RA who fulfil the American College of Rheumatology criteria 1987 are shown in table 1. To analyse whether the ACPA recognition profile would allow for the identification of specific antibody positive subgroups, we wished to perform cluster analyses as well as PCA to investigate whether specific subgroups can be identified on the basis of epitope recognition. If so, such subgroups could then be used to analyse possible clinical association. First, we described patients on the basis of their ACPA status and their ability to recognise one specific citrullinated peptide (ie, cVim59-74 and cEno5-20). This analysis resulted in the identification of different subsets of patients (figure 1A,B). Combining the two reactivities against these two peptides resulted in more different subgroups (figure 1C). Remarkably, when using all nine reactivities included in this study, 64 subgroups could already be identified (figure 1D).

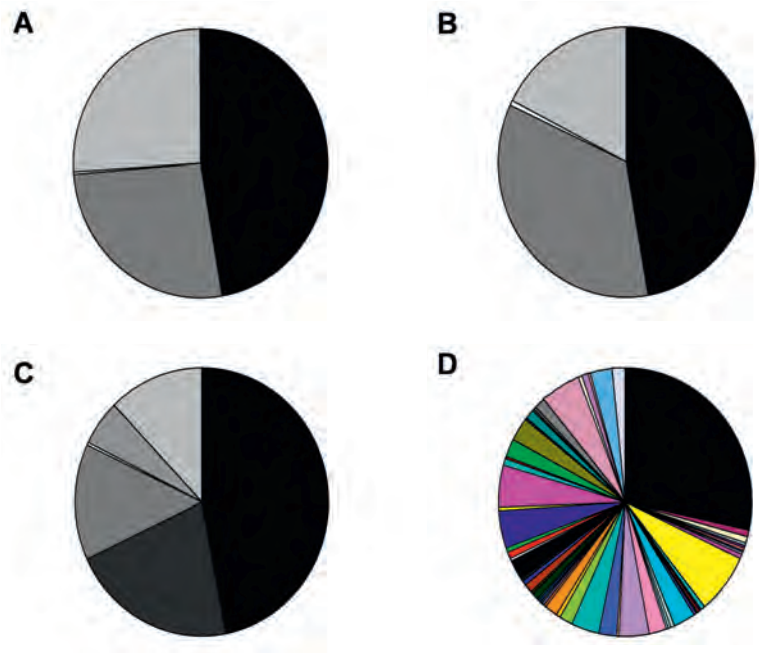

Figure 1. Different subsets of patients based on the epitope recognition profile. Patients were grouped on the basis of recognition of CCP2 and cVim59-74 (depicted in black are patients CCP2-cVim-; in white, CCP2-cVim+; in light grey, CCP2+cVim-; and in dark grey, CCP2+cVim+ (figure 1A), grouped on the basis of recognition of CCP2 en cEno5-20 (depicted in black are patients CCP2-cEno-; in white, CCP2-cEno+; in light grey, CCP2+cVim-; and in dark grey, CCP2+cEno+ (figure 1B)) and on the combination of those two peptides (cVim59-74 and cEno5-20) and CCP2 (figure 1C). Dividing patients based

on all tested reactivities (including cVim1-16, cVim59-74, cFibα27-43, cFibβ36-52, cEno5-20, MBP, MCV, CCP2 and CCP3, resulted in 64 different subsets of patients indicating a heterogeneous epitope recognition pattern. Depicted in black are patients negative for all the tested citrullinated antigens (figure 1D). CCP2, cyclic citrullinated peptide; cEno, citrullinated α-enolase; cVim, citrullinated vimentin; cFib, citrullinated fibrinogen; MBP; myelin-binding protein; MCV, mutated citrullinated vimentin.

	CCP2+ (max n=348)		**CCP2- (max n=313)**	
Age in years, mean ± SD	54.7± 15		58.4± 17	
Female sex	208/311	(66.9%)	163/235	(69.4%)
Shared Epitope status % positive	270/335	(80.6%)	153/293	(52.2%)
Smoking (yes)	170/309	(55.0%)	117/300	(39.0%)
IgM-RF+	267/305	(87.5%)	54/232	(23.3%)

Table 1. Baseline characteristics. CCP, cyclic citrullinated peptide; IgM-RF= IgM-rheumatoid factor.

These data show that within ACPA-positive patients, a large heterogeneity is present in the ACPA recognition profile. More importantly, this large heterogeneity seems to preclude the identification of specific subgroups. To investigate the possibility that specific peptide reactivities would cluster together, we generated a heatmap after an unsupervised hierarchical cluster analysis. A heatmap groups patients who resemble each other the most, based on their fine specificity recognition repertoire. This analysis did not lead to the identification of apparent subgroups of patients harbouring a specific ACPA recognition profile (figure 2). Together, these analyses did not point to the presence of a clear pattern of recognition of citrullinated peptides by ACPA.

Interestingly, when we performed a similar analysis in a supervised manner by ordering patients on anti-CCP2 levels and subsequently analysed the recognition to specific peptides, an association was found between levels of anti-CCP2 antibodies and the number of recognised peptides by ACPA-positive patients with RA (figure 3). The association between levels and the epitope recognition profile was also confirmed with a linear regression analysis ($p<0.001$). As control, the relation to RF or ANF status (figure 3) was also analysed. In this case, no relation between anti-CCP2 levels and the antibody status of RF and ANF was found.

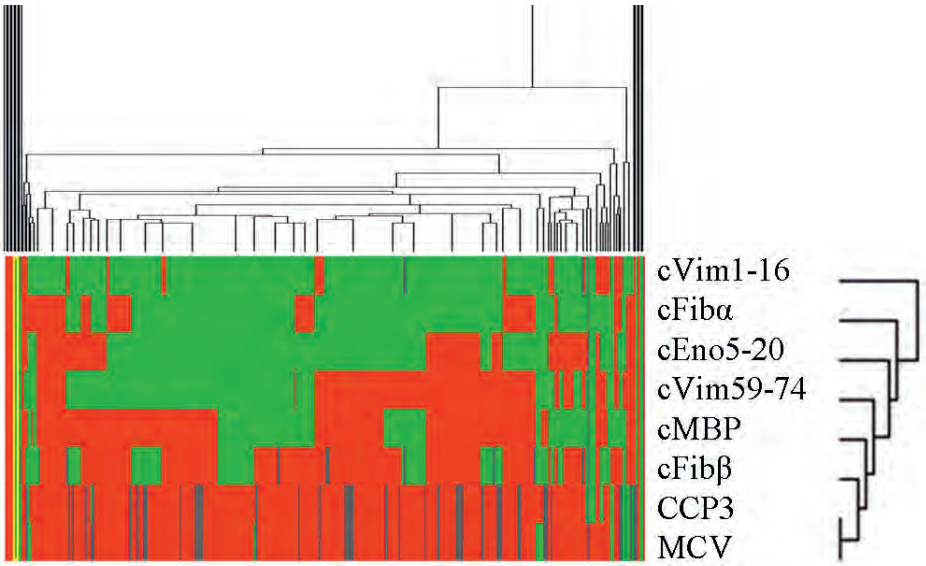

Figure 2. Unsupervised cluster analysis. No apparent clusters of patients were visible after an unsupervised cluster analysis based the recognition of different citrullinated epitopes (including cVim1-16, cFibα27-43, cEno5-20, cVim59-74, cMBP, cFibβ27-43, CCP3 and MCV). Each column represents one patient and each row one tested fine specificity peptide or protein. Depicted in red are patients being positive for the recognition of a specific reactivity, when depicted in green the patients did not recognise that epitope. cEno, citrullinated α-enolase; cFib, citrullinated fibrinogen; MBP; myelin-binding protein; cVim, citrullinated vimentin; MCV, mutated citrullinated vimentin.

Likewise, no association was found between the presence of antibodies against citrullinated epitopes and antibody levels against tetanus (figure 4B) or total IgG levels (figure 4C). Together, these data indicate that anti-CCP2 antibody levels specifically correlate with the number of citrullinated epitopes recognised by ACPA (figure 4A).

As our analyses described above did not identify defined subgroups, we performed PCA thereafter. PCA are an exploratory tool to unravel unknown trends in the data; however, this method also did not provide indications for the presence of patient subsets that can be grouped on the basis of the ACPA recognition profile (data not shown). Together, these analyses show that within these reactivities, no apparent patterns of recognition of citrullinated peptides by ACPA are present.

ACPA fine specificity and clinical characteristics

As outlined above, no specific subgroups of patients could be identified based on the presence of different ACPA characteristics. Therefore, we wished to analyse associations between baseline characteristics and the recognition of specific epi-

topes. To determine whether the baseline characteristics are associated with the recognition of specific citrullinated epitopes, we performed these analyses in the ACPA-positive stratum only. In doing so, we control the possibility that the results described above are explained by the known association between baseline characteristics and ACPA status, rather than by an influence of specific epitope recognition on baseline characteristics (ie, all ACPA reactivities are almost exclusively found within the ACPA-positive stratum).

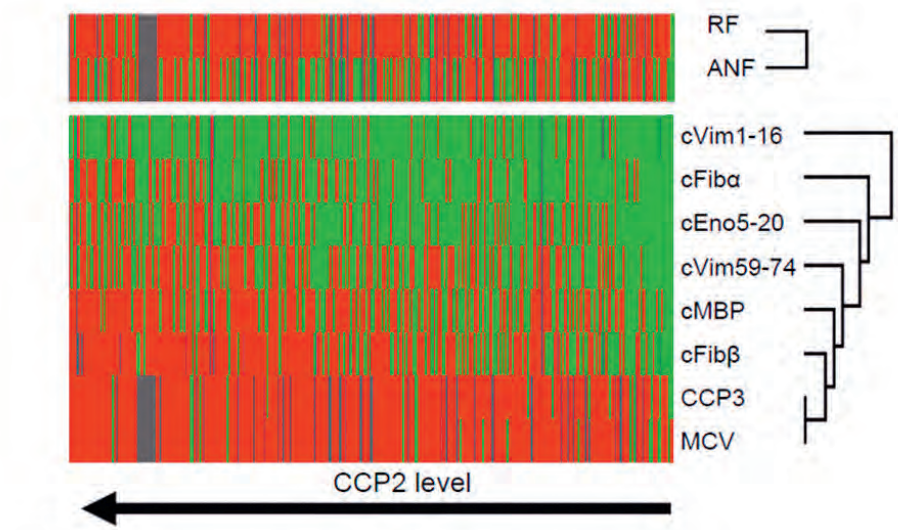

Figure 3. The association between anti-CCP2 levels and the recognition of multiple citrullinated epitopes. A supervised cluster-analysis showed an association between the anti-CCP2 levels and number of recognized peptides (including cVim1-16, cFibα27-43, cEno5-20, cVim59-74, cMBP, cFibβ36-52, CCP3 and MCV). There was no apparent association between levels and RF or ANF. Each column represents one patient and each row, one tested fine specificity peptide or protein. Depicted in red are patients being positive for the recognition of a specific reactivity; depicted in green are the patients who did not recognise that epitope. Depicted in grey are missing values. ANF, anticulear factor; cEno, citrullinated α-enolase; CCP, cyclic citrullinated peptide, cVim, citrullinated vimentin; cFib, citrullinated fibrinogen; MBP, myelin-binding protein; MCV, mutated citrullinated vimentin; RF, rheumatoid factor.

We observed essentially no association with any of the reactivities in relation to any of the baseline characteristics tested (figure 5 and figure 6). Although an association between reactivity against anti-cMBP-reactivity and C reactive protein (CRP) was observed (p=0.008), this association did not remain after the Bonferroni correction for multiple testing (figure 5).

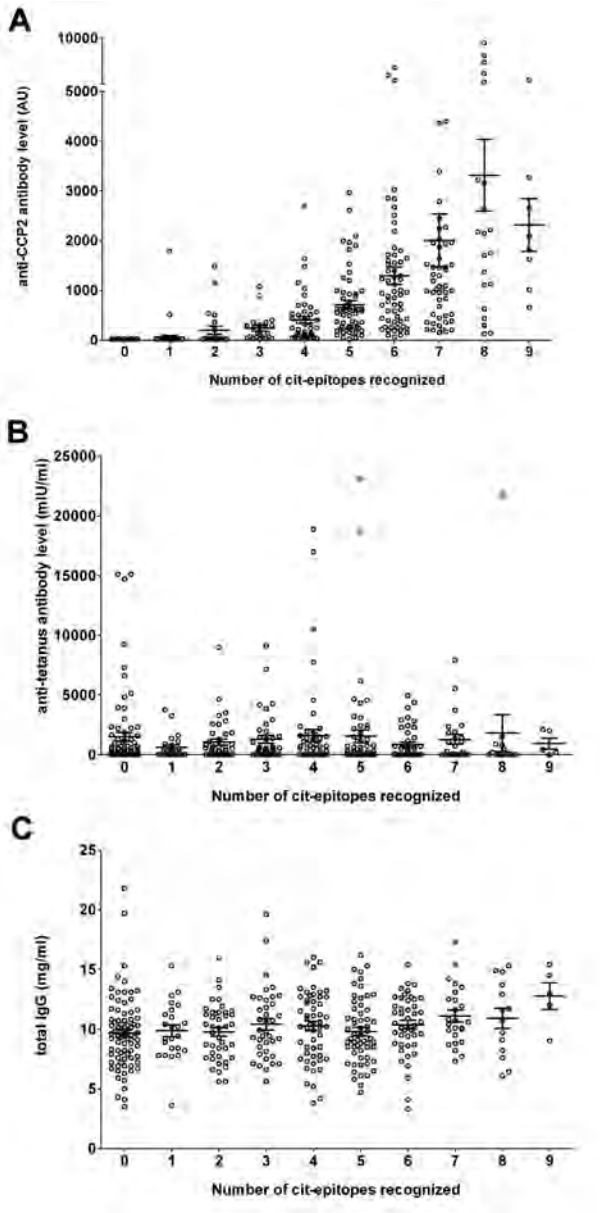

Figure 4. Association between anti-CCP2 levels and number of citrullinated epitopes recognized. The number of epitopes recognized was positively associated with the anti-CCP2 levels (figure 4A). This association is citrulline-specific rather than antibody levels specific, as it was not found for anti-tetanus antibody levels (figure 4B). Also total-IgG levels were not associated with the number of epitopes recognized by RA patients (figure 4C). Graphs are depicted with mean ± standard error of the mean (SEM). Epitopes included in these analyses were: cVim1-16, cVim59-74, cFibα27-43, cFibβ36-52, cEno5-20,

cMBP, MCV, CCP2 and CCP3. CCP, cyclic citrullinated peptide; cEno, citrullinated α-enolase; cFib, citrullinated fibrinogen; MBP; myelin-binding protein; cVim, citrullinated vimentin; MCV, mutated citrullinated vimentin; RA, rheumatoid arthritis.

In line with these data, patients with an extended fine specificity repertoire (defined by the recognition of more than two citrullinated epitopes) also did not display significant differences in baseline characteristics compared to ACPA-positive patients recognising fewer peptides (figure 5), as the difference in CRP levels (p=0.018) could not withstand correction for multiple testing.

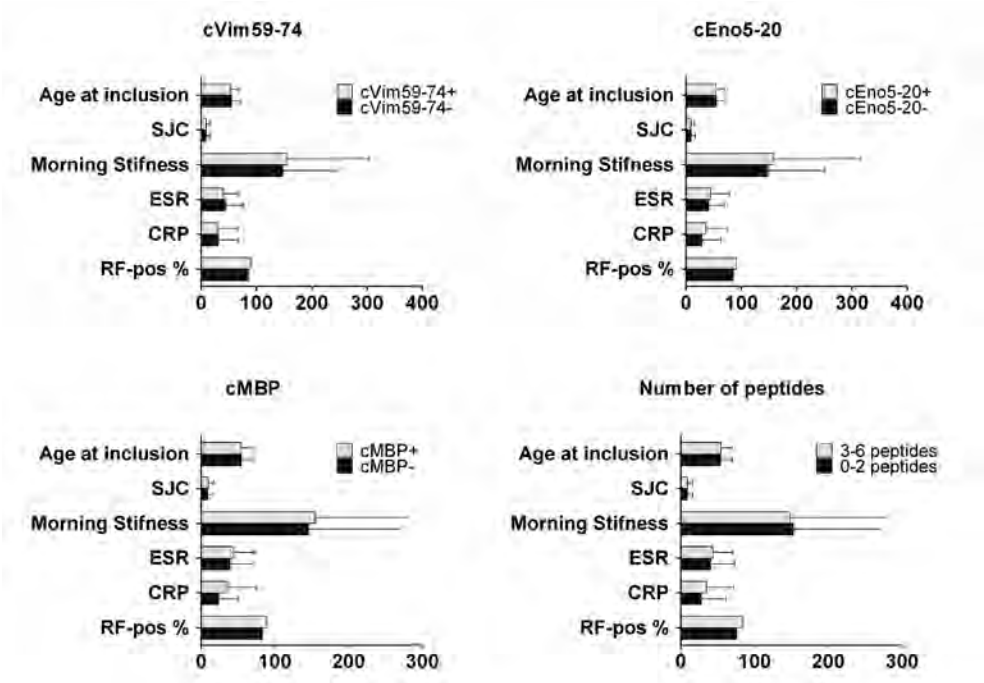

Figure 5. The association between epitope recognition and baseline characteristics. We observed essentially no association with any of the reactivities in relation to any of the baseline characteristics tested. An association was found between the recognition of cMBP and higher CRP levels at baseline (p=0.008) and recognition of more citrullinated peptides with CRP levels (p=0.018). However, after correction for multiple testing, no association between the recognition of specific citrullinated epitopes and baseline characteristics remained. Depicted are the mean and standard deviation between different citrullinated epitopes and baseline characteristics within ACPA positive patients. Age of inclusion is depicted, swollen joint count with number of swollen joints, morning stiffness in min, ESR in mm/h, CRP in mg/l and RF in percentage positivity. ACPA, anticitrullinated protein antibodies; cMBP, citrullinated myelin-binding protein; CRP, C reactive protein; ESR, erythrocyte sedimentation rate; RF, rheumatoid factor; SJC, swollen joint count.

