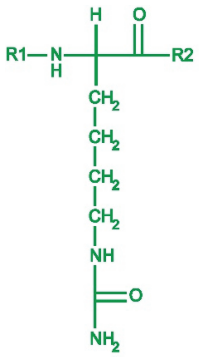
Anti-Carbamylated Protein Antibodies in Rheumatoid Arthritis



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The universe is full of magical things patiently waiting for our wits to grow sharper.
1918, A Shadow Passes by Eden Phillpotts

To:
Leendert Trouw
René Toes
Tom Huizinga

1 Introduction

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Carbamylation and antibodies against carbamylated proteins in autoimmunity and other pathologies
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Introduction

Rheumatoid arthritis, pre-rheumatoid arthritis stages and juvenile idiopathic arthritis

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease that principally affects synovial joints. RA is present in 0,5% to 1% of the global population. The incidence of RA is higher in women than in men and increases with age (1). RA can affect any joint but preferably small joints in hands and feet (2). The symptoms of RA include pain, swelling, stiffness, redness, warmth and can finally lead to loss of joint functions (2). The systemic symptoms of RA include fatigue, malaise, loss of appetite and muscle ache. Next to the joints RA can affect other organs such as skin, lungs, heart and blood vessels (3). RA can be classified using the 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for rheumatoid arthritis (2010 ACR/EULAR criteria) (4). This is a quantitative system in which scores can be obtained from: joint involvement, serologic markers, inflammation markers and duration of symptoms (4).

Development of RA can be acute or can be preceded by pre-RA stages. Yet a uniform and precise definition of pre-RA stages does not exist. Pre-RA stages generally refer to patients who have clinical symptoms involving joints but not fulfill the diagnostic criteria of RA or other forms of arthritis (5). Arthralgia and undifferentiated arthritis (UA) are two common types of pre-RA stages. Arthralgia patients may have symptoms such as joint pain, psychological distress, muscle cramps, abnormal skin sensations, stiffness, loss of motor control, weakness, fatigue and sleeping difficulties but have no clinically apparent joint swelling/inflammation (6-8). UA patients, to the opposite, have clinically apparent joint swelling suggestive of inflammation (9). Patients in these two stages can progress to RA but also have high chance of remission (8,9). The likelihood of progression to RA for arthralgia and UA patients is partially predictable by clinical and serologic variables. The arthralgia and UA patients share the predictable variables: morning stiffness and the presence of anti-cyclic citrullinated peptide antibodies (10,11). The arthralgia patients specific prediction variables are: rheumatoid arthritis in a first degree family member, alcohol non-use, duration of symptoms <12 months, presence of intermittent symptoms, arthralgia in upper and lower extremities, visual analogue scale pain≥50, and history of swollen joints as reported by the patient (10). The UA patients specific prediction variables are: sex, age, localization of symptoms, the tender joint count, the swollen joint count, the C-reactive protein level, rheumatoid factor positivity, and magnetic resonance imaging (MRI)/ultra sound pattern (11-14). Since these associations apply at a group level additional biomarkers are needed that will allow a more personalized medicine approach in these pre-RA patients.

Arthritis can also occur in children and is referred to as juvenile idiopathic arthritis (JIA), the most common rheumatic disease in children (0,01%-0,4%) consisting of eight heterogeneous subgroups (15). A common feature of JIA is joint inflammation resulting in pain, loss of function, and morning stiffness (16). Unlike adult RA, JIA patients have higher chances of remission and a lower prevalence of autoantibodies (17,18). Rheumatoid factor (RF) positive polyarticular JIA is the subgroup which resembles most the clinical and immune-genetic characteristics of adult RA patients (15).

Autoantibodies in RA, pre-RA stage and JIA patients

RA patients are a heterogeneous group of patients with pronounced differences in disease activity and outcome. This heterogenous group can be subdivided by the presence of autoantibodies. Autoantibody positive and negative RA patients were found to have different genetic background, disease development processes and responses to treatments (2). Currently, RF and anti-citrullinated protein antibodies (ACPA) are two major autoantibody systems in RA patients. The identification of RF can be dated back to 1940, reported by Waaler et al (19). RF is a polyclonal antibody system which mainly recognizes the Fc part of IgG (20). RF is present in about 50%-90% of RA patients but can also be present in other rheumatic or non-rheumatic diseases (21). IgM-RF is the most frequently detected isotype in RA patients but IgG and IgA isotypes also exist (22). The possible functions of RF include helping immune complex (IC) formation and clearance as well as facilitating antigen presentation (23). RF was the most important diagnostic marker in RA before ACPA were discovered and it is still included in the newest 2010 ACR/EULAR RA classification criteria in the same way as ACPA (4). However, the clinical relevance of RF was shown to be limited. Given that IgM-RF can be detected in sera of blood bank donors who developed to RA several years later but not in those who did not develop to RA (24-26), a cross-sectional screening study detected only one RA patient in each 10 RF-positive subjects (27). The presence of IgM-RF predicts future development of RA in UA patients independent of ACPA (28). However, the presence of IgM-RF did not have such predictive value in arthralgia patients, no prognostic value for joint damage and for the chances of disease modifying anti-rheumatic drug (DMARD) free remission in early RA patients in the disease course independent of ACPA (7,29,30).

The presence of ACPA and their prognostic value in RA patients were first reported around 50 years later than RF (31). ACPA bind to citrullinated epitopes of auto-antigens. The sensitivity of ACPA (~67%) was comparable to IgM-RF (~69%) but its specificity (~95%) was much higher than IgM-RF (~85%) (32). The presence and the level of ACPA contribute 2 points in the newly made 2010 ACR/EULAR RA classification criteria (4). The presence of ACPA in early RA patients is associated with worse disease development and a lower chance of reaching DMARD free remission (29,30). The presence of ACPA is also associated with a higher risk of cardiovascular disease and a higher mortality in RA patients (33,34).

In addition, the presence of ACPA is associated with more severe extra-articular manifestations in RA patients (35). ACPA positive UA and arthralgia patients have an increased chance of further developing to RA (7) while IgM-RF negative arthralgia patients almost do not develop to arthritis (36). ACPA are present in 24% of UA patients in the Leiden early arthritis cohort (EAC) and the positive predictive value for developing to RA is 67% (28). In addition, around 30% of RA patients developed ACPA before the appearance of any clinical symptom (24,25) The presence of ACPA before the onset of RA in asymptomatic blood donors is associated with the development of erosive RA (26).