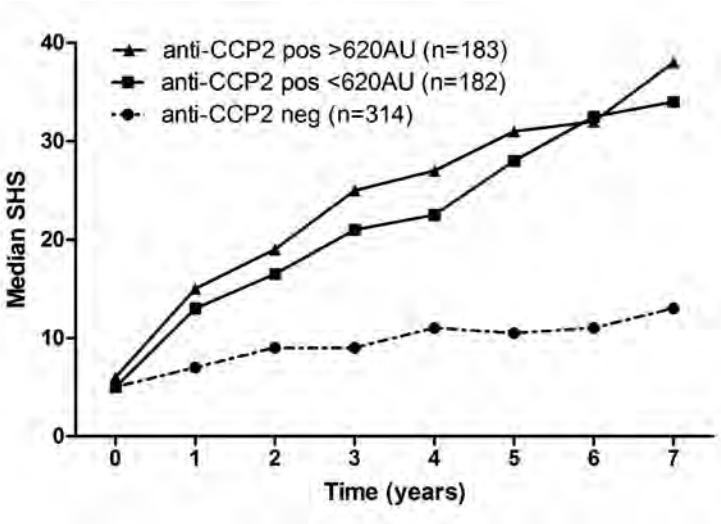

Figure 6. The association between epitope recognition and baseline characteristics. Depicted are the mean and standard deviation between different citrullinated epitopes and baseline characteristics within ACPA positive patients. Age of inclusion is depicted, swollen joint count with number of swollen joints, morning stiffness in min, ESR in mm/h, CRP in mg/l and RF in percentage positivity. ACPA, anticitrullinated protein antibodies; cMBP, citrullinated myelin-binding protein; CRP, C reactive protein; ESR, erythrocyte sedimentation rate; RF, rheumatoid factor; SJC, swollen joint count.

Another goal for making subgroups is to identify patients who will reach remission or will develop more joint destruction over time. Therefore, we wished to analyse sustained DMARD-free remission and joint damage over time within the ACPA-positive stratum. Only nine out of 306 ACPA-positive patients with RA achieved remission. As a consequence of this low number of patients, we did not perform further analyses into the association of the ACPA recognition profile and remission. Recently, we have shown that different fine specificities or an extended ACPA recognition profile was not associated with joint destruction over time.[38] We now extended these findings by analysing additional citrullinated epitopes, but this analysis also did not indicate an association between the recognition of a specific citrullinated epitope with radiological progression. Nonetheless, it is possible that an association exists for another, not in our study tested, epitope. Our data show a strong correlation between anti-CCP2 levels and the number of peptides recognised by patients (figures 3 and 4). Therefore, we wished to use anti-CCP2 levels as a proxy for the peptides that we did not analyse. Intriguingly, we did not observe an association between rate of joint destruction over time and the anti-CCP2 levels within the ACPA-positive patients (p=0.66) (figure 7).

Together, our data indicate that although the ACPA recognition profile is highly diverse, the number of epitopes recognised correlates with ACPA level. Interestingly, the recognition of these citrullinated peptides, irrespective of their differences in peptide-backbone structure, all correlated with similar clinical characteristics.

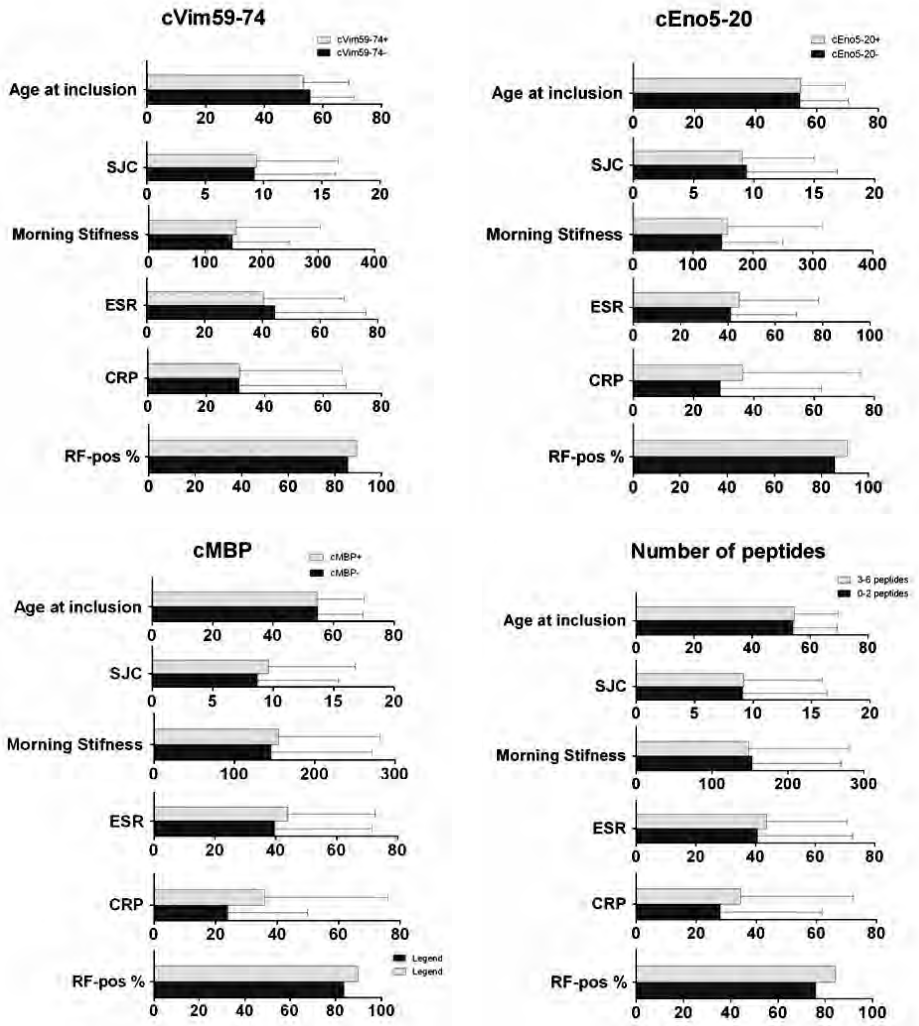

Figure 7. Rate of joint destruction over time and anti-CCP2 levels. Anti-CCP2 positive patients developed significantly more joint destruction over time ($p < 0.001$) compared to anti-CCP2 negative patients. Within the anti-CCP2 positive patients that rate of joint destruction between patient with high levels (>620AU) and low levels (<620AU) did not differ ($p=0.66$). The ACPA positive patients were divided into 2 groups based on the median of 620 AU. ACPA, anticitrullinated protein antibodies; CCP, cyclic citrullinated peptide.

DISCUSSION

Epitope spreading is a phenomenon in which the (auto)immune response is extended to include new epitopes within the same molecule or towards different molecules and is thought to be involved in disease onset of autoimmune disorders. For example, pemphigus is one of the most clearly defined autoimmune diseases mediated by autoantibodies. The involvement of epitope spreading in pemphigus pathogenesis was first demonstrated in endemic pemphigus foliaceous.[26] Subjects in the preclinical stage harbour antibodies recognising different epitopes on desmoglein 1 compared to patients after disease onset.[39] In several cases of pemphigus, it has been described that intermolecular epitope shifting occurs along with the transition of pemphigus phenotype.

In RA, the association between epitope spreading in a predisease phase with RA development had been described as well. For example, it has been shown that epitope spreading of the ACPA response occurs before clinical disease onset and that it is associated with the disease course of early arthritis.[23] Likewise, at time of disease onset, ACPA-positive patients with undifferentiated arthritis who will subsequently develop RA and those that will not already have an immunological distinct immune response, although these differences were not confined to specific citrullinated epitopes. The relevance of epitope spreading before disease onset raised the question as to whether a different epitope recognition pattern would be associated with different clinical phenotypes as has been described in pemphigus. This question was also based upon the findings that HLA shared epitope alleles predispose for the development of antibodies against some but not for other citrullinated epitopes, suggesting that reactivity towards some epitopes plays a more relevant role than others in disease pathogenesis.[30,40,41]

Here, we investigated whether specific subsets of RA patients could be distinguished on the basis of an autoimmune response to specific citrullinated epitopes and investigated the effects of the ACPA fine specificity on clinical features of RA. The analyses were performed within the ACPA-positive stratum to exclude the influence of ACPA status on disease outcome, as published before.[42]

As subgrouping patients based on their epitope recognition profile could be potentially useful to get more homogenous patient groups, we analysed different recognition profiles in detail. Unsupervised clustering of patients based on their epitope recognition resulted in many different subgroups, making it unrealistic to analyse all those subgroups in detail. Therefore we analysed the data with the use of several methods to obtain indications as to whether specific patient groups can be formed based upon the (cumulative) occurrence of specific antibody reactivities. The PCA did not point to specific reactivities as a discriminative factor within the variables. Likewise, the cluster analysis also did not result in clustering of specific patients. These data suggest that the recognition profile of patients with RA displays a large

heterogeneity and that patients are not characterised by a unique and specific epitope recognition pattern.

The data described above might not be surprising, given the observation that even the baseline differences between ACPA- positive and ACPA-negative patients with RA are rather small. Nonetheless, these latter subgroups differ considerably with respect to disease course as measured by radiological progression.[42] We feel that it is unlikely that ACPA fine specificity within ACPA-positive disease will have a similar impact as found for ACPA status within RA. This notion is supported by our observation that a similar rate of joint destruction is observed between ACPA-positive patients with high- and low-baseline anti-CCP2 levels as proxy for the extent of epitope recognition. Likewise, recent observations made by our group and others analysing the possible connection between reactivity against a specific citrullinated epitope provided similar indications.[38,43]

Remarkably, these findings contrast observations made in early/predisease RA, as it has been shown that ACPA-positive subjects who are still healthy or have early arthritis are more likely to develop arthritis when harbouring a more extended generalised citrullinated epitope recognition pattern.[24] The reason why 'maturation' of the ACPA response with respect to its epitope recognition profile is associated with transition to disease but, once disease is established, not with disease outcome, is not known. However, it is tempting to speculate that once a certain threshold is reached, disease manifestations become apparent. In case ACPA would be involved in disease pathogenesis, it is conceivable that over this threshold, higher levels or a more extended recognition profile does not contribute further to disease progression, as the response is already maximally involved in creation of the harmful inflammatory milieu underlying the signs and symptoms associated with RA.

Although our data indicate that no clear link exists between clinical features and autoimmunity against specific citrullinated antigens, we cannot exclude that antibodies against other citrullinated antigens will correlate with clinical outcome once the disease has emerged. Obviously this could lead to two phenotypically different subgroups. However, we feel that it is more likely that inclusion of more citrullinated epitopes will result in more subgroups as analysing nine reactivities already led to a division into 64 subgroups, each displaying a unique ACPA recognition profile. Likewise, we cannot exclude that associations could be found with a more extended samples size; however, based on our sample size, the effect would still remain small, and increasing sample size further will unlikely result in a meaningful clinical difference.

Altogether, our data indicate that the epitope recognition profile is highly diverse. The recognition of different citrullinated peptides at baseline correlated with similar clinical characteristics, irrespective of differences in peptide-backbone structure, indicating that the breaking of tolerance towards citrullinated proteins as such provides more information than the recognition of a particular peptide or set of peptides.

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CHAPTER 8

The window of opportunity in ACPA positive Rheumatoid Arthritis is not explained by ACPA-characteristics

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Very early therapy of Rheumatoid Arthritis (RA) with disease-modifying anti-rheumatic drugs is associated with lower levels of joint destruction and a higher chance on achieving remission.[1-5] Having symptoms for >12 weeks at treatment initiation is a strong and independent risk factors for a persistent disease course.[1, 3-5] These observations have led to the concept of the 'window of opportunity'.[2] This hypothesis presumes that underlying disease processes are not fully matured in the very early stage of arthritis, making modulation more successful. However, putative biological mechanisms remain unexplored.

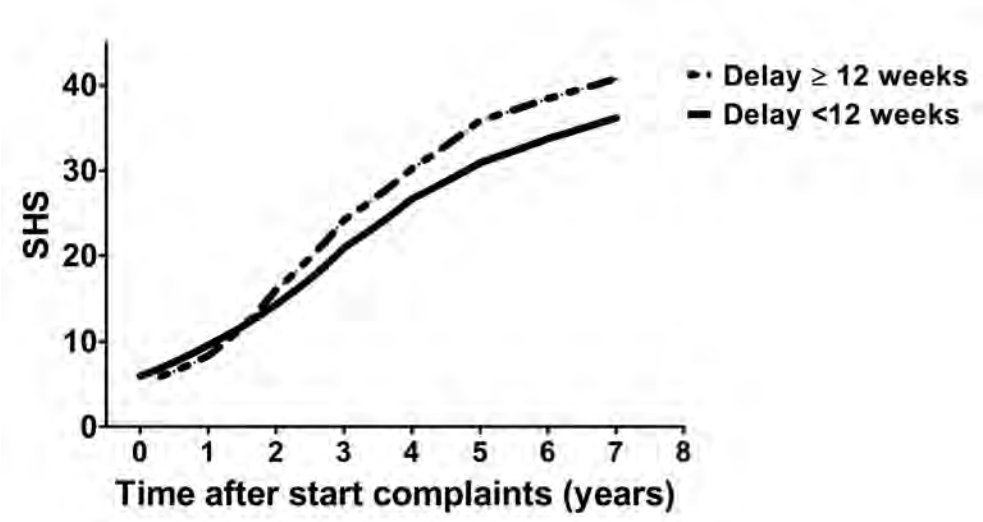

Figure 1 Joint destruction (Sharp/van der Heijde scores) over time in ACPA-positive RA patients with <12 or ≥12 weeks of symptoms at first presentation at the rheumatologist. The date of symptom onset is used as starting point. 70 ACPA-positive patients (22.7%) presented <12 weeks (median after 8 weeks of symptoms) and 239 ACPA-positive patients presented after ≥12 weeks of symptoms (median symptom duration at first presentation at 27 weeks). The RA patients studied were included in the Leiden Early Arthritis Clinic between 1993 and 2006. ACPA, anti-citrullinated protein antibodies; RA, rheumatoid arthritis; SHS, sharp/van der Heijde score.

Anti-citrullinated protein antibodies (ACPA) precede arthritis development and are associated with a severe disease course.[6] We hypothesized that the ACPA response broadens within the very early phase of RA and in doing so limits the 'window of opportunity'. Therefore, it was examined whether patients who were assessed within 12 weeks of symptom onset have a less broadened ACPA response than patients with longer symptom duration.

Three hundred and nine ACPA-positive patients (defined by anti-CCP2-positivity) fulfilling the 1987 ACR criteria for RA and included in the Leiden Early Arthritis

Clinic[7] were studied on the association between symptom duration and the progression in joint destruction over 7.5 years, with symptom onset as starting point.[3] Yearly radiographs of hands and feet were scored according to the Sharp-van der Heijde method.[7] A repeated measurement analysis was used with a random person and time effect; the fixed effect of time was modeled with linear spline functions with knots at each year.[3] Adjustments were made for age, gender and treatment strategy. RA-patients that presented with <12 weeks or ≥12 weeks of symptoms were compared for level, isotype-usage and fine specificity of ACPA at inclusion. Antibody reactivity against peptides derived from human proteins (the citrullinated (Cit) and the uncitrullinated form of two linear peptides derived from vimentin (Vim1-16:STCitS VSSS SYCitCit MFGG and Vim59-74:VYAT CitSSA VCitLCit SSVP), two linear peptides derived from fibrinogen (Fibα27-43:FLAE GGGV Cit GPR VVER H and Fibβ36-52:NEEG FFSA CitGHR PLDK K), one linear peptide derived from α-enolase (Eno5-20:KIHA CitEIF DSCitG NPTV) and Myelin Basic Protein (MBP)) were determined by ELISA and described previously.[3, 7-9] Anti-CCP3 and anti-MCV were also measured by ELISA (Quanta Lite CCP version 3.1 for IgG/IgA, Inova Diagnostics San Diego, USA and Orgentec Diagnostika, Mainz, Germany).

ACPA-positive RA patients who presented <12 weeks of symptom onset had less progression in joint destruction over 7.5 years (p=0.04) (Figure 1). Patients with symptoms <12 weeks revealed no differences in anti-CCP2 level, isotype usage or fine-specificity recognition profile compared to patients with longer symptom duration (Table 1).

To our knowledge this is the first study investigating ACPA-characteristics in relation to the so-called 'window of opportunity'. Recently published data showed a trend for less joint destruction in ACPA-positive RA patients presenting with symptoms <12weeks.[3] In the present study the radiographic data were extended. No clear differences were observed with respect to ACPA characteristics in relation to symptom duration. Although it cannot be excluded that other ACPA-characteristics, such as glycosylation patterns or other 'fine-specificities', would show differences, our data indicate that the 'window of opportunity' is not reflected in the maturation of the ACPA-response.

A longitudinal study-design with regular assessments of ACPA characteristics within the same patients would be more appropriate than a cross-sectional study. However, as ACPA-positive RA patients often present relatively late (only 22.7% of the ACPA-positive RA-patients visited a rheumatologist <12 weeks of symptom onset), it will be difficult to obtain adequate patient numbers.

In conclusion, ACPA-positive RA patients with symptoms <12 weeks have less progressive disease than patients with a longer symptom duration. However, the broadness of the ACPA response is not different between these groups, indicating that maturation of the autoantibody response occurs even earlier.[10]

	<12 weeks		≥12 weeks			
Anti-CCP2 levels*					P-value	
Median (AU)	766		642		0.5	
IQR	285-1711		215-1560			
Fine-specificity	<12 weeks		≥12 weeks		OR	95% CI
cVim1-16-	54	87.1%	177	87.2%	0.99	0.42-2.32
cVim1-16+	8	12.9%	26	12.8%		
cVim59-74-	30	48.4%	100	48.5%	0.99	0.56-1.75
cVim59-74+	32	51.6%	106	51.5%		
cFib-α –	40	64.5%	156	75.5%	0.58	0.32-1.07
cFib-α +	22	35.5%	50	24.3%		
cFib-β –	13	21.3%	60	30.6%	0.61	0.31-1.22
cFib-β +	48	78.7%	136	69.4%		
cEno5-20 –	40	64.5%	139	67.5%	0.88	0.48-1.59
cEno5-20 +	22	35.5%	67	32.5%		
MBP –	19	30.6%	74	35.9%	0.79	0.43-1.45
MBP +	43	69.4%	132	64.1%		
MCV –	3	4.9%	10	5.0%	0.99	0.26-3.71
MCV +	58	95.1%	191	95.0%		
CCP3 –	2	3.3%	14	6.9%	0.46	0.10-2.06
CCP3 +	59	96.7%	188	93.1%		
0-4 peptides**	24	40.7%	97	51.3%	0.65	0.36-1.18
5-8 peptides**	35	59.3%	92	48.7%		
ACPA isotype usage***					OR	95% CI
IgM-ACPA –	13	33.3%	52	33.8%	0.98	0.47-2.07
IgM-ACPA +	26	66.7%	102	66.2%		
IgA-ACPA –	14	35.9%	50	32.5%	1.17	0.56-2.43
IgA-ACPA +	24	64.1%	104	67.5%		
IgG1-ACPA –	0	0%	2	1.3%	N/A	N/A
IgG1-ACPA +	39	100%	152	98.7%		
IgG2-ACPA –	3	7.7%	26	16.9%	0.41	0.12-1.43
IgG2-ACPA +	36	92.3%	128	78.0%		
IgG3-ACPA –	16	41.0%	63	40.9%	1.01	0.49-2.05
IgG3-ACPA+	23	59.0%	91	59.1%		
IgG4-ACPA –	0	0%	6	3.9%	N/A	N/A
IgG4-ACPA +	39	100%	148	96.1%		
0-4 isotypes	14	35.9%	55	35.7%	1.01	0.49-2.10
5-6 isotypes	25	64.1%	99	64.3%		

Table 1. ACPA characteristics at inclusion of ACPA-positive RA patients with symptoms for <12 or ≥ 12 weeks. Fine specificity data were assessed in patients included between 1993 and 2006. Fine-specificity data were missing for 61 patients. Isotype data were determined previously in patients included between 1993 and March 2004 and

are therefore missing in 116 patients. * Difference in anti-CCP2 levels was analyzed using Mann-Whitney test. **8 peptides were included for the high versus low recognition analyses: cVim1-16, cVim59-74, cFib-α, cFib-β, cEno5-20, MBP, MCV, CCP3. ***ACPA isotypes were measured using anti-CCP2 peptides. cEno5-20, citrullinated enolase; cFib, citrullinated fibrinogen; cVim, citrullinated vimentin; MBP, myelin basic protein; MCV, mutated citrullinated vimentin.

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CHAPTER 9

The fine specificity of IgM anti-citrullinated protein antibodies (ACPA) is different from that of IgG ACPA

Arthritis Res Ther. 2011; 13(6): R195

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ABSTRACT

Introduction

The antigen recognition pattern of immunoglobulin M (IgM) could, when directed against protein antigens, provide an indication of the antigenic moieties triggering new B cells. The half-life of IgM is short and memory B cells against T-cell-dependent protein antigens typically produce IgG and not IgM antibodies. In this study, we analyzed whether a difference exists between the fine specificity of IgM versus IgG anti-citrullinated protein antibodies (ACPAs).

Methods

We determined the fine specificity of IgM and IgG ACPAs in 113 ACPA-positive rheumatoid arthritis patients with IgM cyclic citrullinated peptide 2 (CCP2) levels above 100 AU/ml. Fine specificity was assessed by performing ELISA using citrullinated peptides derived from vimentin, fibrinogen-β, fibrinogen-α and α-enolase, as well as citrullinated proteins fibrinogen and myelin basic protein. The arginine counterparts were used as controls.

Results

Recognition of defined citrullinated antigens by IgM ACPA was confined to samples that also displayed recognition by IgG ACPA. However, the IgM ACPA response displayed a more restricted antigen recognition profile than IgG ACPA (OR = 0.26, $P < 0.0001$).

Conclusion

Our data show that several defined citrullinated antigens are recognized only by IgG ACPA, whereas others are also recognized by IgM ACPA. These observations suggest that not all citrullinated antigens are able to activate new B cells despite concurrent recognition by IgG ACPA.

INTRODUCTION

Anti-citrullinated protein antibodies (ACPA) may be involved in the disease pathogenesis of rheumatoid arthritis (RA). ACPA can be found early in the disease course[1], even before disease onset[2], and the presence of ACPA at the time of diagnosis can predict disease course.[3] Moreover, ACPA can contribute to disease pathogenesis by activating immune cells[4,5] and the complement system.[6] The ACPA response likely represents a T-cell-dependent B-cell response, given the protein nature of the antigen recognized and the strong association with the human leukocyte antigen shared epitope alleles. The evolution of such a response is typically characterized by a first wave of IgM antibodies after the first antigen contact, quickly followed by the presence of IgG. After repeated antigen exposure, the IgG responses are further boosted while the IgM peak declines. The latter observation is explained by the presence of Ig-switched, affinity matured, memory B cell that are formed in the presence of CD4+ T cells. These T cells provide the helper activity required for affinity maturation, isotype switching and memory cell formation. When such T-cell help cannot be provided, as in the case of hyper-IgM syndrome, IgG, IgA and IgE antibody levels are absent or severely reduced.[7] The presence of IgG, IgA and IgE ACPAs[5,8], therefore, provides another line of evidence for the T-cell-dependent nature of ACPA responses.

To the best of our knowledge, IgM-producing memory B cells against T-cell-dependent antigens have not been described, in contrast to T-cell-independent B-cell responses against, for example, repetitive sugar residues on bacteria.[9,10] For these reasons, it is most conceivable that the presence of IgM ACPA suggests that activation of recently recruited naïve B cells recognize citrullinated antigens because the half-life of circulating IgM is short. In this study, we hypothesized that there might be certain antigens which drive the ACPA IgM response in RA. We therefore sought to determine whether there is a difference in the fine specificity of IgG and IgM ACPA.

MATERIAL AND METHODS

Fine specificity of anti-citrullinated protein antibody immunoglobulin M

We determined the fine specificity of ACPA IgM and IgG in 113 serum samples of anti-cyclic citrullinated peptide (CCP2) IgG and IgM double-positive RA patients collected from the Leiden Early Arthritis Clinic (EAC), an inception cohort of recent-onset arthritis that was initiated at the Department of Rheumatology at Leiden University Medical Center in 1993.[11] We selected those patients who had a relatively high titer of IgM CCP2 (levels ≥ 100 AU/ ml) to ensure that differences between IgG and IgM reactivity could not be explained by differences in the sensitivity associated with the detection of IgG or IgM antibodies by ELISA. For the determination of ACPA status and ACPA levels of IgM anti-CCP2, we used a commercial immunoassay kit (Euro-Diagnostica, Malmö, Sweden) with minor modifications

for the detection of IgM. The collection and use of patient samples was approved by the local medical ethics committee and in compliance with the Declaration of Helsinki. All patients provided their written informed consent.

Demographic data (age, gender, disease duration and radiographic damage) of ACPA IgG-positive RA patients who had ACPA IgM ≥ 100 AU/ml were not different from patients displaying ACPA IgM levels < 100 AU/ml. The peptides used in this study are linear citrulline (Cit)-containing peptides, which are known ACPA IgG epitopes in RA, as well as their arginine counterparts[12]. Specifically, we used vimentin (Vim) 59 to 74: VYAT CitSSAVCitLCitSSVP; fibrinogen-α (Fib-α) 27 to 43: FLAEGGGVCitGPRVVERH; Fib-β 36 to 52: NEEGFFSA CitGHRPLDKK; and α-enolase 5 to 20: KIHACitEIFDS CitGNPTV.

In addition, we tested citrullinated protein antigens (Fib and myelin basic protein (MBP)) and all of their arginine counterparts. As described previously, the presence of anti-Cit-MBP antibodies on ELISA is clinically equivalent to the original anti-Sa (Cit-Vim) on Western blot assays.[13]

Fine specificity assays of ACPA IgG were performed essentially as described before[12], with minor modifications for ACPA IgM.[8] In brief, for IgM-, Cit- and arginine-containing peptides (10μg/ml) were incubated on streptavidin coated plates. After being washed, sera were incubated at 1:25 dilution at 37°C for 1 hour, bound antibodies were detected using rabbit anti-human IgM (Dako Denmark A/S, Glostrup, Denmark), followed by washing with horseradish peroxidase-labeled goat anti-rabbit IgG (Dako Denmark A/S). 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) was used as a substrate, and absorbance was determined at 415 nm. In each ELISA plate, we included on the Cit-containing peptide eight representative gout controls and used these to calculate the cut-off for positivity.[8] These eight gout controls represent a set of fifty controls as established before[8] and were used on each plate to minimize plate-to-plate variation. This was defined as the mean ± 2 SD of the absorbance on the Cit-containing peptide. In addition, we verified that the difference in absorbance between wells coated with the Cit- and the arginine-containing peptide was at least 0.1 as previously described[8,12] Therefore, sera that fulfilled both criteria were considered positive.

Stability of the anti-citrullinated protein antibody I response

Samples from 18 patients were used to analyze the specific reactivity of IgM against CCP2, Fib-α and Fib-β over time. We used serum samples obtained at baseline and at 1, 2 and 5 years of follow-up. Samples were tested by ELISA as described above. Samples were considered positive when they displayed an absorbance value higher than the cut-off and a difference of at least 0.1 absorbance units when comparing the reactivity against the Cit-containing peptide and its arginine-containing control peptide.

Statistical analysis

Differences between groups were analyzed using the Mann-Whitney U test or analysis of variance. The associations between ACPA IgM and ACPA IgG positivity were evaluated by using 2 × 2 tables for the estimation of ORs and 95% CIs. The correlation between ACPA IgM and ACPA IgG responses were determined using Spearman's rank-correlation coefficients. The c2 test was used to evaluate differences in the frequency distribution of ACPA IgG and IgM. All data were analyzed with GraphPad Prism version 4.0 software (GraphPad Software, San Diego, CA, USA) or SPSS for Windows release 16.0 software (SPSS Inc, Chicago, IL, USA). In all tests, $P < 0.05$ was considered significant.

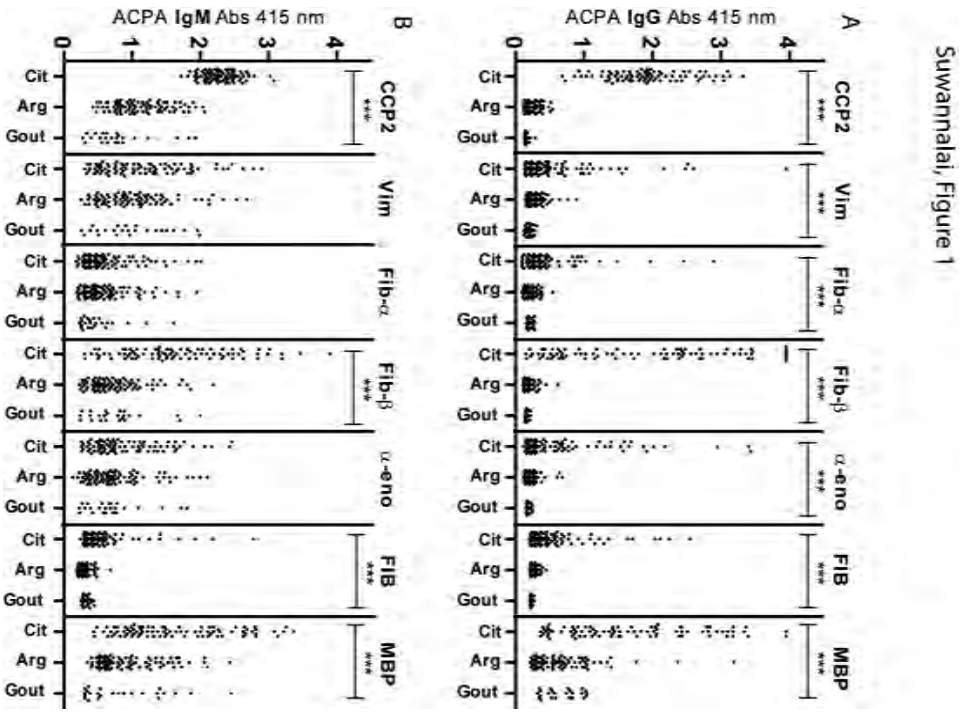


Figure 1. The fine specificity of immunoglobulin G (IgG) and IgM anticitrullinated protein antibodies (ACPAs). The absorbance (Abs) at 415 nm of ACPA IgG **(A)** and IgM **(B)** ACPA fine specificity. ***P < 0.001. Data for gout controls from three ELISA plates are depicted. α-eno = α-enolase; Arg = arginine; CCP2 = cyclic citrullinated peptide 2; Cit = citrulline; Fib = fibrinogen; MBP = myelin basic protein; Vim = vimentin.

RESULTS

Anti-citrullinated protein antibody fine specificity is different from IgG fine specificity

It has been shown that IgM ACPA can be detected in the sera of RA patients with established disease, suggesting an ongoing recruitment of new B cells into the ACPA response.[8] If IgM is the result of triggering of naïve B cells, then the fine specificity of ACPA IgM might conceivably differ from ACPA IgG, as IgM will mainly be directed against the antigens that have recently been detected by B cells.[14] Therefore, we determined the ACPA IgM and IgG responses against a set of citrullinated peptides from Fib, enolase and Vim, as well as the responses against two citrullinated proteins (Figures 1A and 1B). Our data show that some but not all citrullinated epitopes are recognized by IgM ACPA (Table 1 and Figure 1B). These data indicate a restricted epitope recognition profile by IgM ACPA, as some epitopes were not recognized by IgM in all analyzed patient sera.

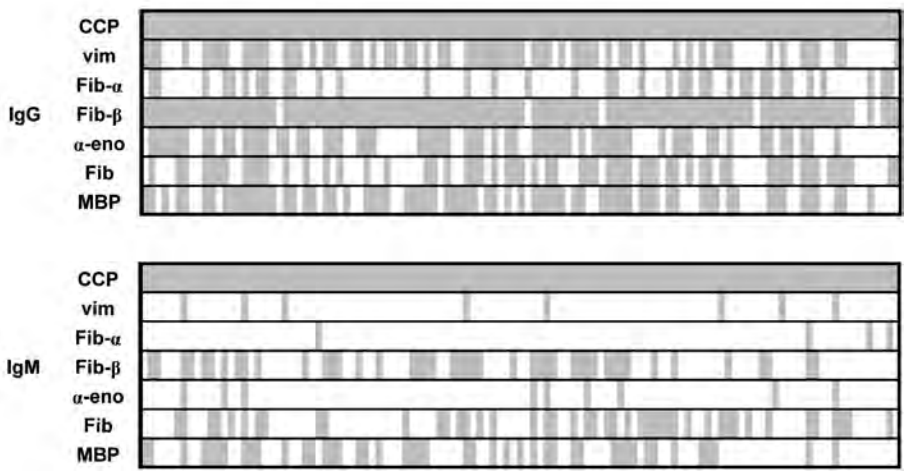
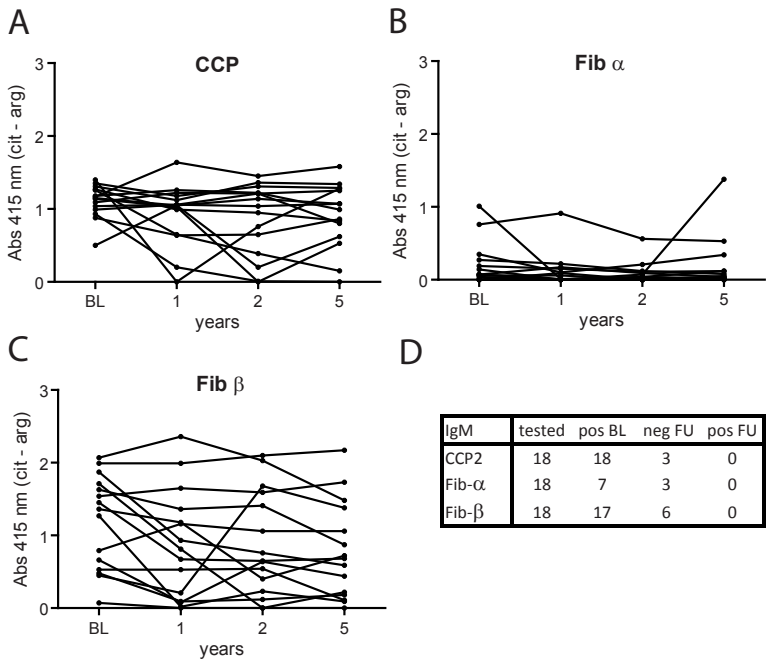


Figure 2. Positivity of immunoglobulin G (IgG) and IgM anticitrullinated protein antibodies (ACPAs). The citrullinated epitopes recognized by IgG and IgM ACPAs in each individual. The gray area represents positive reactivity, and the white area represents lack of reactivity. α-eno = α-enolase; CCP = cyclic citrullinated peptide; Fib = fibrinogen; MBP = myelin basic protein; Vim = vimentin.

We next determined the recognition profile of IgG ACPA. Although not all patient sera recognized all citrullinated epitopes, the IgG ACPA epitope recognition pattern was clearly much broader than the epitope recognition profile of IgM ACPA (Figures

1A and 2). In fact, when all ACPA reactivities were analyzed as a group, the chance of having an IgM-positive response was four times lower compared to IgG (OR = 0.26, P < 0.0001). For those reactivities where, next to IgG ACPA, IgM ACPA responses also were apparent, a correlation between the titers of IgG and IgM ACPAs was observed between the positive samples (data not shown).

When reactivity patterns were directly compared at an individual level, we observed that IgM ACPAs against some citrullinated epitopes, but not others, were present. However, in all cases, IgM positivity was paralleled by the presence of IgG recognizing the same epitopes. In contrast, patients can display certain IgG ACPA reactivities in the absence of IgM reactivities against the same antigen (Figure 2). Together, these data indicate that IgM ACPAs display a more restricted antigen recognition profiles as compared to IgG ACPAs.