Next to the presence of ACPA, several aspects of the ACPA responses, such as titer, isotype usage, epitope spreading and avidity (37-39), are associated with the clinical outcome of RA. An increased ACPA isotype usage was observed in RA patients compared to UA and healthy individuals who are also ACPA positive (37,40). High titer ACPA positive UA patients have a higher risk of developing to RA and a more acute development compared to low titer ACPA positive UA patients (38). The number of recognized citrullinated epitopes is positively associated with the risk of developing to RA and negatively associated with the chance of reaching DMARD free remission in ACPA positive arthralgia and UA patients (28,41,42). ACPA positive RA patients who have the lowest quartile avidity (binding strength to antigens) also have the most joint damage in their disease course (39). Above mentioned associations suggest that the autoantibody response is a developing process and it may trigger other symptoms/pathogenic effects of RA.

The autoantibody profile in JIA patients is quite different from adult RA patients. Antinuclear antibodies (ANA) have a lower prevalence and diagnostic value in RA patients compared to RF (43). But in JIA patients the presence of ANA is associated with a higher risk of uveitis and increased T and B cells infiltration (44,45). The prevalence of RF or ACPA in JIA patients is less than in adult RA patients and is predominantly confined in RF-positive polyarticular JIA subgroup (17,46). Like in adult RA, ACPA were confirmed as an independent risk factor associated with worse disease outcome in JIA patients (47,48).

In summary, both ACPA and RF are diagnostic markers for RA patients and predictive markers for future development of RA in pre-RA stage patients. ACPA are also a prognostic marker in RA patients.

Genetic predisposing and ACPA

As described before, RA patients can be divided into 2 distinct subsets which, at a group level, have different disease courses and genetic risk profiles based on ACPA positivity (49). This again suggests that the production of ACPA is a key intermediate step between the predisposing genetic risk and the development of RA. Eventhough the heritability of

ACPA negative and positive RA patients are both estimated to be about 66% (50), yet no predominant human leukocyte antigen (HLA) association and far less non-HLA risk single-nucleotide polymorphisms (SNPs) were found in association with ACPA negative patients (51-53). The most important genetic risk factors of ACPA positive RA reside in the Human Leukocyte Antigen (HLA) class II locus, more specifically the shared epitopes (SE) on HLA-DR beta 1 (HLA-DRβ1). SE consists of three homologous amino acid sequence variants in residues 70–74 of the HLA-DRβ chain: QKRAA, QRRAA and RRRAA (54). HLA SE alleles contribute 18% of the susceptibility of ACPA-positive RA patients (50). In non-HLA genes which have SNPs associated with higher risk of developing to ACPA-positive RA (52), many genes encode proteins which are potentially contributive to autoantibody production or immune complex activated pathways. Thus both HLA and non-HLA genetic risk factors of RA suggest the pathogenesis of ACPA positive RA patients is perhaps predominantly initiated via adaptive immunity.

Environmental risk factors and ACPA

Beside genetic risk factors, also environmental risk factors such as cigarette smoking and infection are associated with a higher risk of developing to RA. Cigarette smoking is dose dependently associated with the susceptibility of RA and disease progression (55-57). It is also strongly associated with the combined presence of ACPA positivity and SE (55,56,58). Increased expression of peptidyl-arginine deiminase (PAD) induced by smoking is one hypothesis to explain the effect of smoking in RA patients and its association with ACPA positivity and SE (59). Periodontitis (PD), an infectious disease, is also associated with a higher incidence and severity of RA (60-62). Treatment of PD in established RA patients decreased the severity of RA (63-65). The presence of PD and the level of antibodies against P. gingivalis, a pathogen of PD, are associated with the presence of ACPA and RF-IgM (66,67). NETosis of neutrophils is another mechanism which can be triggered by the pathogens of PD (68). NETosis, a process in which the nuclear content of cells is extruded from the cell, will release intracellular PAD4, which offers another source for extracellular citrullination (69).

Pathological functions of ACPA

To explain the potential contribution of ACPA to the pathogenesis of RA, the functions of ACPA have been studied in several aspects. ACPA, citrullinated antigens and their immune complex (IC) were reported to induce the TNF-α production of several cell types including macrophage, monocyte, fibroblast-like synoviocytes and osteoclast precursors (70-72). TNF-α is a key cytokine in the pathogenesis of RA (73). Furthermore, ACPA stimulate osteoclastogenesis and osteoclast mediated bone erosion. In addition ACPA have been shown to activate the classical and alternative pathways of complement (74). ACPA may appear in mice with collagen induced arthritis without immunization of citrullinated

antigens depending on their genetic background (75). However, whether ACPA play a role in the progression of the disease in DBA1 and Balb/c mice is in debate (76-78).

Carbamylation

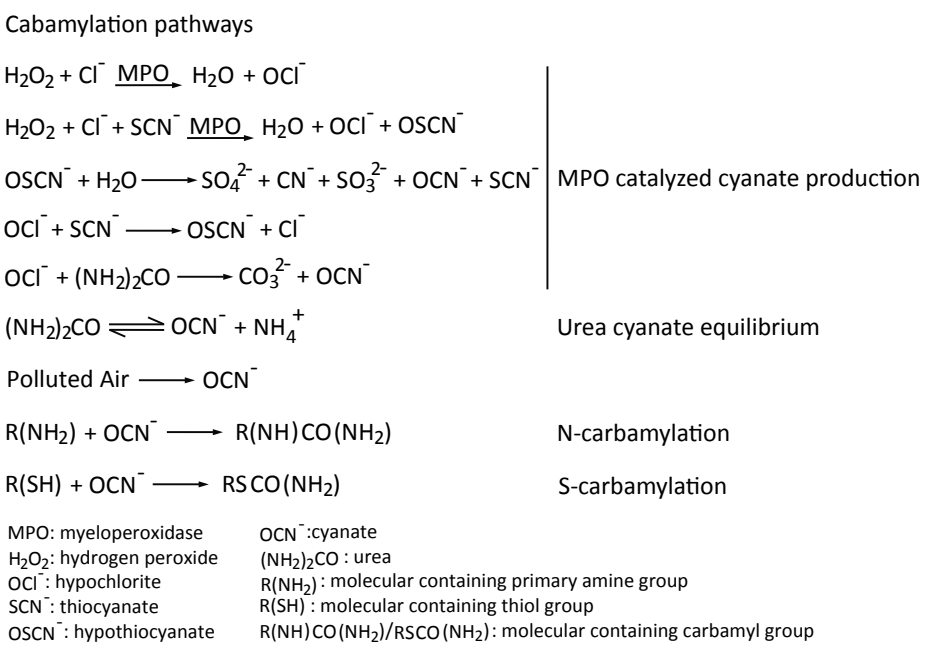
As ACPA are only present in about 67% of the RA patients and as part of the ACPA negative RA patients also have severe joint damage, there is a need for additional biomarkers to identify ACPA negative patients in need of a more aggressive intervention (79). In an attempt to identify additional biomarkers to be used for such identification we addressed the presence of antibodies directed against proteins modified by another form of post-translational modification, carbamylation. Homocitrulline is an amino acid with a high structural similarity to citrulline and therefore we hypothesized that anti-homocitrulline containing (carbamylated) protein antibodies may also be present in some RA patients and we tested this hypothesis following previous studies on carbamylation as described in detail below.