IgM	tested	pos BL	neg FU	pos FU
CCP2	18	18	3	0
Fib-α	18	7	3	0
Fib-β	18	17	6	0

Figure 3. Stability of the ACPA IgM response. Sera of 18 patients obtained at baseline (BL) and at 1, 2 and 5 years of follow up were analyzed by ELISA for reactivity against CCP2, Fib-α and Fib-β. (A,B,C) graphs display the IgM reactivity to the indicated peptides over time. Shown is the absorbance of the citrulline reactivity minus the absorbance of the arginine reactivity. (D) table shows the number of positive patients at baseline (BL) as well as the conversion towards negative or towards positive during one of the follow-up (FU) time points analyzed.

Stability of the anti-citrullinated protein antibody I response

To address the question whether the observed differences between IgG and IgM reactivity are limited to baseline samples only or whether they are also present at later follow-up, we analyzed ACPA IgM reactivity against three peptides over time. The reactivity of ACPA IgM against CCP2, Fib-α and Fib-β at baseline and at 1, 2 and 5 years of follow-up was analyzed in sera of 18 patients. We observed that the ACPA IgM levels against these peptides appeared rather stable (Figures 3A to 3C). Some patients whose sera reacted with CCP2, Fib-α and Fib-β at baseline may over time become IgM-negative (Figure 3D). Importantly, none of the patients tested became IgM-seropositive against either Fib-α or Fib-β peptides. These data indicate that the observations made using the base- line samples (Figures 1 and 2) would also be made at any of the follow-up time points. Collectively, these data therefore indicate that the ACPA IgM response is narrower than the ACPA IgG response, an observation not applicable only to the baseline results but also during follow-up.

	IgG (%)	IgM (%)
CCP2	100	100
Vim	56	7
Fib-α	33	4
Fib-β	94	40
α-enolase	55	8
Fib	53	40
MBP	63	40

Table 1. Specific positivity of anticitrullinated protein antibody immunoglobulin G and immunoglobulin M in 113 rheumatoid arthritis patients directed against a set of defined specificities. CCP2 = cyclic citrullinated peptide 2; Fib = fibrinogen; IgG = immunoglobulin G; IgM = immunoglobulin M; MBP = myelin basic protein; Vim = vimentin.

DISCUSSION

In this study, we observed that ACPA IgM responses are different from ACPA IgG responses, as they display a more restricted antigen recognition pattern. These data are intriguing, as they indicate that the regulation of the IgM ACPA response differs from the regulation of B cells producing IgG-directed against citrullinated antigens. Although the reason for this difference is not known, we think it is most conceivable that these findings are explained by a limited recruitment of new B

cells into the ACPA response that is driven by some, but not other, citrullinated antigens. Given the short half-life of circulating IgM and the lack of memory B cells producing IgM against protein antigens, we think that the IgM ACPAs detected in this study are produced by new B cells that are recruited into the ACPA response against certain citrullinated antigens. Even in the case where ACPA IgM-producing memory B cells exist, it is still interesting that such cells are present only against certain citrullinated antigens. For this study, we selected patients who are double-positive for IgG and IgM CCP2 reactivity. To exclude the possibility that differences between IgG and IgM may be explained by differences in the sensitivity associated with the detection of IgG or IgM antibodies by ELISA, we selected only patients with an IgM level of at least 100 AU/ml.

Indeed, in the setup we used, we observed that, although the IgG responses against the different citrullinated antigens were rather similar in absorbance units, IgM reactivity was detected only against some antigens but not others. This observation was made at baseline but appears also to be present at follow-up. To ensure that we did not introduce additional bias into our data, we compared the demographic data (age, gender, disease duration and radiographic damage) of ACPA IgG-positive RA patients who had ACPA IgM ≥ 100 AU/ml with those who had ACPA IgM < 100 AU/ml and observed no differences.

Since IgM rheumatoid factor (RF) could also be a confounding factor, we compared RF levels between the patients who displayed ACPA IgM ≥ 100 AU/ml with those who had ACPA IgM < 100 AU/ml and observed that in RA patients with ACPA IgM ≥ 100 AU/ml, the levels of RF IgM were higher than in RA patients who had ACPA IgM < 100 AU/ml (RF IgM 50 AU/ml (25 to 118) and 28 AU/ml (12 to 60), respectively). To exclude the possibility that our findings were influenced by IgM RF, we analyzed ACPA IgM specificities in relation to IgM RF. We observed that IgM RF-positive samples can be negative for IgM ACPA reactivities and that, in the absence of IgM RF, IgM ACPA reactivities can be detected readily (data not shown). RF-positive samples that have IgG reactivity against all fine specificity epitopes may have IgM ACPA against only some antigens and not others, confirming that our assay did not merely detect IgM RF. Previously, we addressed this issue experimentally.[8] When RFs were depleted from RF IgM-positive, ACPA IgM-positive and ACPA IgG-positive sera using IgG-coated Cyanogen bromide-activated-Sepharose beads (Sigma-Aldrich, St Louis, MO, USA), there was no reduction of ACPA IgM levels. Moreover, after mixing sera which were highly positive for RF IgM but negative for ACPA IgG and ACPA IgM with sera which were RF IgM- and ACPA IgM-negative but ACPA IgG-positive, ACPA IgM could not be detected.[8] Collectively, these observations support the notion that true IgM ACPA and not IgM RF bound to ACPA IgG were detected by the methods employed.

Although it is tempting to speculate, these studies should not be taken as an argument for the involvement of the antigens analyzed here in the recruitment of

new B cells into the ACPA response. Peptides are unlikely to reflect correctly the three-dimensional structure of citrullinated proteins that form the epitope for antibodies. Moreover, ACPA IgG is cross-reactive to multiple citrullinated antigens[15,16], and therefore recognition of a citrullinated antigen by ACPA IgG does not indicate that this antigen is necessarily involved in the induction of B-cell responses. Nonetheless, our data do show that the IgM ACPA response is significantly more restricted than that of the IgG ACPA present in the same patient.

How IgM can be formed in the presence of an active IgG response against the same antigen is not clear. In other situations, as exemplified by the prophylactic administration of anti-Rhesus D antigen antibodies to pregnant women carrying a Rhesus D-positive child, the presence of IgG against a certain antigen will prevent the induction of a novel IgM response. The mechanism behind this protective measure is thought to be mediated by either the capture and clearance of circulating Rhesus D antigen and/or by IgG-Rhesus D immune complexes that inactivate new Rhesus D-reactive B cells through FcgRIIB, the inhibitory Fcg IIB receptor[17]. Clearly, IgG ACPAs do not inhibit the activation of IgM-positive, citrullinated antigen-reactive B cells. The reason for this finding is not known but could possibly be explained by the low avidity of the ACPA[18], conceivably resulting in "nonstable" immune complexes unable to trigger FcgRIIB.

CONCLUSIONS

Collectively, our data show that the immune response against one citrullinated antigen is different from the immune response against another citrullinated protein. Some responses are dominated by IgG, whereas both IgM and IgG responses were found for other ACPA antigens. Elucidation of the mechanism behind this observation could be of relevance to the identification of those citrullinated antigens that drive ACPA responses and could provide clues to how the continuous recruitment of new B cells can be halted.

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CHAPTER 10

The concentration of anticitrullinated protein antibodies in serum and synovial fluid in relation to total immunoglobulin concentrations

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*: The first two authors contributed equally

ABSTRACT

Background

Anticitrullinated protein antibodies (ACPA) are one of the best predictors for the development of rheumatoid arthritis. Nonetheless, relatively little information is present on the absolute concentration of ACPA in relation to total immunoglobulin (Ig) concentrations. Such information would be of relevance to compare ACPA levels to other antibody levels. Here, we estimated the relative abundance of ACPA immunoglobulins in serum and synovial fluid using a quantitative approach.

Methods

ACPA were purified using HiTrap Streptavidin columns coupled with biotinylated cyclic citrullinated peptide (CCP2). Total Ig and anti-CCP2 isotype reactivities were measured by ELISA.

Results

ACPA were successfully isolated as substantial antibody amounts were eluted from sera of ACPA positive patients and neglectable antibody amounts were eluted from sera of ACPA negative patients. Up to 1 in 80 IgG-molecules were estimated to be ACPA. Strikingly, IgM-ACPA was most abundant in synovial fluid (with the highest enrichment in the range of 1 IgM-ACPA for every 8 IgM-antibodies).

Conclusion

ACPA-IgG levels are estimated to be within the range of peak levels of protective antibody responses against recall antigens. IgM-ACPA is abundantly present in synovial fluid, suggesting the presence of a continuous ongoing autoimmune response in the synovial compartment.

INTRODUCTION

Anti-citrullinated protein antibodies (ACPA) have been shown to initiate and enhance disease in murine models of arthritis[1, 2] and to activate both FcR-positive cells[3, 4] and the complement system,[5] arguing that they could play a role in disease pathogenesis. ACPA are highly specific for RA and can be detected years before clinical manifestations.[6, 7] Activation of naïve B-cells upon the first antigen encounter results in proliferation and differentiation in IgM-secreting cells. During their differentiation upon further contact with T cells, some B cells undergo isotype switching and affinity maturation. The ACPA response utilize all isotypes.[4, 8-10] Intriguingly, an expanded ACPA-isotype profile associates with more severe radiographic damage, indicating that the extent of the ACPA-response impacts on disease progression.[11]

It has been shown that anti-CCP2-antibody levels are higher in synovial-fluid than serum.[12-14] However, limited information on absolute ACPA levels in either synovial fluid or serum is present as the levels are generally expressed as arbitrary units. Nonetheless, information on the absolute concentration of ACPA is of interest as it allows the comparison of the ACPA response to other antibody responses in quantitative terms. To quantitate ACPA levels it is required to isolate ACPA. Here we present data on the estimation of ACPA quantities in serum and synovial-fluid.

MATERIALS AND METHODS

Patient population

Patients analyzed were derived from the Leiden Early Arthritis clinic (EAC) cohort.[15] All patients fulfilled the American College of Rheumatology 1987 revised criteria for the classification of RA.[16] Serum samples of 10 ACPA-positive and 2 ACPA-negative RA patients and 2 healthy controls were obtained for ACPA purification. Knee synovial-fluid was obtained at the time of therapeutic arthrocentesis. Samples were obtained from 5 ACPA-positive (including 2 paired synovial-fluid-serum samples selected for high IgM-ACPA levels) and 1 ACPA-negative RA patient and 1 patient with osteoarthritis, attending the outpatient clinic of the rheumatology department in Leiden. The protocols were approved by the relevant local ethics committee and all participants provided informed consent.

Affinity-purification of ACPA

Synovial-fluid samples were treated with hyaluronidase type IV from bovine testes (Sigma Aldrich; 100 μg/ml) and protease-inhibitor (Sigma Aldrich; 1:50). ACPA from synovial-fluid and serum were purified using HiTrap-streptavidin HP 1 ml columns (GE-Healthcare) coupled with biotinylated CCP2 peptides as described previously.[17] The CCP2 peptide was obtained from Dr Drijfhout, Department of

IHB, LUMC, The Netherlands. Antibodies were eluted with 0.1M Glycine Hydrogen Chloride pH2.5 and neutralized with 2 M Tris. A control column coated with CCP2 arginine was attached before the CCP2 citrulline column. This was necessary to control for non-specific adherence of antibodies to the bead material. We observed that, indeed, a considerable amount of IgG (mean: 10ug/ml) adhered to the control column (data not shown). After running the sample the columns were disconnected and eluted separately to guarantee the citrulline specificity of the purified antibodies.

Anti-CCP2 assays

Anti-CCP2 IgG was measured by ELISA (Immunoscan RA Mark2; Eurodiagnostica, Arnhem, The Netherlands). Samples with values > 25 units/ml were considered positive according to the manufacturer's instructions. Anti-CCP-positive individuals were considered ACPA-positive.

Quantitative IgA, IgM, IgG ELISA

Quantitiative Ig analyses were performed using the Human IgA-ELISA, IgG-ELISA and IgM-ELISA Quantitation Set (Bethyl Laboratories, Inc, USA) according to the manufacturer's instructions.

RESULTS

Affinity-purification of ACPA

ACPA were successfully isolated using HiTrap Streptavidin columns loaded with CCP2 peptides as substantial amounts of antibodies were eluted from sera of ACPA positive RA patients and neglectable amounts from sera of ACPA-negative patients or healthy controls (range: 0-2.1 µg/ml) (table 1). Two affinity-columns, one coated with biotinylated CCP2 arginine and one coated with biotinylated CCP2 citrulline, were used to purify ACPA (figure 1A). The CCP2-arginine coated column was essential in the purification procedure, as considerable amounts of, most likely, non-specific antibodies were eluted from this control column (mean: 10µg IgG/ml). The flow-through was collected and antibodies were eluted with glycine-HCl and directly neutralized. The flow-through was completely devoid of anti-CCP2-reactivity, except for 4 high-titer samples (figure 1B), probably due to overloading of the column. Recovery of ACPA-activity was not complete. In some samples only 20% of ACPA-activity could be detected. The recovery rate of ACPA of the different immunoglobulins (Ig) after purification was comparable. Together, these data indicate that ACPA can be purified from ACPA-positive sera and indicate that numbers of absolute ACPA levels are possibly an underestimation as not all ACPA activity originally present in the samples has been recovered.

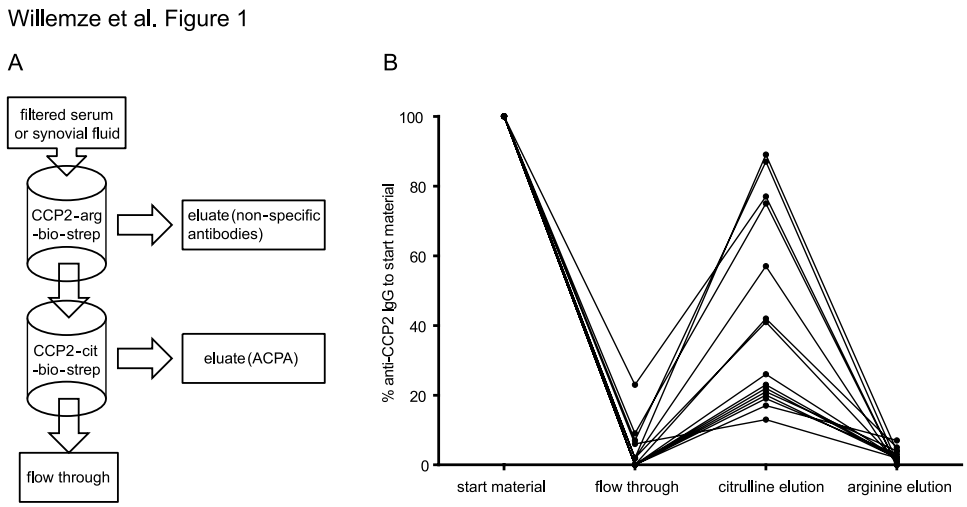

Figure 1. Affinity-purification of ACPA. ACPA was purified using 2 affinity-columns. A control column coated with CCP2-arginine was attached to the column with CCP2-cittrulline to guarantee the citrulline specificity of the purified antibodies (A). In the elution step the two columns were disconnected and the antibodies were eluted off separately. Start material (serum or synovial-fluid) (1), flow through (2), elution CCP2-arginine (3) and elution CCP2-citrulline (4) were measured for ACPA presence and total immunoglobulin content. ACPA were effectively purified using HiTrap Streptavidin columns (B). Hardly any ACPA remained in the flow-through (FL). However, not all ACPA-activity could be recovered as in some patients only 20% of the ACPA-activity present in the start material were present in the elution, indicating an underestimation of the amount of quantified ACPA. The elution of the arginin-column contained barely any ACPA. The percentage in the graph represents the amount of ACPA present in relation to the startmaterial. The different fractions were measured with an anti-CCP2 ELISA and the amount was measured in AU/ml.

Quantification of ACPA immunoglobulins present in serum and synovial-fluid

After affinity purification, ACPA-IgG levels of ACPA-positive patients were quantified by measuring the amount of IgG present. In sera of ACPA-positive patients, we measured up to 60 µg/ml IgG-ACPA (mean: 28µg/ml). As hardly any IgG could be isolated and measured from sera of ACPA-negative RA patients (mean: 1.5 µg IgG/ml), we conclude that considerable amounts of ACPA are present in ACPA-positive sera (table 1). These levels were estimated to be on average almost 1 in 183 IgG-antibodies. Within patients with high levels even up to 1 in 80 antibodies were ACPA (figure 2). IgA-ACPA is less abundant, only 4 patients displayed a level higher than 1 µg/ml in serum. The abundance of IgA-ACPA appeared, therefore, limited

	ACPA-status	IgG-ACPA AU/ml	Total IgG mg/ml	Total IgA mg/ml	Total IgM mg/ml	anti-CCP2 IgG µg/ml	anti-CCP2 IgA µg/ml	anti-CCP2 IgM µg/ml
RA1-serum	+	195	6,2	1,6	0,4	6	0	0
RA2-serum	+	1121	4	1,1	1,2	18,2	0	0
RA3-serum	+	273	7,8	2,4	1,2	8,4	0	0,2
RA4-serum	+	752	3,7	2,2	0,3	31,8	5,6	0,7
RA5-serum	+	59	4,7	1,2	0,4	7,8	0	0
RA6-serum	+	407	4,4	3	0,6	17,5	1,1	0,9
RA7-serum	+	514	6	5,4	1.0	20,4	0,5	2,8
RA8-serum	+	826	5,1	1,2	1.0	59,2	0,7	1,3
RA-P1-serum	+	2957	3,9	1,5	0,8	46,7	5,4	85,2
RA-P2-serum	+	3859	4,8	1,8	1,0	60,3	5,9	98,6
RA9-serum	-	<25	4,8	1,6	1,6	2,1	0	0,9
RA10-serum	-	<25	6,6	1,8	0,8	0,8	0	0
HC1-serum	-	<25	4,6	1,5	3,4	0	0	0
HC2-serum	-	<25	5,8	2	3,8	0,5	0	0,8
RA11-SF	+	423	6,2	1,8	0,6	3,3	0,6	7,3
RA12-SF	+	176	7,3	1,2	0,4	16,5	0,2	2,8
RA13-SF	+	2929	10	1,6	0,3	103,2	1,1	9,6
RA-P1-SF	+	1691	2,5	0,8	0,8	58,2	4,3	101,4
RA-P2-SF	+	2710	2,7	0,9	0,7	54,2	4,4	88,8
RA14-SF	-	<25	2,2	0,6	0,4	0	0	0,5
OA1-SF	-	<25	0,8	0	0	0	0	0

Table 1. Relative abundance of ACPA-immunoglobulins in serum and synovial fluid in relation to total immunoglobulin levels. IgG-ACPA (AU/ml), total IgG, total IgA and total IgM were measured in serum or synovial fluid before purification of ACPA. Purified ACPA was measured for the IgG, IgA, and IgM concentration (µg/ml). The paired samples (P1 and P2) were selected for their high IgM ACPA levels (~5000 AU/ml). ACPA= anti-citrullinated protein antibody. SF= synovial Fluid. RA=rheumatoid arthritis. OA=osteoarthritis. P= paired sample. HC= healthy control

with a maximum of 0.36% of all IgA present in serum. IgM-ACPA was abundantly present in some subjects, in 2 patients around 10% of the serum IgM appeared to be ACPA.