Pathways that induce carbamylation of proteins

Carbamylation: cyanate reacting to primary amino or thiol groups

Carbamylation is a post-translational modification in which cyanate reacts to primary amino or thiol groups. The reaction of cyanate to either amino groups or thiol groups is specified as N-carbamylation or S-carbamylation. Beside the N-terminus of all proteins, the amino acids lysine, arginine and cysteine contain side chains that can react with cyanate (80,81). However, since carbamylation on side chains of cysteine and arginine, the N-terminus of proteins and free amino acids is rarely reported we therefore here refer to carbamylation as cyanate reacting on peptidyl-lysine without further specification. Urea is a source of cyanate in all individuals and is present in body fluids in equilibrium with ammonium cyanate (Fig. 1). The equilibrium ratio between cyanate and urea has been suggested to be around 1 to 500.000 (82). Despite the low concentration of cyanate, trace amount of carbamylation can be detected in healthy individuals (83,84). As expected, elevated carbamylation was extensively reported among patients with renal dysfunction and elevated blood urea nitrogen (BUN) levels (85-87).

Figure 1. Pathways involved in in vivo cyanate generation



Inflammation increases the level of cyanate

In addition to renal insufficiency, inflammation is another factor which can stimulate the degree of carbamylation. Wang and Holzer et al. demonstrated that inflammation can enhance carbamylation via a mechanism which depends on myeloperoxidase (MPO) (88,89). MPO is mainly stored in granules of neutrophils (90) and it can generate cyanate using hydrogen peroxide and thiocyanate as substrates. Thiocyanate, derived from e.g. food or smoke exposure (88), can be oxidized by hydrogen peroxide with the help of MPO, resulting in the formation of hypothiocyanate (84) which decomposes to cyanate and other ions (91). In addition MPO can also catalyze the reactions between hydrogen peroxide and chloride (90) that via a series of reactions leads to increased levels of cyanate (Fig. 1) (89). The marked increased levels of MPO in inflammation (92) therefore stimulates the formation of cyanate. These findings indicate that MPO released from neutrophils can further increase the level of carbamylation during inflammation.

Direct inhalation of cyanate

Cyanate (~200 parts-per-trillion volume) can also be directly inhaled from urban air. A five times higher concentration, one parts-per-billion volume of cyanate in inhaled breath, can

already generate an aqueous solution of 100 μM (93). This concentration is equal to or higher than the effective dose of cyanate which is able to cause notable effects in several in vitro studies (94-96). Cyanate in air can be derived from various sources such as biomass burning, coal burning, biofuel usage, cooking, tobacco usage and wild fire. Even in the absence of inflammation the direct exposure to air borne cyanate can be sufficient to generate low levels of carbamylation.

Lysine carbamyltransferase converts free amino acid lysine to homocitrulline

Beside above mentioned mechanisms, carbamylation on free amino acids can also be catalyzed by the enzyme lysine carbamyltransferase. This enzyme converts the free amino acid lysine and carbamyl phosphate to homocitrulline (97,98). Whether this enzyme is able to catalyze the reaction between carbamyl phosphate and peptidyl-lysine has, to the best of my knowledge, not been studied. Carbamyl phosphate injected in rats caused extensive carbamylation (99). Thus leaking of carbamyl phosphate synthetase or lysine carbamyltransferase due to apoptosis or necrosis of cells might potentially be a currently unexplored source of introducing carbamylation. At this stage, protein carbamylation mediated by enzymes seems unlikely but clearly requires further investigation.

Effects of carbamylation in (patho)physiology

Consequences of carbamylation have been reported to occur at the protein, cellular and systemic level. Decreased activity upon carbamylation has been reported for several enzymes and hormones, (100-105). Altered binding affinity to target ligands upon carbamylation has also been reported on hemoglobin A and human serum albumin (106-109). Other reported effects of carbamylation on proteins include changing their polymerization ability (collagen and actin), sensitivity to proteinases (collagen and glutamate dehydrogenase) and antibody antigen binding avidity (blood group specific glycoprotein) (110-112).

Not surprisingly, carbamylation of proteins and small molecules has an impact on normal cellular functions. Exposure to relatively high concentrations of cyanate is cytotoxic, which has been reported for e.g. human erythrocytes (114,115). Lower levels of carbamylation may also change cellular functions in several ways as described below. For example, in vitro incubation with cyanate dose-dependently decreased protein synthesis of rat bone marrow cells (108), insulin secretion of pancreatic β cells (95), the respiration rate of rat mitochondria (116) and ROS production of human neutrophils (117). Next to a role of cyanate on cellular functions also the interaction with carbamylated proteins has an impact on cellular functions. For example, carbamylated BSA increased collagen production of mesangial cells (118-120) and adhesion of monocytes onto carbamylated collagen was significantly enhanced (121).

As a consequence of the effects on protein and cellular functions, carbamylation may also trigger systemic effects. Cyanate is one of the agents which have been used to treat sickle cell anemia patients (122,123). However, neurotoxicity and cataract were observed as side effects. More than half of sickle cell anemia patients who received cyanate treatment developed nerve conduction abnormalities (124). Similar to neurotoxicity, cataract was also reported as a side effect in sickle cell disease patients treated with cyanate (125).

As mentioned above, patients suffering from uremia have increased levels of carbamylation due to high level of urea (126-128). The quantity of carbamylation is also associated with other renal function markers such as creatinine, cystatin C and estimated glomerular filtration rate (129-131). Since the degree of carbamylation is associated with these other markers of renal function, it is not easy to judge the independent contribution of carbamylation to kidney dysfunction. Two recent findings regarding the prognostic value of carbamylated proteins in end stage renal disease (ESRD) may suggest that carbamylation is an independent risk factor in the progression of renal dysfunction (131). ESRD is the last stage of chronic kidney disease (CKD) in which kidney failure is permanent and medical interventions (dialysis or kidney transplantation) are compulsory for patients to survive. Baseline percentage of carbamylated Lys549 on albumin appeared to be the risk factor with the highest hazard ratio for mortality in two independent ESRD cohorts after correcting for all other known risk factors (132). This was recently independently confirmed (131).

Similar to CKD, increased levels of carbamylation are also observed in cardiovascular disease (CVD) which is associated with adverse clinical events. Increased carbamylation levels of plasma protein were found in patients with atherosclerotic CVD and systolic heart failure compared to healthy controls and was associated with developing adverse clinical events of these patients even after correcting other risk factors (88,130). The degree of carbamylation on high density lipoprotein (HDL) in the lesions of atherosclerosis patients is also correlated with the severity of the lesions and MPO mediated oxidative stress (133).

Induction of antibody responses against carbamylated proteins

Post-translationally modified proteins have been described to have the capacity to break immunological tolerance and induce autoantibody responses (134-136). The notion that this can also occur in the setting of carbamylation was initiated by Steinbrecher et al. who reported immunization experiments with carbamylated proteins (137). This hypothesis was also supported by the presence of anti-CarP antibodies as described for animal models previously. Both rabbits (138) and mice (139,140) were shown to be able to develop antibody responses against homocitrulline containing antigens upon immunization with peptides containing homocitrulline or carbamylated proteins. Besides, the presence of antibody reactivity against carbamylated proteins was suggested in a small RA cohort.(140) Following

these previous studies, we aimed to investigate the presence of anti-CarP antibodies in RA, pre-RA stages and JIA patients and whether they have similar diagnostic, predictive and prognostic value in these patients as ACPA and RF.

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Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage

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2

Abstract

Autoimmune responses against posttranslationally modified antigens are a hallmark of several autoimmune diseases. For example, antibodies against citrullinated protein antigens (ACPA) have shown their relevance for the prognosis and diagnosis of rheumatoid arthritis (RA), and have been implicated in disease pathogenesis. It is conceivable that other autoantibody systems, recognizing other posttranslationally modified proteins, are also present in RA. Here, we describe the presence of an autoantibody system that discriminates between citrulline- and homocitrulline-containing antigens in the sera of RA-patients. IgG antibodies recognizing carbamylated (homocitrulline-containing) antigens were present in sera of over 45% of RA-patients. Likewise, anticarbamylated protein (anti-CarP) IgA antibodies were observed in 43% of RA-sera. ACPA and anti-CarP antibodies are distinct autoantibodies because, in selected double-positive patients, the anti-CarP antibody binding to carbamylated antigens could be inhibited by carbamylated antigens, but not by control or citrullinated antigens. Similarly, ACPA-binding to citrullinated antigens could only be inhibited by citrullinated antigens. In line with this observation, 16% of ACPA-negative RA-patients, as measured by a standard ACPA assay, harbored IgG anti-CarP antibodies, whereas 30% of these patients tested positive for IgA anti-CarP antibodies. The presence of anti-CarP antibodies was predictive for a more severe disease course in ACPA-negative patients as measured by radiological progression. Taken together, these data show the presence of a unique autoantibody system recognizing carbamylated, but not citrullinated, protein antigens. These antibodies are predictive for a more severe clinical course in ACPA-negative RA-patients, indicating that anti-CarP antibodies are a unique and relevant serological marker for ACPA-negative RA.

Introduction

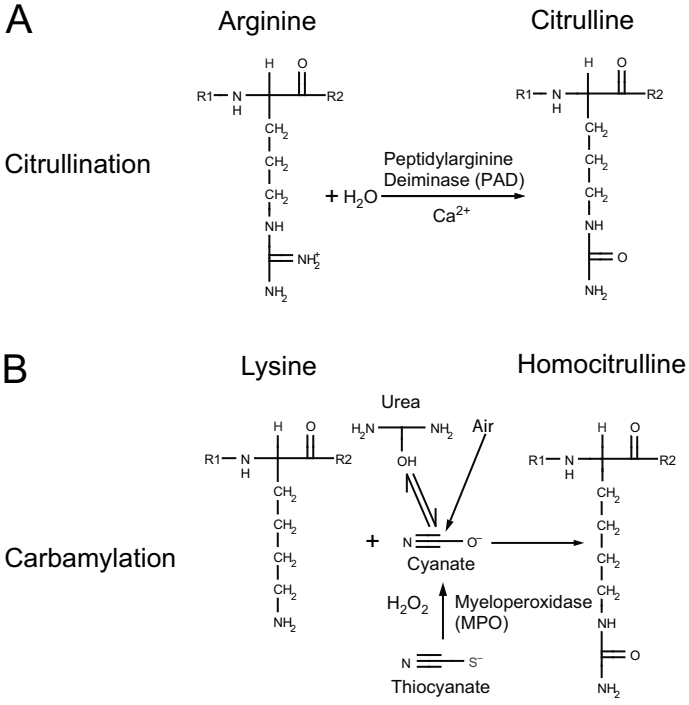
The identification of anticitrullinated protein antibodies (ACPA) has contributed significantly to the understanding of rheumatoid arthritis (RA) (1). Significant differences between ACPA-positive and -negative disease have been reported with respect to the contribution of genetic and environmental risk factors, as well as disease progression and remission (2–5). Over the past few years important insight has been gained into the occurrence and etiophathology of ACPA-positive RA. However, less information is available on ACPA-negative RA. This lack of information is partly because of the absence of robust biomarkers characterizing this manifestation of RA.

The posttranslational modification of arginine into citrulline by peptidyl arginine deiminase (PAD) enzymes is essential for the generation of citrullinated antigens that are recognized by ACPA (1). Under physiological circumstances, citrullination is involved in tissues like hair and skin because of its role in terminal epithelial differentiation (6). In the nucleus citrullination plays a role in epigenetic regulation (7) and condensation of chromatin, and has been reported to be involved in translation (6) and the host defense against pathogens (8). Under pathological conditions where cell death may overwhelm the phagocytic capacity of phagocytes, necrotic cells may release PAD into the extracellular space, where higher calcium concentrations now also allow the citrullination of other proteins located outside the cell (6). These proteins may be targeted by ACPA, possibly leading to inflammation and arthritis.

Citrulline highly resembles homocitrulline (Fig. 1), another posttranslationally modified amino acid (9). Homocitrulline is one methylene group longer, but similar in structure (9). Homocitrulline is generated from a lysine residue following a reaction of cyanate, which is present in the body in equilibrium with urea. Under physiological conditions the urea concentration may be too low to allow extensive carbamylation but the conversion process leading to the formation of homocitrulline from lysine in proteins does occur in vivo. In conditions of renal failure, the urea concentration increases and carbamylation of many proteins can be readily detected. However, most carbamylation is believed to take place during inflammation when myeloperoxidase is released from neutrophils (10). This enzyme converts thiocyanate to cyanate, now allowing more carbamylation to occur (11). It has been shown recently that homocitrulline-containing proteins are present in the RA joint and that they may affect T-cell triggering and possibly autoantibody formation in rodents (9, 12). Although highly similar, carbamylation differs from citrullination as, next to their structural difference, lysine is modified instead of arginine. Therefore, homocitrulline will, by definition, be located at other positions in proteins than citrulline. Because of the similarity between citrulline and homocitrulline, In this chapter we analyzed whether

autoantibodies against carbamylated proteins are present in RA and whether these antibodies differ from ACPA with respect to antigen binding and clinical associations.

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.