Next we compared ACPA levels in paired serum- and synovial-fluid samples. A trend towards higher levels of ACPA-IgG in synovial fluid as compared to serum was observed, however this difference does not reach statistical significance (p=0.07). In contrast, a significant increase in IgM-ACPA was noted in synovial fluid as compared to serum (Fig 2a). To quantify these ACPA-levels we next purified ACPA from synovial-fluid to investigate the local presence of the ACPA isotypes in the joint. Surprisingly, IgM-ACPA was most abundantly present in synovial fluid, where up to 13% of IgM-antibodies were estimated to be ACPA (figure 2). These 2 patients displayed high IgM ACPA levels in synovial-fluid (~5000 AU/ml). Up to 101 µg/ml IgM-ACPA was present in synovial-fluid (range: 2.8-101 µg/ml).

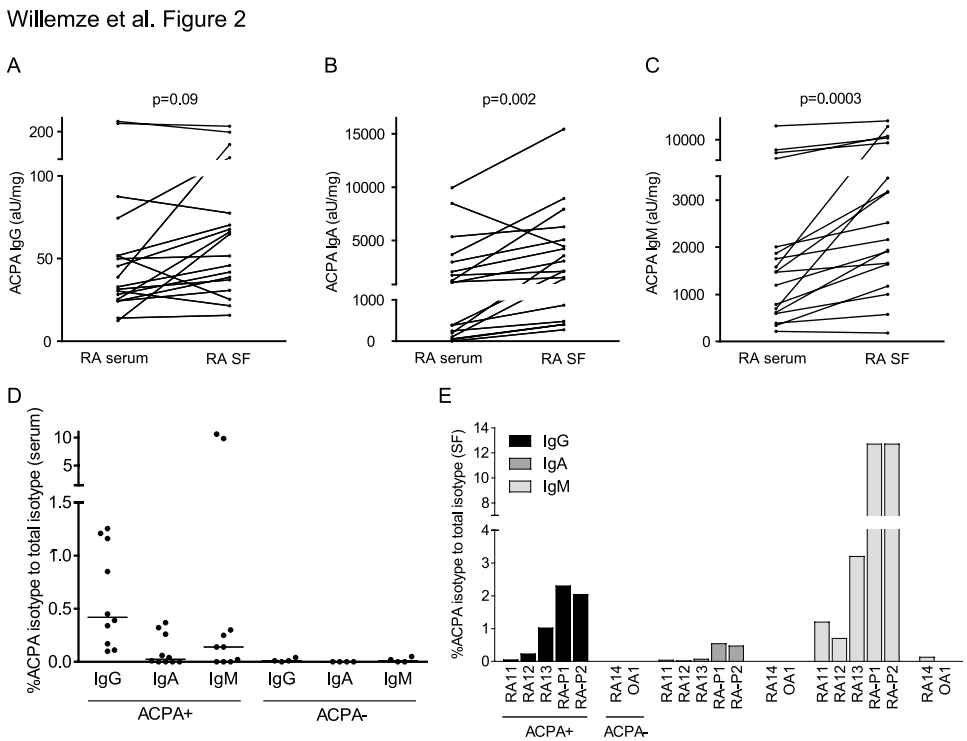

Figure 2. ACPA immunoglobulins in serum and synovial fluid of RA patients. ACPA IgG in relation to total IgG (aU/mg total IgG) is not significantly higher in synovial fluid as compared to serum, only a trend was found (Wilcoxon signed-rank test p=0,09) (A). ACPA IgA (aU/mg total IgA) and ACPA IgM (aU/mg total IgM) are significantly enriched in synovial fluid as compared to serum (Wilcoxon signed-rank test: IgA, p=0,002, IgM, p=0,0003)

(B-C). Percentage ACPA IgG, IgA and IgM of total IgG, IgA and IgM in serum are depicted for 10 ACPA positive RA patients, 2 ACPA negative RA patients and 2 healthy controls (D). Percentage ACPA IgG, IgA and IgM of total IgG, IgA and IgM in synovial fluid are depicted for 5 ACPA positive RA patients, 1 ACPA negative RA patient and 1 osteoarthritis patient (E).

IgG-ACPA was found in synovial fluid up to 103 µg/ml (table 1), based on the total amount of IgG present, this indicates that in synovial-fluid up to 2.3% of total IgG is ACPA.

In line with the small amount IgA-ACPA in serum, also little IgA-ACPA was present in synovial fluid. We included synovial fluid of one ACPA-negative patient with RA and one patient with osteoarthritis patient as controls. In these samples, no IgA-ACPA and IgG-ACPA were detected. The purified sample of the ACPA-negative patient contained 0.5µg/ml IgM.

DISCUSSION

In this study, we quantified the abundance of different ACPA-immunoglobulins in serum and synovial-fluid of RA patients. IgG-ACPA is present in relatively high concentrations in serum and synovial-fluid as up to 1 out of 80 IgG-antibodies can be ACPA. These findings extend a previous observation describing the estimation of IgG-ACPA amounts using 3 RA sera.[18] Furthermore, we now report that IgM-ACPA can be abundantly present in synovial-fluid (up to 13% of total IgM). Next to IgM-ACPA, also IgG-ACPA was found in relatively high concentrations in synovial-fluid (up to 2.3% of total IgG). The estimations presented are conceivably an underestimation of the true quantity as not all ACPA-activity could be recovered after purification possibly as a result from the isolation procedure. Nonetheless, we feel that the estimations presented provide a good reflection of total Ig ACPA levels as also evidenced by the good correlation between ACPA-IgG levels in serum and purified ACPA-IgG concentrations (Spearman's rank correlation coefficient: 0.931. $p<0.01$).

The amounts of IgG-ACPA (mean: 28 µg/ml) present in the sera are remarkably in line with the peak levels of IgG directed against tetanus following repetitive vaccinations (20-28 µg/ml).[19] Protective antibody titers after vaccination have been described as titers above 1 µg/ml against for example Haemophilusinfluenza type-b and group-B streptococci.[19-22] Surprisingly, the ACPA-concentrations found exceed these protective antibody titers and are in the same range as the amount of antibody present shortly after vaccination. The relatively high ACPA antibody levels might be related to the continuous presence of citrullinated antigens in the joint, which could activate ACPA producing B cells.

Furthermore, the results indicate the abundant presence of IgM-ACPA in synovial

fluid, as up to 1 in 8 IgM antibodies can be ACPA in patients with high ACPA-levels. IgM responses against T cell dependent antigens are, in general, not continuously present. In the setting of vaccination, for example, levels of antigen specific IgM decrease within weeks after immunization against rabies.[23] Therefore the high concentrations of IgM-ACPA in sustained disease is intriguing and suggest the continuous local production and conceivably persistence of autoreactive B cell clones at the site of the inflamed joint. Previously, we reported that some IgG-ACPA positive patients harbour IgM-ACPA 7 years after the initial presence of IgG-ACPA.[10] Since IgM-antibodies have a half-life of days and long-lived plasma cells producing IgM against protein antigen have not been described in humans, the continuous presence of IgM against T cell dependent antigens, indicate the continuous triggering of newly generated B cells. This suggests also that novel IgM producing B-cells are continuously recruited into the ACPA-response, indicating that the ACPA-response is continuously reactivated during the course of arthritis.[10]

Nonetheless we feel that the data presented on IgM-ACPA levels should be taken with caution as we can formally not exclude that the measured IgM-ACPA levels are influenced by IgM-rheumatoid factor (RF) bound to ACPA. However, previously reported data by our group showed that the depletion of IgM-RF did not result in reduction of IgM-ACPA levels. Furthermore mixing sera of RF-positive with IgM-ACPA-negative patients did not change the reactivity.[10] Likewise, not all IgM-ACPA positive patients included in this manuscript were IgM-RF-positive, excluding a contribution of IgM-RF, at least in these patients.

In conclusion, high concentrations of ACPA are present in serum and synovial fluid of ACPA-positive RA patients, exceeding protective antibody levels against recall-antigens. Furthermore, the abundance of IgM-ACPA in synovial-fluid indicates ongoing recruitment of new B cells into the ACPA response, reflecting a continuous (re)activation of the RA specific ACPA response during the course of arthritis.

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CHAPTER 11

Recognition of citrullinated and carbamylated proteins by human antibodies: specificity, crossreactivity and the 'AMC-Senshu' method

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Recently, a novel family of autoantibodies in rheumatoid arthritis (RA) patients was described: anti-carbamylated protein (Anti-CarP) antibodies, which target carbamylated (homocitrulline-containing) epitopes.[1, 2] Since citrulline and homocitrulline have a similar structure, we wished to determine to what extent human autoantibodies can differentiate between them. Unlike human antibodies, the anti-modified citrulline (AMC) antibody developed by Dr Senshu[3-7] is able to recognise citrullinated epitopes irrespective of the neighbouring amino acids. Thus, we also aimed to verify whether the AMC assay could distinguish between these two amino acids.

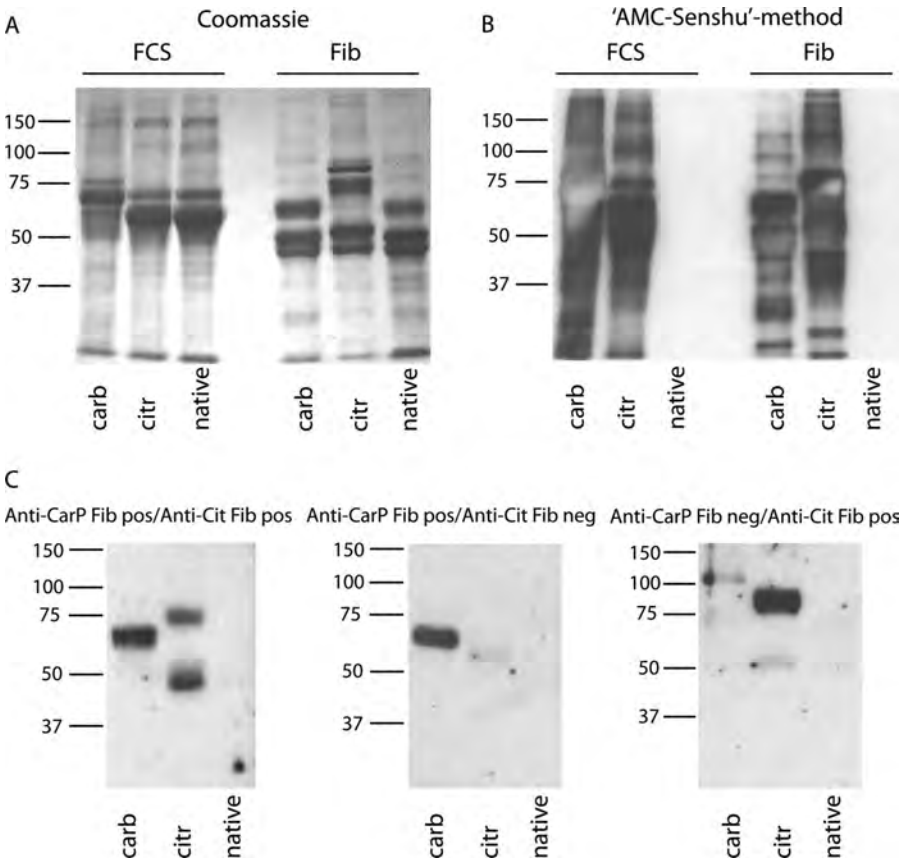

Figure 1 The 'Anti-Modified Citrulline (AMC)-Senshu' method does not discriminate citrullinated and carbamylated antigens, while human autoantibodies do. (A) Coomassie blue staining showed equal loading of foetal calf's serum (FCS), Ci-FCS, Ca-FCS, fibrinogen (Fib), Ci-Fib and Ca-Fib. (B) The 'AMC-Senshu' antibody used according to the protocol of the manufacturer, did not recognise FCS and Fib, but strongly recognised Ci-FCS, Ca-FCS, Ci-Fib and Ca-Fib. (C) Three selected rheumatoid arthritis sera can recognise both Ci-Fib and Ca-Fib, or only one of the modifications specifically.

To address this, we loaded gels with citrullinated, carbamylated and non-modified forms of foetal calf's serum (FCS) and human fibrinogen (Fib). Gels loaded with equal amounts of these protein preparations (figure 1A) were used for western blotting and staining with the 'AMC-Senshu' method. Both the citrullinated and the carbamylated forms of the proteins tested were strongly recognised, whereas, the non-modified form did not reveal any staining (figure 1B). Staining similar western blots with selected human sera[2] revealed that sera-positive for anti-citrullinated protein antibodies (ACPA) and anti-CarP stained both citrullinated (Ci) and carbamylated (Ca) forms of Fib, whereas, single positive sera stained only one form of modified Fib (figure 1C). These data indicate that although the 'AMC-Senshu' method does not discriminate between these two modifications, human sera of RA patients are able to.

In the double positive sera, the antibody response may either be cross-reactive, or harbour two separate reactivities. Four double positive sera were tested in inhibition assays in which the sera were incubated with Fib, Ci-Fib or Ca-Fib at 4°C overnight before detecting binding to Ci/Ca-Fib. Fib did not inhibit sera binding to Ci/Ca-Fib (figure 2A,B). In the sera analysed, binding to Ca-Fib can be inhibited by Ci-Fib to various degrees, whereas, binding to Ci-Fib could be inhibited by Ca-Fib to approximately 30% (figure 2A,B). These data indicate that part, but not all ACPA and anti-CarP antibodies are cross-reactive.

In addition, we performed ACPA depletion studies in which double positive sera were depleted of ACPA. Biotinlylated CCP2 peptide and its arginine control were loaded separately onto HiTrap Streptavidin HP Columns. ACPA/anti-CarP double positive sera were applied to one CCP2 arginine-loaded column connected to one CCP2 citrulline-loaded column. The starting material and flow-through were tested on anti-CarP FCS and CCP2 ELISAs. After ACPA depletion, more than 98% of ACPA in the sera was depleted (figure 2C), while more than half the anti-CarP antibodies remained in the flow-through in five out of seven samples (figure 2C), confirming the data presented in figure 2A,B by showing that part, but not all anti-CarP antibodies are cross-reactive to citrullinated epitopes.

As suggested before, we found the 'AMC-Senshu' method cannot differentiate citrullination and carbamylation.[8, 9] Interestingly, part of the human sera can make this distinction. Anti-CarP antibodies and ACPA are often detected together, and here we show that double positive samples harbour anti-citrullinated epitope-specific antibodies, anti-carbamylated epitopes specific antibodies and cross-reactive antibodies. The finding that the 'AMC-Senshu' method recognises both citrullinated and carbamylated proteins does not argue against the notion that citrullinated proteins are present in synovial fluid and tissues, since a number of studies confirmed the presence of citrullinated proteins by mass spectrometry fingerprinting.[3-5, 7]

However, our study suggests that the extent and nature of citrullination and carbamylation in the joint should be re-evaluated especially in light of our recently described anti-CarP response that is, at least in part, not cross-reactive with citrullinated proteins.

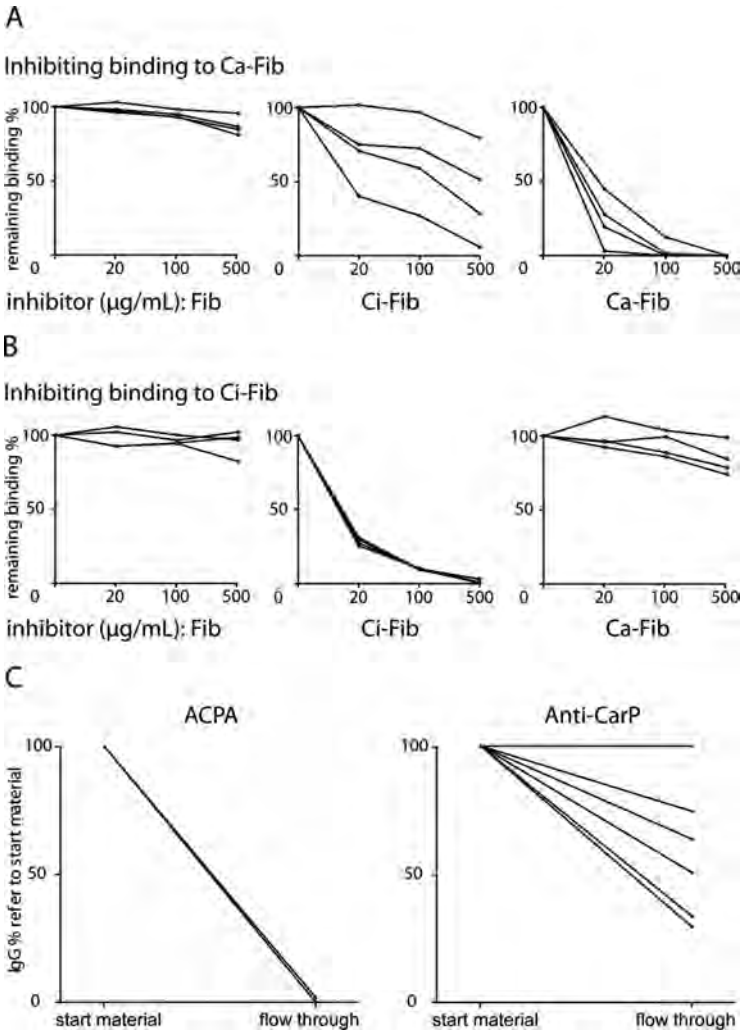

Figure 2 Anti-carbamylated protein (Anti-CarP) antibodies and ACPA represent two families of autoantibodies. (A) Inhibition studies on sera double positive for ACPA and anti-CarP antibodies. Fibrinogen (Fib) does not inhibit sera binding to Ca-Fib. Ci-Fib can partially inhibit sera binding to Ca-Fib, whereas, Ca-Fib can completely inhibit binding to itself. (B) Fib does not inhibit sera binding to Ci-Fib, whereas, Ci-Fib can inhibit more than 97% of binding to itself. Ca-Fib can only inhibit less than 30% of sera binding to Ci-Fib. (C) After ACPA depletion using CCP2 loaded columns, more than 98% of ACPA was depleted from the sera, while more than 50% of anti-CarP antibodies remained in five out of seven samples.