Results

Anticarbamylated protein antibodies and ACPA are different antibody families

To detect antibodies against carbamylated proteins (anti-CarP antibodies), we developed an ELISA using carbamylated FCS (Ca-FCS) and nonmodified FCS as antigens. Analyzing sera of 40 RA patients and 40 controls, we observed that sera of RA-patients reacted with Ca-FCS compared with sera obtained from healthy subjects with both IgG (Fig. 2 A and B) and IgA (Fig. 2 D and E) reactivity. The enhanced reactivity of RA sera to Ca-FCS is further emphasized after subtraction of the reactivity against unmodified FCS (Fig. 2 C and F). Because citrulline and homocitrulline are two rather similar amino acids (Fig. 1), we next wished to determine whether ACPA also recognizes

homocitrulline when located at the same position as citrulline in a peptide. For this purpose we performed ELISAs using a citrullinated Fibrinogen (Fib) peptide known to be recognized by ACPA (13). Within this peptide backbone, a citrulline, an arginine, a homocitrulline, or a lysine residue was introduced for further analysis. Analyzing a set of 76 RA sera, we observed that ACPA only recognized the citrullinated peptide, but not the arginine-containing or the homocitrulline-containing peptide (Fig. 3A). These data indicate that ACPA can discriminate between citrulline and homocitrulline presence within the same peptide backbone. Next, we wished to analyze whether there is cross-reactivity between anti-CarP antibodies and ACPA for binding to posttranslationally modified proteins. Therefore, we performed inhibition studies using sera that were reactive to both citrullinated and carbamylated antigens. We analyzed the binding of anti-CarP antibodies to Ca-FCS–coated plates following preincubation with Ca-FCS, citrullinated FCS (Ci-FCS), native FCS, or by citrullinated peptides used to detect ACPA (cyclic citrullinated peptide-1, CCP1). Following preincubation, we observed that anti-CarP antibody binding to Ca-FCS can only be inhibited by Ca-FCS but not by Ci-FCS, native FCS, or by peptides used to detect ACPA (Fig. 3B). We also performed the reverse inhibition experiment where we analyzed the binding of ACPA to plates coated with Ci-FCS following the same preincubation procedure. We observed that ACPA binding to Ci-FCS could only be inhibited by Ci-FCS and the citrullinated peptide but not by Ca-FCS, nonmodified FCS, or the arginine form of the peptide (Fig. 3C). Taken together, these data indicate that anti-CarP antibodies and ACPA are not, or only limited, cross-reactive and specifically directed against homocitrulline or citrulline-containing antigens, respectively. Because all observations described above were made using ELISA, we also wished to confirm our findings using a different technique. For this reason we performed a Western blot-analysis using FCS, Ca-FCS, and Ci-FCS on reduced gels, followed by Western blotting. The different blots were incubated with sera of individuals that were either anti-CarP–positive and ACPA-negative or anti-CarP–negative and ACPA-positive. We observed a positive staining of the anti-CarP–positive sample only on Ca-FCS but not on Ci-FCS or FCS (Fig. 3D). In contrast, the anti-CarP–negative, ACPA-positive sample reacted to Ci-FCS, but not to Ca-FCS and FCS (Fig. 3D). To confirm the presence of anti-CarP antibodies we repeated these experiments using a more defined protein, human Fib, as a target antigen. Fib was citrullinated by PAD (Ci-Fib) or carbamylated by cyanate (Ca-Fib). The nonmodified form (Fib), Ci-Fib, and Ca-Fib were used as antigens in ELISA. Similar to the observations for FCS, we observed significant binding of antibodies to the Ci-Fib and the Ca-Fib but not to the Fib-coated wells (Fig. 4A). This finding was largely restricted to the RA sera and not the controls ($P \leq 0.0001$). To analyze cross-reactivity we also performed inhibition studies, as described above. ELISA analyses confirmed that ACPA and anti-CarP antibodies are largely noncross-reactive (Fig 4B). To ensure that reactivity toward carbamylated proteins is mediated by the antigen-binding part of the antibodies, we generated F(ab′)2. As expected, F(ab′)2, generated from anti-CarP IgG-positive samples but not from negative samples display anti-CarP reactivity (Fig. 4 C and D).

As observed using intact antibodies, F(ab′)2-reactivity toward Ca-Fib could also be inhibited specifically by Ca-Fib, whereas F(ab′)2-reactivity toward Ci-Fib could only be inhibited specifically by Ci-Fib (Fig. 4E).

Figure 2. Antibodies against carbamylated proteins are present in sera of RA patients. The reactivity of IgG (A and B) and IgA (D and E) from sera of healthy controls (NHS) or RA patients (RA) to wells coated with nonmodified FCS (FCS) or carbamylated FCS (Ca-FCS) is depicted. Data expressed as absorbance at 415 nm. (C and F) Absorbance units of FCS were subtracted from the absorbance units of Ca-FCS, representing the specific anticarbamylated protein response. ***$p < 0.0001$ for a t test comparing NHS and RA.

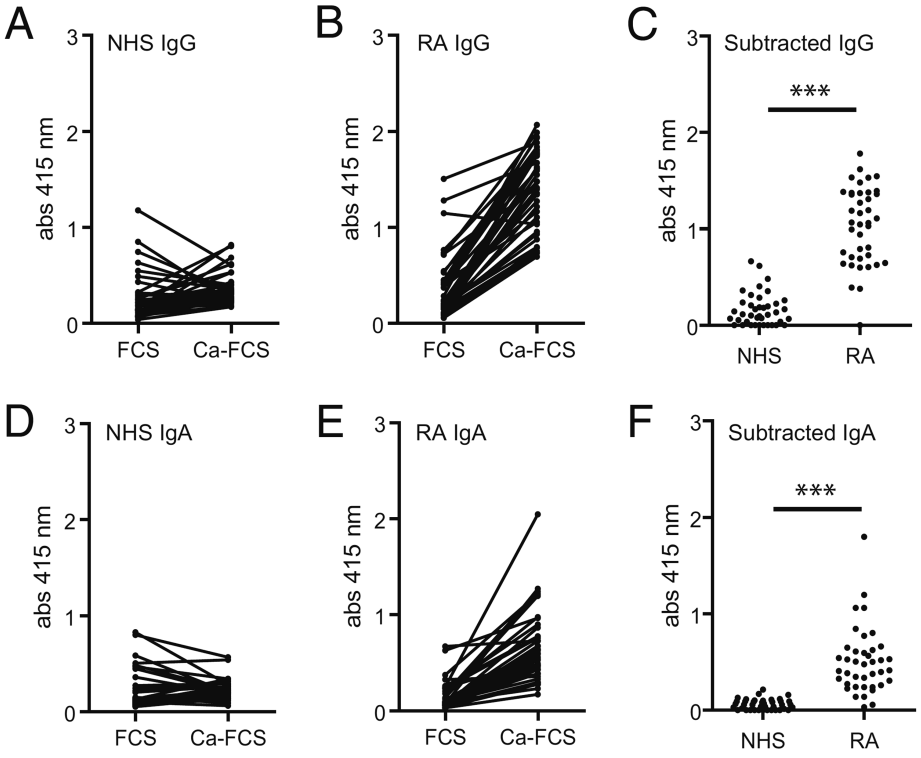


Figure 3. Anti-CarP antibodies and ACPA are two separate autoantibody systems. IgG reactivity of 76 sera from RA patients, toward several forms of a Fib peptide is depicted. (B and C) Antibody binding to Ca-FCS or Ci-FCS was inhibited using preincubations with fluid-phase inhibitors. (D) FCS, Ca-FCS, and Ci-FCS were separated by SDS-PAGE gels and blotted. The presence of antibodies reactive to proteins on the blots was analyzed by incubating these blots with either anti-CarP–positive ACPA-negative and anti-CarP–negative ACPA-positive sera.

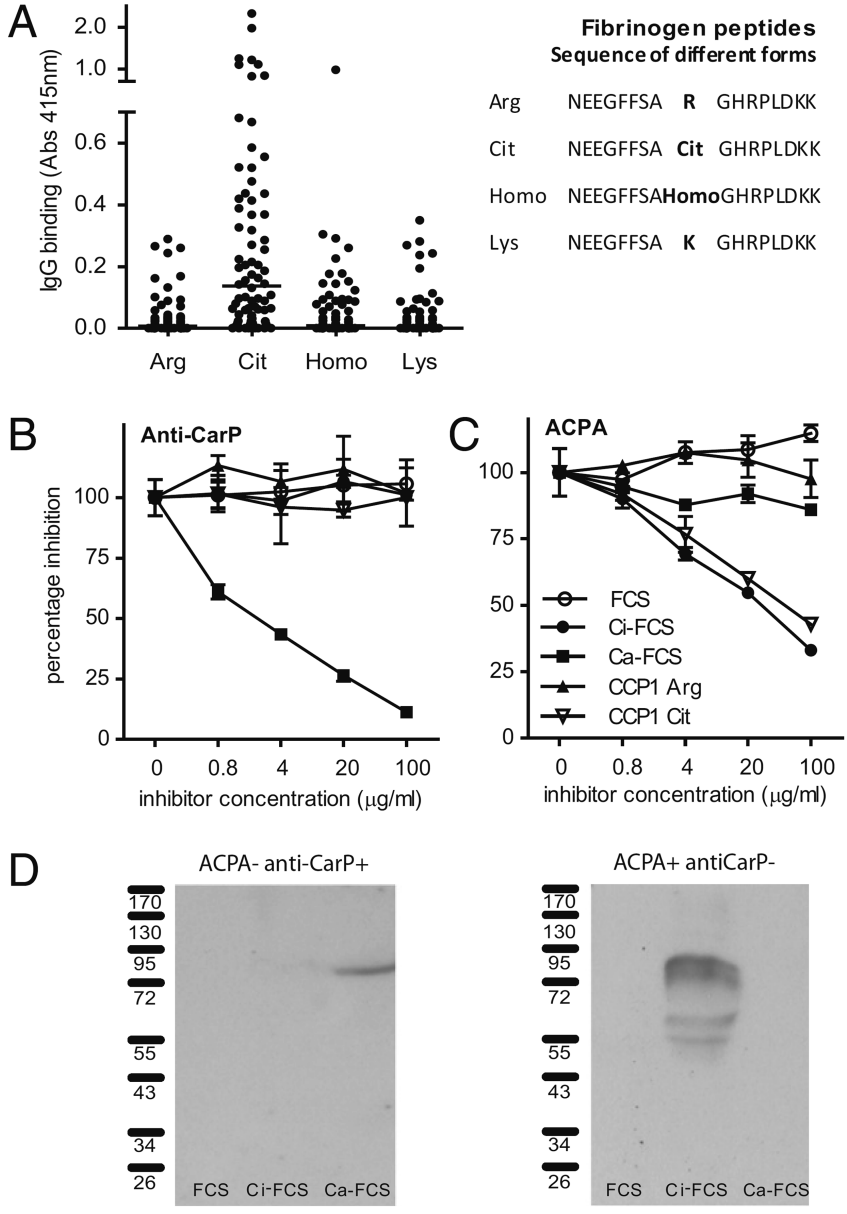
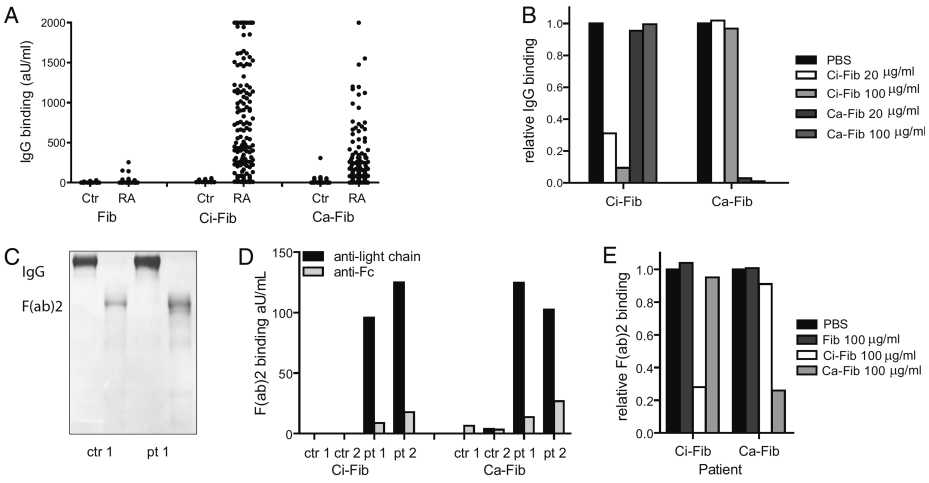


Figure 4. Anti-CarP antibodies bind to Ca-Fib via variable domains. (A) IgG reactivity against Fib, Ci-Fib, and Ca-Fib of 54 healthy controls and 214 RA patients was analyzed by ELISA. (B) Specificity of anti–Ca-Fib reactivity was confirmed using inhibition studies. One sample is shown, where data are expressed relative to inhibition with PBS. (C) The molecular nature of purified IgG and F(ab′)2 was confimed by Coomassie-stained SDS-PAGE gel. (D) F(ab′)2 fragments were generated from purified IgG of 2 anti-CarP–positive patients and two negative controls. Only F(ab′)2 from patients reacted with Ci-Fib and Ca-Fib. (E) Inhibition experiments confirm that also F(ab′)2 are not necessarily cross-reactive between Ci-Fib and Ca-Fib.