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CHAPTER 12

Summary and discussion

Rheumatoid arthritis (RA) is a common chronic auto-immune disorder, of which persistent synovitis, bone erosions and auto-antibody formation are characteristic features.[1] Although it poses a considerable health problem, relatively little remains known about the disease pathogenesis and etiology. In the past decade anti-citrullinated protein antibodies (ACPA) have emerged as suspects in the development and/or progression of RA.[2-4] Citrullinated proteins-containing the amino acid citrulline, generated post-translationally from arginine-are found in the joints of patients with RA, but are not specific for the disease.[5, 6] This situation contrasts with the presence of ACPA, which are mostly found in individuals with RA. Intriquingly, ACPA can also be found in individuals before symptom onset.[7, 8] In these instances the ACPA response seems to be in its infancy, recognizing only a few citrullinated antigens and not using the full isotype repertoire. The characteristics of the ACPA response mature before clinical disease precipitates.[9, 10] Evidence is emerging that ACPA status can further characterize the heterogeneous RA phenotype, not only with respect to outcome, but perhaps also with respect to intervention.

In this thesis, we studied potential roles of ACPA in RA pathogenesis, the mechanisms that underlie the occurrence, and evolution of the ACPA response, as well as the relationship of these autoantibodies with clinical phenotypes and disease outcome.

The prevalence of the ACPA response in Caucasians is estimated to be 1-2% of the population; therefore little information about ACPA in unaffected individuals is present in the literature. In **chapter 2** we investigated the presence and characteristics of ACPA in healthy individuals and RA patients. A North American native population in Central Canada is known to have a high predisposition to RA. Healthy family members from this population had a high prevalence of ACPA. However, the ACPA response in these healthy individuals was less mature as compared to the ACPA response of patients with RA, with a much lower isotype usage and less epitope recognition. More strikingly, the ACPA and rheumatoid factor (RF) responses were associated in patients with RA, but were discordant in their healthy relatives. Our data indicate the presence of an interaction between these risk factors, with high odds for having RA when both antibodies are present. Furthermore the presence of ACPA was associated with RA irrespective of RF status, while the association of RF with disease relied on its interaction with ACPA.

We choose to investigate characteristics of ACPA in individuals from this cohort because North American Natives have previously been reported to have a younger age at disease onset and an increased prevalence and severity of RA. Genetic studies have also revealed a higher prevalence of HLA shared epitope (SE) alleles in North American Natives and increased frequency of RF positivity in patients with RA in several North American native populations.[11] The data so far present about ACPA in healthy individuals originates from retrospective studies, collecting serum samples from blood donors with RA before disease onset.[7, 8] These studies, how-

ever did not investigate the characteristics of the ACPA response in health versus disease. The most striking finding in our study, the interaction between RF and ACPA, implies that these autoimmune responses may converge to precipitate disease. Although the biological mechanism underlying this observation is unknown, this association could be explained by a model proposed in a study from Wipke et al, suggesting that that autoantibody-mediated articular inflammation in mice may be facilitated by soluble immune complexes that enable the access of pathogenic antibodies into the joint.[12] Because RF antibodies recognize IgG molecules, they can form soluble immune complexes, which could facilitate access of ACPA joints. An alternative explanation is that RF could amplify the effector mechanisms induced by ACPA in the joint, thereby exacerbating joint inflammation.

Although we detected a relatively high prevalence of ACPA in the population of healthy relatives, our data indicate that the mere presence of ACPA is not enough to induce disease. The limited ACPA isotype usage in the healthy relatives is consistent with a relatively immature autoantibody response. In patients with RA, chronic exposure to citrullinated antigens in the joint conceivably results in continuous (re)activation of antigen-specific B cells and favors isotype switching. This hypothesis is also supported by a low frequency of IgM ACPA in healthy relatives as IgM is indicative of ongoing immune responses. The fine specificity data indicate that ACPA recognize at least partially different antigens in patients with RA and their healthy relatives, with responses against citrullinated fibrinogen and citrullinated vimentin being present in more than half of the patients while being virtually undetectable in their healthy relatives. These citrullinated antigens are present in the inflamed joint and may serve as an important source of continuous antigen stimulation in RA synovium.[13, 14] In contrast, the antigens that stimulate the ACPA response in healthy individuals and are responsible for the initial loss of immune tolerance are currently not known. Considering that the unaffected relatives are, on average, younger than patients with RA, it is conceivable that disease will develop in the future in at least some of the ACPA positive relatives. Therefore, we speculate that the relatively limited ACPA response in healthy individuals will change over time, leading to disease manifestations. These changes likely involve broader isotype usage and/or epitope spreading and could be facilitated by RF antibodies that may allow access of ACPA into the joint or amplify their effects. Understanding the pathways responsible for the diversification of the ACPA response in RA is important, because such an understanding could provide new treatment possibilities for targeting the pathological autoimmune response before disease becomes manifest.

As described above, the transition from asymptomatic autoimmunity to clinically detectable arthritis is not well understood. In **chaper 3** we therefore studied an individual patient from a multi-case RA family from a North American Native

population. This young female had initially asymptomatic autoimmunity and subsequently developed clinical features suggestive of early RA. These data suggest that onset of clinical synovitis was heralded by an expansion in ACPA isotype usage, along with epitope spreading to citrullinated fibrinogen-associated antigens. Interestingly, the autoantibody levels continued to rise despite disappearance of the clinical symptoms with pregnancy. This observation implies that the clinical improvement typically seen during RA pregnancy may not necessarily reflect an attenuation of the underlying autoimmune mechanisms, at least at this early stage of the process. At the time of the study, it was not possible to determine with certainty whether this patient's synovitis represents the earliest clinical evidence of RA, since she had yet to meet the American College of Rheumatology criteria set. On the other hand, it is possible that the very early use of antimalarials may indeed serve to prevent the full expression of the clinical RA syndrome. This has been shown with the use of methotrexate in anti-cyclic citrullinated peptide-positive patients with undifferentiated arthritis.[15] The serological evolution and synovial features are consistent with the hypothesis that isotype expansion and epitope spreading of ACPA responses to synovial autoantigens are associated with the onset of synovitis in an individual who is genetically susceptible to RA.

ACPA positive and ACPA negative disease have been shown to be associated with different genetic and environmental risk factors, fuelling the hypothesis that different pathways mechanisms underlie these two separate disease subsets.[16-18] For example, ACPA negative RA associated with HLA-DR3[19, 20], whereas the HLA SE alleles predispose to ACPA positive disease.[21] Likewise, the contribution of smoking to disease risk is mainly confined to the ACPA positive HLA SE positive group.[18] The HLA SE alleles are also involved in shaping the fine specificity response, as these alleles predispose to the development of antibodies against citrullinated vimentin but not aginast citrullinated fibrinogen.[22] This would indicate that HLA SE alleles influence the magnitude and the specificity of the ACPA response. It has even been suggested that a specific interaction between environment (that is, smoking) and genetic background (HLA expression profile) might explain the reactivity against a specific citrullinated antigen.[23] Since specific interaction between these two risk factors had been postulated in association with autoimmunity to citrullinated a-enolase peptide (CEP-1)[23], it has been investigated whether the interaction was truly specific for this antigen or if it might also exist for other peptides. Indeed, a more recent paper from our group revealed that this interaction was not specific for CEP-1, but rather extended to other citrullinated antigens as well, for example with antibodies directed against citrullinated vimentin.[24] These data indicated that the interaction between HLA SE alleles and smoking extended to several citrullinated autoantigens, which might even be explained by a general predisposition to ACPA development.

In **chapter 4** we wished to explore whether this effect of interaction would still be present in ACPA positive patients. These findings could be of relevance, since interactions between genotype, smoking, and autoimmunity to certain citrullinated antigens could further expand our understanding of RA pathogenesis. However, no stratification for ACPA status was performed in previous studies, allowing the possibility that the interaction effects were not explained by their influence on the formation of autoimmune reactions against specific citrullinated antigens, but rather by their influence on the formation of ACPA. Therefore, we reasoned that if such interaction effects only shaped the response to certain specific epitopes and not to other citrullinated epitopes, these effects should still be observed among the subset of ACPA-positive patients. However, after stratification for ACPA, there was no gene-environment interaction present in the shaping the reactivity against specific citrullinated antigens. This indicates that the association found is caused by the presence of ACPA rather than by the presence of an autoimmune reaction to specific citrullinated epitopes.

Taken together, these data indicate that the gene–environment interaction between tobacco exposure and HLA shared epitope alleles does not influence the reactivity of the ACPA response. Rather, the presence of HLA shared epitope alleles seems to be the main factor in shaping the antigen recognition of the ACPA response. This is evidenced by the fact that the association between HLA shared epitope alleles and certain fine specificities, such as citrullinated vimentin 59–74 and citrullinated α-enolase, remained after stratification for ACPA status, as has been demonstrated previously.[22] Thus, these results suggest that ACPA fine specificity recognition is mainly dependent on HLA shared epitope status and that tobacco exposure and the interaction between HLA shared epitope alleles and tobacco exposure contribute little.

As discussed above, citrullinated proteins are found at inflamed sites in healthy individuals as well as in patients.[5, 6] However, antibodies directed against citrullinated proteins are very specific for RA. One of the most abundant proteins in the joint is fibronectin. In **chapter 5** we characterized the citrullination of fibronectin in the joint of patients with RA. Furthermore, we studied the prevalence, fine specificity en HLA association of autoantibodies direct against citrullinated fibronectin in patients with RA. Our study revealed that fibronectin can be citrullinated at least at five positions. Together with the flanking amino acids, three of these citrullinated residues comprise two eptiopes recognized by RA Autoantibodies. An epitope containing two adjacent citrullines at positions corresponding to residues 1035 and 1036 appeared to be most frequently recognized by RA sera. The data presented in chapter 5 not only showed that antibodies against citrullinated FN are present in RA patients, but also demonstrated that the anti-FN antibodies represent a subgroup of anti-CCP2 antibodies and that they can already be detected very early in

the disease. Anti-citrullinated fibronectin antibodies recognizing anti-FN-$Cit_{1035,1036}$ were associated with HLA SE alleles, however not with clinical features of RA.

The fact that RA sera were only reactive with two of the found peptides is consistent with the results of other studies showing that the amino acids flanking the citrulline residue contribute to the formation of auto-epitopes.[6] Several studies with synthetic citrullinated peptides (derived from vimentin, fibrinogen and α-enolase) showed that not all peptides containing citrullinated residues are recognized by patient sera, indicating that not only the citrulline is important, but also the amino acids surrounding the citrullinated residue.[25-27] A large overlap of anti-FN-$Cit_{1035,1036}$ with reactivities to other fine specificity peptides was observed, as might be expected from previous data.[28, 29] These data display the large heterogeneity of the ACPA response in RA and indicate that the anti-FN-$Cit_{1035,1036}$ antibodies are an abundant ACPA subclass that can be detected with synthetic peptides derived from citrullinated synovial proteins. The association of the recognition of anti-FN-$Cit_{1035,1036}$ with HLA SE alleles as such is not surprising as it was show before that HLA SE alleles are associated with not only the magnitude but also with the fine specificity of the ACPA response.[22] One could imagine that the selection of citrullinated epitopes presented to T cells are restricted by the HLA-DRB1 SE alleles (or the HLA-DQ alleles that are genetically linked to the SE alleles).

As antibodies directed against some citrullinated peptides are associated with HLA SE alleles[22, 30], one would expect that reactivity towards some epitopes plays a more relevant role than others in disease pathogenesis.[22-24] Indeed, it has been described that ACPA eptitope spreading occurs over several years prior to the diagnosis of RA. The initial auto-immune response is mostly directed towards only one auto-antigen, but this is not always the same autoantigen.[10] Furthermore, in patients with undifferentiated arthritis (UA), a more extended epitope recognition profile was found in individuals that develop RA over time.[29] The relevance of epitope spreading before disease onset raised the question as to whether a different epitope recognition pattern would be associated with different clinical phenotypes. Therefore, we wished to study the relation between the fine specificity profile of RA patients with clinical phenotype in **chapter 6** and **chapter 7**.

We first studied the association of the ACPA fine specificity and the rate of joint destruction over time, under the influence of HLA SE alleles in **chapter 6**. Further insights into which ACPA fine specificities might be associated with disease severity could therefore have prognostic value and contribute to our understanding of disease pathogenesis. In this study, however, we could not detect an association between ACPA fine specificities and radiographic joint damage. An anti-citrulline immune response to 3 out of 6 of the epitopes studied developed preferentially in patients harboring SE alleles, but this did not translate into more severe ra-

diographic outcome. Also, the number of citrullinated epitopes recognized by an individual patient did not influence the degree of joint destruction. We addressed this issue by using SE alleles as a surrogate marker for those ACPA fine specificities that develop under the influence of SE alleles. After stratification for ACPA, SE alleles no longer contributed to joint damage. Based on this finding, we consider it unlikely that a SE associated ACPA fine specificity can be identified that predicts disease course in RA. If such a predictive recognition profile exists, antibodies recognizing this epitope are likely to be generated independent of SE alleles.

These findings are relevant for strategies aimed at identifying patients that are at risk for rapidly progressive disease and provide evidence that the recognition profile of the ACPA response is unlikely to have a relevant impact on radiographic progression. HLA SE alleles are instrumental in shaping the ACPA repertoire. However, ACPA fine specificities formed under the influence of SE alleles do not seem to affect joint destruction.

As we found no influence of the ACPA fine specificity repertoire on the rate of joint destruction over time, we extended our search towards other clinical characteristics of RA in **chapter 7**. In this chapter we investigated whether specific subsets of RA patients could be distinguished on the basis of an autoimmune response to specific citrullinated epitopes and investigated the effects of the ACPA fine specificity on clinical features of RA. The analyses were performed within the ACPA-positive stratum to exclude the influence of ACPA status on disease outcome, as published before.[31] Our data showed that the ACPA response is highly diverse with respect to recognition of specific citrullinated epitopes. We found a strong association between the number of ACPA fine specificities and the anti-CCP2 (cyclic citrullinated peptide-2) levels.

Furthermore, the recognition of different citrullinated peptides at baseline correlated with similar clinical characteristics, irrespective of differences in peptide-backbone structure, indicating that breaking of tolerance towards citrullinated proteins as such provides more information than the recognition of a particular peptide or set of peptides. As subgrouping patients based on their epitope recognition profile could be potentially useful to get more homogenous patient groups, we analysed different recognition profiles in detail. These data suggested that the recognition profile of patients with RA displays a large heterogeneity and that patients are not characterised by a unique and specific epitope recognition pattern. The lack of association between ACPA fine specificities and clinical characteristics might not be surprising, given the observation that even the baseline differences between ACPA-positive and ACPA-negative patients with RA are rather small. Nonetheless, these latter subgroups differ considerably with respect to disease course as measured by radiological progression.[31] We feel that it is unlikely that ACPA fine specificity within ACPA-positive disease will have a similar impact as

found for ACPA status within RA. This notion is supported by our observation that a similar rate of joint destruction is observed between ACPA positive patients with high- and low-baseline anti-CCP2 levels as proxy for the extent of epitope recognition. Likewise, the data presented in chapter 6 and a recent observations by Fisher et al analysing the possible connection between reactivity against a specific citrullinated epitope provided similar indications.[32]

Remarkably, these findings contrast observations made in early/predisease RA, as it has been shown that ACPA-positive subjects who are still healthy or have early arthritis are more likely to develop arthritis when harbouring a more extended generalised citrullinated epitope recognition pattern.[29] The reason why 'maturation' of the ACPA response with respect to its epitope recognition profile is associated with transition to disease but, once disease is established, not with disease outcome, is not known. However, it is tempting to speculate that once a certain threshold is reached, disease manifestations become apparent. In case ACPA would be involved in disease pathogenesis, it is conceivable that over this threshold, higher levels or a more extended recognition profile does not contribute further to disease progression, as the response is already maximally involved in creation of the harmful inflammatory milieu underlying the signs and symptoms associated with RA. Obviously this could lead to two phenotypically different subgroups. Altogether, the data in chapter 7 indicate that the epitope recognition profile is highly diverse. The recognition of different citrullinated peptides at baseline correlated with similar clinical characteristics, irrespective of differences in peptide-backbone structure, indicating that the breaking of tolerance towards citrullinated proteins as such provides more information than the recognition of a particular peptide or set of peptides. Thus, although studies investigating ACPA characteristics in relation to clinical phenotypes have not yet resulted in further refinement of the ACPA-positive subgroup, it is clear that stratifying patients with RA on the basis of ACPA status has resulted in the identification of more homogenous patient groups, with respect to both disease course and response to treatment.

Although the ACPA fine specificity repertoire seems not to play a role in the rate of joint destruction in RA patients, the presence of ACPA does play an important role.[31, 33-35] The observation that ACPA status is, to some extent, related to therapeutic outcome in early disease is intriguing. For a number of diseases, such as diabetes mellitus, it has been suggested that a critical period exists in which interventions might reverse the disease process.[36] For RA, such a 'window of opportunity' might also exist, because symptom duration >12 weeks at treatment initiation is a strong and independent risk factor for a persistent disease course with more joint destruction.[37-40] Although this observation could be explained by the assumption that acute-versus-insidious symptom onset characterizes the manifestation of different disease subsets, it could also be indicative of a window of opportunity.