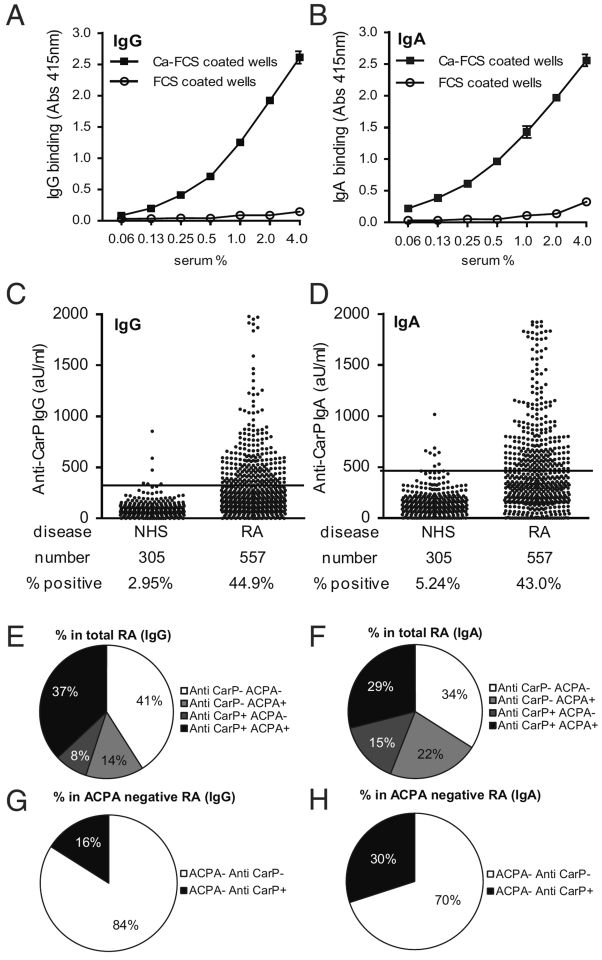
Collectively, these data indicate that anti-CarP antibodies and ACPA recognize different antigens, one recognizing citrullinated proteins (ACPA) and the other carbamylated proteins (anti-CarP). Likewise, these data indicate that antigen-recognition is most likely mediated via the variable domains present in the F(ab′)2 fragments.

Anti-CarP antibodies are present in RA

Following the identification of anti-CarP antibodies as an autoantibody family separate from ACPA, we wished to quantify the presence of these anti-CarP antibodies in a large population of RA patients and controls. For this reason, we first generated a standard, comprising of a pool of anti-CarP antibody-positive sera. This standard displayed a specific, dose-dependent binding of both IgG and IgA to Ca-FCS but no binding to unmodified FCS (Fig. 5 A and B). For this analysis, we again used the FCS-based assay in an attempt to capture as many anti-CarP reactivities as possible. We established a cutoff for positivity using sera of 305 healthy individuals, as described in Materials and Methods. Using this approach, we observed that 45% of the sera of RA patients analyzed are positive for IgG

anti-CarP antibodies (Fig. 5C). Likewise, 43% of sera from RA patients tested are positive for IgA anti-CarP antibodies (Fig. 5D).

Figure 5. Anti-CarP IgG and IgA antibodies are present in RA sera. (A and B) Dose-response curves of the anti-CarP antibody-positive standard (IgG and IgA) on Ca-FCS and FCS in ELISA. (C and D) ELISA was performed for the detection of anti-CarP IgG and IgA in sera of healthy controls (NHS) and RA patients. A cut-off was established using the mean plus two times the SD of the healthy controls, as described in the Materials and Methods. Reactivity is depicted as arbitrary units per milliliter. The number of samples tested and the percentage of positivity is indicated below the graph. (E and F) Pie charts showing the percentage of RA patients positive and negative for anti-CCP2 and anti-CarP antibodies. (G and H) Pie charts showing the percentage of anti-CarP IgG- or IgA-positive patients negative for anti-CCP2.



Anti-CarP antibodies are also present in sera of anti-CCP2–negative RA patients

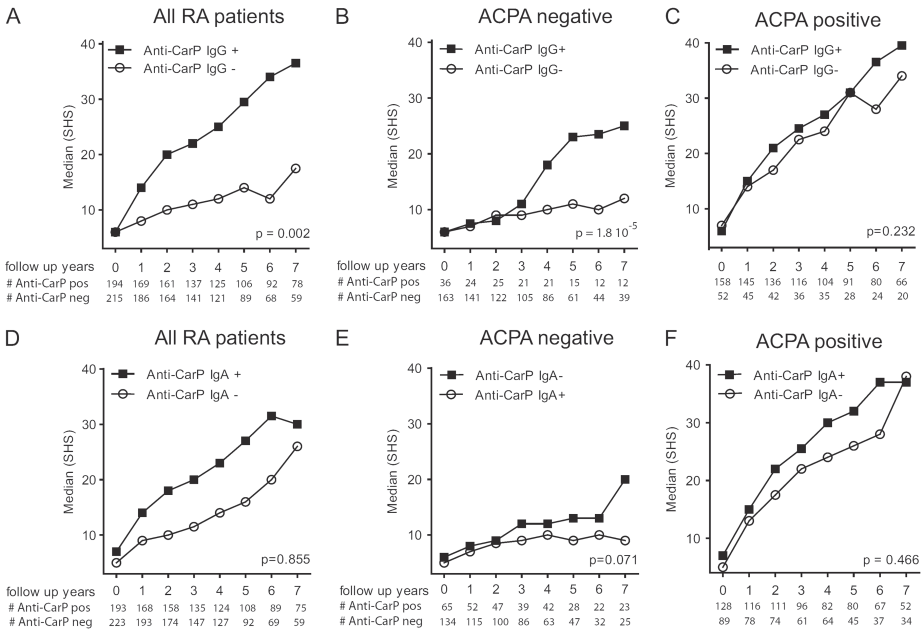
The group of RA patients analyzed in this study consisted of both ACPA-positive and ACPA-negative individuals, as measured by the CCP2 assay. Therefore, we analyzed next the association between anti-CarP antibodies and anti-CCP2 antibodies. The presence of anti-CarP antibodies and anti-CCP2 antibodies showed a limited degree of correlation when analyzing the entire RA population (r2 = 0,27, p < 0,001 for anti-CarP IgG or r2 = 0,15, p < 0,001 for IgA). However, we also identified substantial numbers of RA patients that are only positive for anti-CCP2 antibodies, as well as a group of patients that is only positive for anti-CarP antibodies (Fig. 5 E and F). We observed that ~16% of the anti-CCP2–negative RA patients displayed anti-CarP IgG antibodies, whereas 30% of the anti-CCP2–negative RA patients tested positive for anti-CarP IgA (Fig. 5 G and H). These data indicate that the presence of anti-CarP antibodies overlaps with the occurrence of anti-CCP2 antibodies, but that this overlap is not absolute, as over 30% of the anti-CCP2–negative patients harbor anti-CarP antibodies. In total, more than 35% of all anti-CCP2–negative patients have either anti-CarP IgG or IgA antibodies.