The difference in outcome in relation to symptom duration raised the question of whether a difference in ACPA characteristics could be involved. However, in **chapter 8**, we show that ACPA-positive patients with symptoms of RA for <12 weeks display no difference in the specificity and isotype repertoire of their ACPA response compared with patients with longer symptom duration. These findings indicate that the ACPA-characteristics analyzed do not have an impact on the putative window of opportunity and emphasize further that maturation of the autoantibody response occurs at an early stage, before the first signs and symptoms of disease appear.[41]

In the previously mentioned studies we investigated to role of the IgG ACPA fine specificity repertoire. As IgM producing memory B cells have not been described, the presence of IgM ACPA suggests that activation of recently recruited naïve B cells recognize citrullinated antigens because the halflife of circulating IgM is short. Therefore, in **chapter 9** we sought to find certain epitopes that shape the ACPA IgM response in RA. We observed that ACPA IgM responses are different from ACPA IgG responses, as they display a more restricted antigen recognition pattern. These data are intriguing, as they indicate that the regulation of the IgM ACPA response differs from the regulation of B cells producing IgG-directed against citrullinated antigens. Although the reason for this difference is not known, we think it is most conceivable that these findings are explained by a limited recruitment of new B cells into the ACPA response that is driven by some, but not other, citrullinated antigens. Given the short half-life of circulating IgM and the lack of memory B cells producing IgM against protein antigens, we think that the IgM ACPAs detected in this study are produced by new B cells that are recruited into the ACPA response against certain citrullinated antigens. Even in the case where ACPA IgM-producing memory B cells exist, it is still interesting that such cells are present only against certain citrullinated antigens.

To exclude the possibility that our findings were influenced by IgM RF, we analyzed ACPA IgM specificities in relation to IgM RF. We observed that IgM RF-positive samples can be negative for IgM ACPA reactivities and that, in the absence of IgM RF, IgM ACPA reactivities can be detected readily. RF-positive samples that have IgG reactivity against all fine specificity epitopes may have IgM ACPA against only some antigens and not others, confirming that our assay did not merely detect IgM RF. Collectively, these observations support the notion that true IgM ACPA and not IgM RF bound to ACPA IgG were detected by the methods employed. Although it is tempting to speculate, these studies should not be taken as an argument for the involvement of the antigens analyzed here in the recruitment of new B cells into the ACPA response. Peptides are unlikely to reflect correctly the three-dimensional structure of citrullinated proteins that form the epitope for antibodies.

Moreover, ACPA IgG is cross-reactive to multiple citrullinated antigens[15,16], and

therefore recognition of a citrullinated antigen by ACPA IgG does not indicate that this antigen is necessarily involved in the induction of B-cell responses.

Nonetheless, our data do show that the IgM ACPA response is significantly more restricted than that of the IgG ACPA present in the same patient. How IgM can be formed in the presence of an active IgG response against the same antigen is not clear. In other situations, as exemplified by the prophylactic administration of anti-Rhesus D antigen antibodies to pregnant women carrying a rheusus D-positive child, the presence of IgG against a certain antigen will precent the induction of a novel IgM response. The mechanism behind this protective measure is thought to be mediated by either the capture of clearance of circulating Rhesus D antigen and/or by IgG-Rhesus D immune complexes that inactivate new Rhesus D-reactive B celles trough FcγRIIB, the inhibitory FcγRIIB receptor.[42] Clearly, IgG ACPAs do not inhibit the activation of IgM-positive, citrullinated antigen-reactive B cells. The reason for this finding is not known but could possibly be explained by the low avidity of the ACPA[18], conceivably resulting in "nonstable" immune complexes unable to trigger FcγRIIB. Collectively, the data in chapter 9 show that the immune response against one citrullinated antigen is different from the immune response against another citrullinated protein. Some responses are dominated by IgG, whereas both IgM and IgG responses were found for other ACPA antigens. Elucidation of the mechanism behind this observation could be of relevance to the identification of those citrullinated antigens that drive ACPA responses and could provide clues to how the continuous recruitment of new B cells can be halted.

Previously it has been shown that the levels of ACPA are higher in synovial-fluid than serum.[5, 54, 55] However, very limited information on absolute levels of ACPA in either synovial fluid or serum is present as the levels are generally expressed as arbitrary units. Nonetheless, it is interesting to obtain information on the absolute concentration of ACPA as this would allow the comparison of the ACPA response to other antibody responses in quantitative terms. In order to quantitate ACPA levels it is required to isolate ACPA. In **chapter 10**, we quantified the abundance of different ACPA immunoglobulins in serum and synovial fluid of RA patients. We found that IgG ACPA is present in relatively high concentrations in serum and synovial-fluid as up to 1 out of 100 IgG-antibodies can be ACPA. These findings exceed a previous observation describing the estimation of the amount of IgG ACPA using 3 RA sera, most likely due to the current, more efficient purification method.[30] Furthermore, we now reported that IgM ACPA can be abundantly present in synovial-fluid (up to 3% of total-IgM). Next to IgM ACPA, also IgG ACPA was found in relatively high concentrations in synovial-fluid (up to 1% of total IgG). The estimations presented are conceivably an underestimation of the true quantity as not all ACPA activity could be recovered after purification.

The amounts of IgG ACPA present in the sera are remarkably in line with the

peak levels of IgG directed against tetanus following repetitive vaccinations.[56] Protective antibody titers after vaccination have been described as titers above 1 µg/ml against for example Haemophilus influenza type b and group B streptococci.[56-59] Surprisingly, the ACPA concentrations found exceed these protective antibody titers and are in the same range as the amount of antibody present shortly after vaccination. The relatively high antibody levels of ACPA might be related to the continuous presence of citrullinated-antigens in the joint, which could activate ACPA producing B-cells.

Furthermore, the results indicate the abundant presence of IgM ACPA in relation to total IgM in synovial fluid, as up to 1 in 33 IgM-antibodies can be ACPA in patients with high ACPA levels. IgM responses against T-cell dependent antigens are, in general, not continuously present. In the setting of vaccination, for example, levels of antigen specific IgM decrease during the weeks after immunization against rabies.[60] Therefore the presence of IgM ACPA in high concentrations in sustained disease is intriguing. Previously published data by our group displayed that some IgG ACPA positive patients, still harbour IgM ACPA 7 years after the initial presence of IgG ACPA.[61] As described above, the continuous presence of IgM against T-cell dependent antigens, indicate the continuous triggering of newly generated B-cells. This suggests also that novel IgM producing B cells are continuously recruited into the ACPA response, indicating that the ACPA response is continuously reactivated during the course of arthritis.[61]

Nonetheless, in line with chapter 9, we feel that the data presented on IgM ACPA levels should be taken with some caution as we can formally not exclude that the measured IgM-ACPA levels are influenced by IgM RF bound to ACPA. However, previously reported data by our group showed that the depletion of IgM RF did not result in reduction of IgM ACPA levels. Furthermore mixing sera of RF-positive with IgM ACPA negative patients did not change the reactivity.[61] Likewise, not all IgM ACPA positive patients included in this chapter were IgM RF positive, excluding a contribution of IgM RF, at least in these patients. Therefore, the increased presence of IgM-ACPA in synovial-fluid indicates ongoing recruitment of new B-cells into the ACPA-response, reflecting a continuous (re)activation of the RA-specific ACPA-response during the course of ACPA-positive arthritis.

Besides antibody responses directed against citrullinated proteins, also antibodies directed against carbamylated proteins (anti-CarP) have recently been shown to be present in RA.[43] Interestingly, these anti-CarP antibodies are also present in around 20% of the ACPA negative RA patients and are associated with more severe joint damage in this group. ACPA recognize proteins only after the enzymatic conversion of the amino acid arginine by PAD enzymes to become the amino acid citrulline. Next to citrullination, also other post-translational modifications are known to occur. Therefore, it is likely that proteins that have undergone a differ-

ent type of post-translational modification are also recognized by autoantibodies. One of these other post-translational processes is the process of carbamylation. In this chemical reaction, mediated by cyanate, the amino acid lysine is changed to become the amino acid homocitrulline. Cyanate, necessary for such carbamylation is naturally present in the body and in equilibrium with urea.[44] In the healthy situation the concentration of urea is rather low. It is likely that under such conditions especially long lived proteins, such as matrix molecules, become carbamylated. Renail failure, a condition with increased urea concentrations, is known to be associated with enhanced protein carbamylation.[45] Also smoking, the most prominent environmental risk factor for RA, enhances carbamylation by increasing the cyanate concentration.[46] Extensive carbamylation is especially thought to occur during (chronic) inflammation, when myeloperoxidase is released from neutrophils as this enzyme shifts the equilibrium of thiocyanate towards cyanate.[46] As smoking and chronic inflammation are important in the context of RA, it is likely that in the inflamed synovium carbamylation is taking place. The post-translationally modified amino acids citrulline and homocitrulline are very similar structures (figure 1).

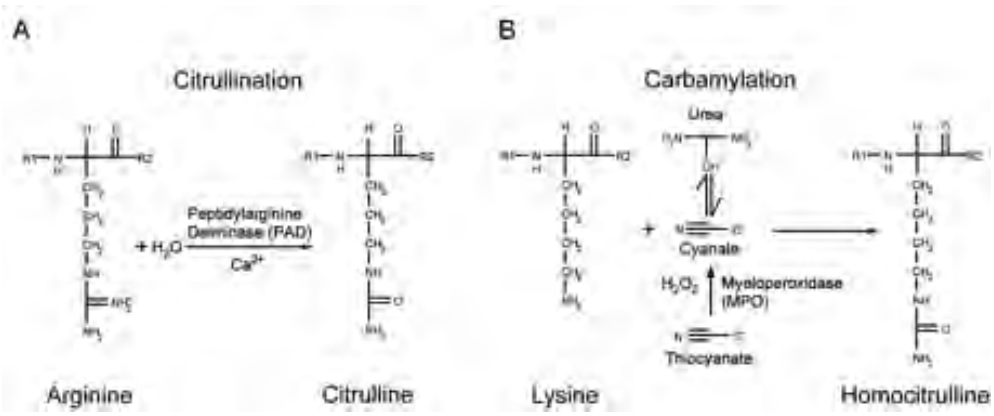

Figure 1. Illustration of citrullination and carbamylation. Citrullination (A) and carbamylation (B) occur on different amino acids via different mechanisms, but yield similar end-products.

In both cases a positively charged amino acid is replaced by a neutral one. The only structural difference is the difference in length; homocitrulline is one CH3 group longer. As these structures are really similar, in **chapter 11** we wished to determine to what extent human antibodies can differentiate between them. Unlike human antibodies, the anti-modified citrulline (AMC) antibody developed by Dr Senshu[47] is able to recognize citrullinated epitopes irrespective of the neighbouring aminoacids. Thus, we also aimed to verify whether the AMC assay could distinguish between those two amino acids.

Both the citrullinated and the carbamylated forms of the proteins tested were strongly recognized, whereas, the non-modified form did not reveal any staining. Staining similar western blots with selected human sera revealed that sera-positive for ACPA and anti-CarP stained both citrullinated and carbamylated forms of Fib, whereas, single positive sera stained only one form of modified Fib. These data indicate that although the 'AMC-Senshu' method does not discriminate between these two modifications, human sera of RA patients are able to. Furthermore, we also showed that part, but not all ACPA and anti-CarP antibodies are cross-reactive.

As suggested before, we found the 'AMC-Senshu' method cannot differentiate citrullination and carbamylation.[48, 49] Interestingly, part of the human sera can make this distinction. Anti-CarP antibodies and ACPA are often detected together, and here we show that double positive samples harbour anti-citrullinated epitope specific antibodies, anti-carbamylated epitopes specific antibodies and cross-reactive antibodies. The finding that the 'AMC-Senshu' method recognizes both citrullinated and carbamylated proteins does not argue against the notion that citrullinated proteins are present in synovial fluid and tissues, since a number of studies confirmed the presence of citrullinated proteins by mass spectrometry fingerprinting.[50-53] However, our study suggests that the extent and nature of citrullination and carbamylation in the joint should be re-evaluated especially in light of our recently described anti-CarP response that is, at least in part, not cross-reactive with citrullinated proteins.

CONCLUSION

The identification of ACPA has been a major breakthrough in the understanding of pathogenesis in RA. It has become clear that this unique autoantibody response identifies more homogenous subsets of patients with RA than those characterized by levels of other autoantibodies, and that differing disease courses possibly reflect the involvement of ACPA in disease pathogenesis. The elucidation of the characteristics of the ACPA response have shown that ACPA are not pathogenic per se, as illustrated by the fact that most patients are ACPA-positive a while before they develop disease. Possibly, a more mature ACPA response—as illustrated by more extensive isotype switching, enhanced antigen-recognition profile and higher titers—might be required for these autoantibodies to contribute to disease pathogenesis. Once RA is established, the ACPA response does not seem to mature. Nonetheless, ACPA status is important for clinical decision making, as it is the factor that is most predictive of disease outcome and associates with the effectiveness of various interventions.

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CHAPTER 13

Nederlandse samenvatting

NEDERLANDSE SAMENVATTING

Reumatoïde artritis

Reumatoïde artritis (RA) is een veel voorkomende chronische auto-immuun ziekte, waarbij ontsteking van de gewrichten het belangrijkste kenmerk is. In de westerse wereld is ongeveer 0.5-1% van de volwassen bevolking aangedaan door de ziekte. Een aanzienlijke geografische variatie in het optreden van RA wordt beschreven, met een zeer hoge prevalentie in inheemse Amerikaanse populaties en een zeer lage prevalentie in de bevolking van Zuidoost Azië. De aandoening komt ongeveer drie keer vaker bij vrouwen dan bij mannen voor en de prevalentie neemt toe met de leeftijd. Aangezien RA een systeem ziekte is, kunnen naast symptomen van vermoeidheid, gewichtsverlies en koorts ook ontstekingsreacties in de inwendige organen optreden zoals in het hart, de longen en de nieren.

De ziekte presenteert zich vaak met gezwollen en pijnlijke gewrichten, waarbij voornamelijk de kleine gewrichten van de handen en voeten meedoen. Een langdurige aanwezigheid van synovitis kan leiden tot beschadiging van kraakbeen en subchondraal bot, uiteindelijk leidend tot misvormingen en invaliditeit. Indien geen of onvoldoende behandeling plaatsvindt, kan RA ertoe leiden dat patiënten steeds meer moeite hebben met hun normale dagelijkse bezigheden en daarbij een verminderde kwaliteit van leven veroorzaken. Voor de samenleving kan dit uiteindelijke leiden tot enorme toename in de kosten van de gezondheidszorg en verlies van arbeidskrachten op de arbeidsmarkt.

Voor patiënten met RA zijn de behandelmogelijkheden de laatste jaren sterk verbeterd. De steunpilaren van de behandeling zijn de 'Disease Modifying Anti Rheumatic Drugs' (DMARDs). Deze vormen een heterogene verzameling van therapeutische middelen waarvan de werkingsmechanismen grotendeels onbekend zijn. Indien de artritis, ondanks het gebruik van DMARDs, onvoldoende onder controle is of als er sprake is van bijwerkingen van deze medicatie, dan spelen de zogenoemde 'biologicals' zoals tumor necrosis factor (TNF) blokkers een belangrijke rol in het effectief onderdrukken van de ziekte. Hierdoor is het tegenwoordig mogelijk om bij een groot deel van de patiënten het ontstekingsproces te remmen en gewrichtsschade te voorkomen.

ACPA

Hoewel RA een aanzienlijk gezondheidsprobleem vormt is relatief weinig bekend over de pathogenese en etiologie van de ziekte. In het afgelopen decennium zijn antilichamen tegen gecitrullineerde eiwitten (anti-citrullinated protein antibodies; ACPA) in beeld gekomen als verdachten voor de ontwikkeling en/of progressie van RA. Gecitrullineerde eiwitten bevatten het aminozuur citrulline, dat onder invloed van het enzym peptidyl arginine deiminase (PAD) uit het aminozuur arginine gevormd wordt. De rol van citrullinatie is nog onduidelijk. Wel is bekend dat gecitrul-

lineerde eiwitten vaker gezien worden bij ontsteking.

Gecitrullineerde eiwitten worden aangetroffen in de gewrichten van patiënten met RA, maar zijn niet specifiek voor de ziekte, aangezien zij ook gevonden worden bij gezonde personen. De aanwezigheid van ACPA is echter wel ziekte specifiek, aangezien deze antilichamen bijna uitsluitend worden gezien bij personen met RA. Voor het meten van ACPA worden verschillende diagnostische testen gebruikt, waarbij gebruik gemaakt wordt van "cyclisch gecitrullineerde peptiden" (cyclic citrullinated peptides; CCP). Indien deze vergeleken worden met het klassieke auto-antilichaam, waarvan al veel langer bekend is dat het geassocieerd is met RA (IgM reumafactor, IgM-RF) blijkt de anti-CCP test een grotere specificiteit te hebben. De sensitiviteit van IgM-RF en anti-CCP is bij patiënten met een gediagnosticeerde RA ongeveer even groot.

ACPA kunnen vele jaren voordat de ziekte zich presenteert gevonden worden bij de op dat moment nog gezonde personen. De ACPA reactie is in dat geval nog niet volledig ontwikkeld. De antilichamen herkennen slechts enkele gecitrullineerde antigenen en hebben daarbij nog niet het volledige isotype profiel. Deze twee eigenschappen van ACPA breiden zich uit alvorens de ziekte echt tot ontwikkeling komt. Verder zijn ACPA een belangrijke voorspeller van bijvoorbeeld gewrichtsschade in RA.

In dit proefschrift onderzochten we de potentiële rol van ACPA bij de pathogenese van RA, de mechanismen die betrokken zijn bij de ontwikkeling van ACPA, en de relatie van ACPA met het klinische fenotype en ziekte uitkomsten.

ACPA in de gezonde populatie

De prevalentie van ACPA wordt in de westerse populatie geschat op 1% van de bevolking. Tot op heden is weinig informatie bekend in de literatuur over ACPA in gezonde personen. In **hoofdstuk 2** hebben we de aanwezigheid en kenmerken van ACPA in gezonde individuen en patiënten met RA onderzocht. Van een Noord-Amerikaanse inheemse bevolking in centraal Canada is bekend dat RA relatief vaak voorkomt. Gezonde familieleden van de deze patiënten hadden een hoge prevalentie van ACPA. De ACPA van deze gezonde personen zijn minder "volgroeid" dan de antilichamen van de patiënten, aangezien ze veel minder epitopen herkennen en een lager isotype gebruik hebben. Alhoewel we een relatief hoge prevalentie van ACPA in de gezonde eerstegraads familieleden zagen, lieten onze data zien dat enkel de aanwezigheid van ACPA onvoldoende is om ziekte te veroorzaken.

Opvallender was dat ACPA en RF bijna alleen samen gezien werden bij patiënten met RA. Bij gezonde familieleden was geen of slechts één van deze autoantilichamen aanwezig. Mogelijk geeft een interactie tussen deze twee risicofactoren een verhoogde hoge kans op RA. Daarnaast was de aanwezigheid van ACPA geassocieerd met RA ongeacht de aanwezigheid van RF, terwijl andersom RF alleen geassocieerd was met RA in de aanwezigheid van ACPA.

De verwachting is dat de ACPA reactie zich in gezonde mensen in de loop van de tijd zal ontwikkelen en tot ziekte kan leiden. Om dit te onderzoeken hebben we in **hoofdstuk 3** een individuele patiënte bestudeerd uit een familie uit de Noord-Amerikaanse inheemse bevolking waar veel familieleden belast waren met RA. Deze jonge vrouw had aanvankelijk asymptomatisch auto-immuniteit en ontwikkelde vervolgens klinische kenmerken die passen bij vroege RA. Bij deze patiënte zagen we dat het ontstaan van een synovitis voorafgegaan werd door de ontwikkeling van ACPA (toename isotype gebruik en meer epitopen die werden herkend). Gedurende het onderzoek voldeed de patiënte niet aan de American College of Rheumatology criteria voor RA, maar zij werd ook in een vroeg fase behandeld met DMARDs, wat wellicht verdere ziekte progressie heeft voorkomen.

ACPA, genetische risicofactoren en omgevingsfactoren

Het is bekend dat ACPA positieve en ACPA negatieve RA geassocieerd zijn met verschillende genetische en omgevingsfactoren. Om die reden wordt gedacht dat een verschillende pathofysiologie ten grondslag ligt aan deze twee ziektebeelden. Iets meer dan de helft van de vroege RA patiënten zijn ACPA positief. ACPA positieve en ACPA negatieve RA zijn geassocieerd met verschillende genen die coderen voor de humane leukocyten antigenen (HLA). ACPA negatieve RA is bijvoorbeeld geassocieerd met HLA–DR3, terwijl de HLA 'Shared Epitope' (HLA SE) allelen geassocieerd zijn met ACPA positieve ziekte. De HLA SE allelen worden gekenmerkt door een vergelijkbare aminozuurvolgorde ter plaatste van positie 70-74 van het HLA-DRB1 molecuul. Deze positie is gelegen in de bindingsplaats van het HLA molecuul. De HLA SE allelen predisponeren niet zo zeer voor RA, maar voor ACPA positieve ziekte.

Daarnaast wordt de bijdrage van het roken aan ziekterisico voornamelijk beperkt tot de ACPA positieve, HLA SE positieve groep. Het is eerder beschreven dat een interactie optreedt tussen roken en HLA SE allelen en de ontwikkeling van ACPA. Later werd gesuggereerd dat dit gold voor de vorming van de auto-immuun reactie gericht op specifieke gecitrullineerde antigenen. Deze bevinding is van belang aangezien interacties tussen genotype, roken en auto-immuniteit gericht tegen bepaalde gecitrullineerde antigenen verder helpen in het begrijpen van de pathogenese van RA. In **hoofdstuk 4** laten we echter zien dat deze gen-omgeving interactie niet de specificiteit van de ACPA reactie bepaalt, maar geassocieerd is met de aanwezigheid van ACPA onafhankelijk van welk antigen herkend wordt. Indien de groepen gestratificeerd werden voor de aanwezigheid van ACPA waren de eerder gevonden associaties niet langer aanwezig.

Gecitrullineerde eiwitten worden gevonden op plaatsen waar inflammatie optreedt, zowel in patiënten als in gezonde individuen. Zoals reeds hierboven beschreven zijn antilichamen gericht tegen gecitrullineerde eiwitten zeer specifiek voor RA. Een van de meest voorkomende eiwitten in het gewricht is fibronectine.

In **hoofdstuk 5** beschrijven we de karakteristieken van gecitrullineerd fibronectine in het gewricht van patiënten met RA. Het eiwit fibronectine kan op 5 verschillende posities gecitrullineerd worden. Twee van deze epitopen van gecitrullineerd fibronectine werden herkend door patiënten. De aanwezigheid van deze anti-gecitrullineerde fibronectine antilichamen zijn geassocieerd met HLA SE allelen, maar niet met klinisch kenmerken van RA.

ACPA fijn specificiteit en klinisch fenotype

Aangezien de aanwezigheid van antilichamen tegen bepaalde gecitrullineerde eiwitten geassocieerd is met HLA SE allelen, zou men verwachten dat de reactiviteit gericht op bepaalde epitopen relevanter zijn voor de pathogenese van RA dan andere. Eerder is inderdaad beschreven dat herkennen van meer epitopen enkele jaren voor de diagnose RA kan plaatsvinden. De oorspronkelijke respons is meestal gericht tegen één autoantigen, maar dit hoeft niet altijd dezelfde te zijn. Verder is eerder aangetoond dat patiënten met ongedifferentieerde artritis met een uitgebreidere epitoop herkenning eerder RA zullen ontwikkelen dan patiënten met een meer beperkte herkenning. In **hoofdstuk 6 en 7** hebben we daarom onderzocht of een verschil in epitoop herkenning (dus een andere fijn-specificiteit) samen zou gaan met een ander klinisch fenotype.

In **hoofdstuk 6** hebben we laten zien dat de fijn-specificiteit van de ACPA reactie (het aantal of de herkenning van specifieke epitopen) niet geassocieerd is met meer gewrichtsschade gedurende follow up.

Aangezien we niet konden aantonen dat de ACPA fijn-specificiteit van invloed was op gewrichtsschade over tijd, hebben we de analyse uitgebreid naar andere klinische karakteristieken van RA. In **hoofdstuk 7** hebben we geprobeerd patiënten in te delen op basis van hun auto-immuunreactie gericht op specifieke gecitrullineerde antigenen en hun effect op klinische eigenschappen. Deze analyse werd uitgevoerd binnen de ACPA positieve groep om het effect van de ACPA status uit te sluiten zoals reeds hierboven beschreven. Zoals men ook zou verwachten waren de concentraties van anti-CCP2 (cyclic citrullinated peptide-2) sterk geassocieerd met het aantal ACPA fijn-specifiteiten aanwezig in serum. De ACPA concentraties, gebruikt als surrogaat marker voor de fijn-specificiteit, waren niet geassocieerd met gewrichtsschade. Herkenning van verschillende gecitrullineerde eiwitten bij binnenkomst van de patiënten ging samen met vergelijkbare klinische karakteristieken onafhankelijk van welke peptiden we bestudeerden. In dit hoofdstuk laten we zien dat de ACPA reactie erg gevarieerd is, maar dat er geen duidelijk associatie aanwezig is tussen herkenning van bepaalde gecitrullineerde epitopen en fenotype van RA.

Deze bevindingen suggereren dat individuen met een bredere ACPA reactie wel een verhoogd risico hebben om RA te ontwikkelen wanneer zij nog gezond zijn of vroege artritis hebben, maar dat wanneer de ziekte eenmaal echt aanwezig is de

verdere ontwikkeling van de antilichaam reactie van minder groot belang is. De reden hiervan is onbekend, mogelijk is een bepaalde grenswaarde aanwezig waar patiënten overheen moeten om ziek te worden. Indien ACPA betrokken zijn bij de pathogenese van ACPA is het mogelijk dat wanneer de ziekte eenmaal volledig aanwezig is, een bredere reactiviteit niet meer extra schade geeft aangezien de schadelijke inflammatoire reactie al is aangezwengeld.

ACPA en de 'window of opportunity'

Het is interessant dat de aanwezigheid van ACPA gerelateerd is aan uitkomst van de ziekte (zoals gewrichtsschade). Voor een aantal ziektebeelden zoals diabetes mellitus wordt gesuggereerd dat een tijdsframe bestaat waarin de ziekte reversibel is. Voor RA wordt ook beschreven dat een dergelijke 'window of opportunity' mogelijk zou bestaan. Dit zou kunnen betekenen dat als je RA binnen de 'window of opportunity' zou behandelen je de ziekte zou kunnen voorkomen. Eerder is aangetoond voor RA dat gewrichtsklachten die langer dan 12 weken bestaan bij het begin van de behandeling een sterke en onafhankelijke voorspeller zijn voor een hardnekkige ziekte met meer gewrichtsschade. Dit zou verklaard kunnen worden door het verschil van een acuut versus sluipend begin van de ziekte, maar ook door de window of opportunity waar een vroege behandeling problemen kan voorkomen. In **hoofdstuk 8** hebben wij onderzocht of patiënten met een verschillende klachtenduur bij presentatie een verschil in ACPA karakteristieken lieten zien. In dit hoofdstuk tonen wij dat patiënten met symptomen passend bij RA < 12 weken, geen andere fijn-specificiteit of isotypen-gebruik van hun ACPA reactie laten zien in vergelijking met patiënten met een langere ziekte duur bij de start van de behandeling. Deze data zijn een indicatie dat de bestudeerde ACPA karakteristieken geen invloed hebben op de window of opportunity en benadrukken dat de ontwikkeling van de antilichaam reactie mogelijk optreedt voor de eerste presentatie van de symptomen van RA.

ACPA immunoglobulines

In de bovengenoemde studies hebben we de rol van de fijn-specificiteit van IgG ACPA bestudeerd. Aangezien IgM ACPA producerende memory B cellen niet eerder beschreven zijn, suggereert de aanwezigheid van IgM ACPA de recente activatie van naïeve B cellen, die gecitrullineerde antigenen herkennen, aangezien de halfwaarde tijd van circulerend IgM kort is.

In **hoofdstuk 9** hebben we verschillende epitopen onderzocht die de ACPA IgM reactie van RA kunnen vormen. Onze data laten zien dat de ACPA IgM reactie anders is dan de ACPA IgG respons. ACPA IgM laat een beperktere antigen herkenning zien. Dit zou kunnen betekenen dat niet alle gecitrullineerde antigenen in staat zijn om nieuwe B cellen te activeren. Het is interessant dat patiënten IgM ACPA kunnen vormen tegen een epitoop waar reeds IgG ACPA aanwezig is.

In zwangere vrouwen worden bijvoorbeeld profylactisch anti-Rhesus D antigen antilichamen (IgG) toegediend om zo te voorkomen dat de moeder een nieuwe IgM respons ontwikkelt. Hierdoor wordt het circulerende Rhesus D antigen geklaard of gevangen en worden geen nieuwe B cellen geactiveerd. IgG ACPA zorgt klaarblijkelijk niet voor inhibitie van de activatie van IgM positieve gecitrullineerde-antigen-reactieve B cellen. Het ontdekken van het mechanisme hierachter zou kunnen helpen bij het ontwikkelen van medicatie betrokken bij het remmen van nieuwe B cellen die betrokken worden in het proces.

Eerdere studies hebben aangetoond dat ACPA concentraties in synovial vocht hoger zijn dan in serum. Er is echter weinig bekend over de absolute concentraties van ACPA in zowel serum als synoviaal vocht, aangezien de levels meestal uitgedrukt worden in 'arbitrary units'. Het is interessant om absolute concentraties te weten van ACPA om de antilichaam reactie kwantitatief te kunnen vergelijken met andere antilichaam reacties. In **hoofdstuk 10** hebben we de kwantitatieve hoeveelheid van de verschillende ACPA immunoglobulines in serum en synoviaal vocht van RA patiënten onderzocht. In serum en synoviaal vocht wordt IgG ACPA in relatief hoge concentraties gevonden, 1 van de 100 IgG antilichamen kan ACPA zijn. IgM ACPA kan overvloedig aanwezig zijn in synoviaal vocht (tot 3% van totaal IgM). Ook IgG ACPA was in relatief hoge concentraties aanwezig in synoviaal vocht (tot 1% van totaal IgG). Deze berekeningen werden gemaakt na zuivering van ACPA. Mogelijk werden niet alle ACPA gemeten na zuivering, dus het zou een onderschatting kunnen zijn. De hoeveelheid IgG ACPA komt opvallend overeen met de piek levels van IgG gericht tegen tetanus na een herhaaldelijke tetanus vaccinatie. De relatief hoge ACPA concentraties kunnen gerelateerd zijn aan de continue aanwezigheid van gecitrullineerde antilichamen in het gewricht, welke ACPA producerend B cellen kunnen activeren.

ACPA en Anti-CarP antilichamen

Naast een antilichaam reactie gericht tegen gecitrullineerde eiwitten zijn recent ook antilichamen gericht tegen gecarbamyleerde eiwitten (anti-CarP) beschreven in RA. Deze anti-CarP antilichamen zijn aanwezig in 20% van de ACPA negatieve RA patiënten en zijn geassocieerd met meer gewrichtsschade in deze groep. ACPA herkennen eiwitten alleen na een enzym reactie waarbij het aminozuur arginine door PAD enzymen wordt omgezet in het aminozuur citrulline. Naast citrullinatie kunnen eiwitten ook andere post-translationele veranderingen ondergaan. Mogelijk worden deze veranderde eiwitten ook herkend door auto-antilichamen. Carbamylatie is een chemische reactie, gemedieerd door cyanaat. In deze chemische reactie wordt lysine veranderd in homocitrulline. Cyanaat is normaal gesproken aanwezig in het lichaam in een lage concentratie en in evenwicht met ureum. Roken kan de cyanaat concentratie verhogen en zorgen voor meer carbamylatie.

Chronische inflammatie kan ook leiden tot meer carbamylatie. De combinatie van deze twee in RA maakt het waarschijnlijk dat in een ontstoken gewricht ten gevolge van inflammatie meer carbamylatie plaats vindt. Homocitrulline en citrulline hebben erg veel overeenkomsten, homocitrulline is slechts één CH_3 groep groter.

Aangezien deze structuren zo overeen komen hebben we in **hoofdstuk 11** bestudeerd of menselijke antilichamen deze twee antigenen kunnen onderscheiden. Een anti-modified citrulline (AMC) antilichaam ontwikkeld door Dr Senshu is in staat om gecitrullineerde epitopen te herkennen ongeacht de omliggende aminozuren. We hebben ook gekeken of deze AMC assay kan differentiëren tussen citrulline en homocitrulline. Zowel de gecitrullineerde als de gecarbamyleerde eiwitten werden sterk herkend door de 'AMC-Senshu' methode en kon dus geen onderscheid maken. De antilichamen uit serum van RA patiënten konden dit echter wel. Anti-CarP antilichamen en ACPA worden vaak samen gezien, maar kunnen ook onafhankelijk van elkaar voorkomen.

Aangezien de 'AMC-Senshu' methode geen onderscheid kan maken tussen citrulline en homocitrulline en deze methode in het verleden gebruikt werd om gecitrullineerde antigenen aan te tonen, zal dit opnieuw evalueerd moeten worden. Met massa spectrometrie is echter wel aangetoond dat gecitrullineerde eiwitten voorkomen in het gewricht.

CONCLUSIE

De identificatie van ACPA is een belangrijke ontwikkeling geweest in het begrijpen van de pathogenese van RA. Het is duidelijk geworden dat deze unieke antilichaam reactie meer homogene groepen kan vormen bij patiënten met RA met betrekking tot klinische presentatie en uitkomst dan eerder het geval was met andere auto-antilichamen. Verder kan mogelijk een verschil in het beloop van de ziekte veroorzaakt worden door de betrokkenheid van ACPA in de pathogenese van de ziekte.

Het ophelderen van de karakteristieken van de ACPA reactie heeft laten zien dat ACPA niet per se pathogeen hoeven te zijn, aangezien ook gezonde individuen ACPA bij zich kunnen dragen. Mogelijkerwijs is een meer ontwikkelde ACPA reactie (door bijvoorbeeld een breed isotype gebruik, toegenomen antigen-herkenning en hogere concentraties) nodig voordat de antilichamen kunnen bijdragen aan het klinisch manifest worden van de ziekte. Wanneer de ziekte eenmaal tot bloei gekomen is, lijkt de ACPA reactie zich niet verder te ontwikkelen. De ACPA status blijft echter wel belangrijk voor het maken van klinisch beslissingen, aangezien het de belangrijkste voorspeller is van ziekte uitkomst en geassocieerd is met de effectiviteit van verschillende interventies.

APPENDIX

Curriculum vitae
List of publications
Dankwoord

CURRICULUM VITAE

Annemiek Willemze werd op 16 juni 1985 geboren in Leiden. Na het behalen van haar VWO diploma in 2003 aan het Visser 't Hooft Lyceum te Leiden is zij in hetzelfde jaar gestart met de studie Geneeskunde te Leiden. Tijdens haar studie deed zij acht maanden onderzoek in het Health Science Centre van de afdeling reumatologie aan de universiteit van Manitoba in Winnipeg, Canada (onder leiding van prof. dr. H.S. El-Gabalawy). Gedurende deze stage in Canada werd gestart met onderzoek naar auto-antistoffen in reumatoïde artritis. In 2010 haalde zij het artsexamen aan de universiteit Leiden. Aansluitend startte zij met het promotieonderzoek op de afdeling Reumatologie van het LUMC onder begeleiding van prof. dr. R.E.M Toes, prof. dr. T.W.J. Huizinga en dr. L.A. Trouw. Hiervoor ontving zij een AGIKO subsidie van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek.

In december 2012 is zij begonnen aan haar opleiding tot reumatoloog (opleider prof. dr. Huizinga), waarvan zij de eerste drie jaren interne geneeskunde in het Medisch Centrum Haaglanden in Den Haag volgt (opleiders dr. A.H. Bootsma en dr. P.H.L.M. Geelhoed-Duijvestijn). Naar verwachting zal haar opleiding tot reumatoloog worden afgerond in 2018 in het LUMC.

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