Anti-CarP antibodies are associated with more severe radiological damage

The presence of ACPA is associated with a more severe clinical disease course as measured by radiological damage. To analyze whether the presence of anti-CarP antibodies are also predictive for a more severe disease course, we compared the extent of joint damage over time between anti-CarP–positive and –negative patients participating in the Leiden Early Arthritis Clinic (EAC) cohort. This cohort is an inception cohort of patients with recent-onset arthritis where X-rays of hands and feet are taken of all RA-patients at yearly intervals to assess radiological damage using the Sharp–van der Heijde method (14). We observed that the presence of anti-CarP IgG strongly associates with a more severe disease progression. Patients positive for anti-CarP IgG had more joint destruction over 7 y than IgG-negative patients without [β = 2,01, 95% confidence interval (CI) 1,68–2,40, p = 8,68 × 10−14] or with correction of ACPA and rheumatoid factor (RF) (β = 1,41, 95% CI 1,13–1,76, p = 0,002) (Fig. 6A). Anti-CarP IgA was associated with more joint destruction over 7 y than anti-CarP IgA-negative patients without correction of ACPA and RF (β = 1,21, 95% CI 1,01–1,45, p = 0,041) but not after correction (p = 0,855) (Fig. 6D). As the analysis described above does not show whether anti-CarP antibodies predict radiological progression in the anti-CCP2–negative, anti-CCP2–positive, or both RA subgroups, we next performed a stratified analysis. Importantly, this analysis revealed that the presence of anti-CarP IgG is associated with a more severe joint damage in the anti-CCP2–negative subgroup (β = 1,86, 95% CI 1,41–2,66, p = 1,8 x 10−5) (Fig. 6B). Likewise, a similar trend toward more joint damage over time was observed for anti-CCP2–negative patients who tested positive

for IgA anti-CarP antibodies (β = 1,25, 95% CI 0,98–1,58, p = 0,071) (Fig. 6E). In contrast, in the anti-CCP2–positive subgroup, which is already characterized by severe joint destruction, no additional increase was observed in individuals who also harbored anti-CarP antibodies (Fig. 6C&F). Taken together, these data indicate that the detection of anti-CarP antibodies at baseline is predictive for a more destructive disease course in anti-CCP2–negative RA as measured by the Sharp–van der Heijde method.

Figure 6. Anti-CarP IgG antibodies are associated with a more severe radiological progression in RA. The extent and rate of joint destruction were analyzed in all RA patients included, or analyzed separately for ACPA-negative or ACPA-positive subgroups. The severity of joint destruction of anti-CarP IgG-positive versus -negative patients is depicted as median Sharp-van der Heijde score (SHS) on the y axis and the follow-up years on the x axis. Below the x axis, the patient number is listed for each time point. (A) Radiological progression for all RA patients analyzed, or for the (B) anti-CCP2–negative, or (C) anti-CCP2–positive patients only. Similarly, the effect of anti-CarP IgA antibodies on severity is depicted for (D) the RA group as a whole, or (E) for anti-CCP2–negative patients only, or (F) anti-CCP2–positive patients only. The P values indicated in the figure are derived from the analysis model following corrections, as described in Materials and Methods and Results.



Discussion

A family of autoantibodies that recognize carbamylated proteins, anti-CarP antibodies, can be detected in sera of RA patients. Both inhibition studies and cohort studies show that anti-CarP antibodies and ACPA represent two different and independent autoantibody families, one recognizing carbamylated proteins and the other citrullinated proteins. Our data show that anti-CarP antibodies and ACPA are, by and large, noncross-reactive although we do not exclude that some cross-reactivity exists at the population level, as is also indicated in recent data obtained in rabbits after vaccination with carbamylated proteins (12). Interestingly, positivity for anti-CarP antibodies is related to clinical outcome, as individuals positive for anti-CarP IgG, but negative for anti-CCP2 antibodies, have a more destructive disease course compared with anti-CarP IgG-negative RA patients.

It is currently unknown which proteins undergo posttranslational modifications like carbamylation. Carbamylation is mediated by cyanate, which is in equilibrium with urea. Increased urea concentrations, smoking, and inflammation have been reported to shift this equilibrium towards cyanate and, hence, enhanced carbamylation (11). Because currently no in vivo relevant targets for anti-CarP antibodies are known, we used a complex protein mixture as an initial source of carbamylated protein antigens for the detection of anti-CarP antibodies. Western blot analyses indicate the recognition by anti-CarP antibodies of at least one dominant protein present in FCS after carbamylation using cyanate (representing high urea concentrations) (Fig. 3D). However, these data are likely not to represent the in vivo situation where carbamylation is a more gradual but constantly occurring process (15). In this respect, it is likely that especially long-lived proteins acquire homocitrulline residues over time, as carbamylation is nearly irreversible and thus will lead to the accumulation of homocitrulline-residues on proteins with a long half-life. Intriguingly, the joint is known for the presence of long-lived proteins, such as collagens and other cartilage-expressed proteins. Therefore, it is conceivable that such matrix-proteins will accumulate homocitrulline residues during life, especially under conditions of inflammation. Indeed, it has been shown that homocitrulline is present in the joint (9), possibly representing the long-lived nature of many joint-derived proteins. It will be interesting to know the identity of these proteins and whether these can serve as a target for anti-CarP antibodies.

The molecular nature of the antigens recognized by ACPA has been identified more than 15-y ago by describing that citrulline is an essential constituent of antigens recognized by these RA-specific antibodies (16, 17). This finding has made considerable impact, as it has opened up the way to relevant and novel insights into RA-diagnosis and etiopathology (1). For example, ACPA are now part of the new American College of Rheumatology/ European League Against Rheumatism criteria for RA (18), and have been implicated in RA-pathogenesis, both in animal models (19–21) and in ex vivo human studies (22–25).

Importantly, the description of ACPA has led to the realization that RA constitutes at least two clinical syndromes that share many clinical features, but differ with respect to genetic background, predisposing environmental factors and clinical progression/remission (3, 4, 26–28). Although it is clearly too early to allow any firm conclusions, it is tempting to speculate that anti-CarP antibodies also contribute to disease pathogenesis and display diagnostic value, given the similar nature of the antigens recognized and their presence in ACPA-negative disease.

The presence of anti-CarP antibodies in anti-CCP2–negative disease is highly intriguing, as it could potentially represent a unique biomarker that positively identifies at least part of this manifestation of RA. To gain further insight into this possibility, it is important to establish whether the presence of anti-CarP antibodies is specific for RA or also found in other rheumatic diseases, as well as whether their presence predict the development of (ACPA-negative) RA in patients suffering from early unclassified RA and joint complaints, such as arthralgia.

To establish a cut-off to define a positive sample, we have analyzed the presence of IgG and IgA directed against Ca-FCS and FCS in sera of healthy controls. All samples were tested for reactivity toward Ca-FCS and FCS, and absorbance values were converted into arbitrary units per milliliter using an anti-CarP antibody-positive standard present on the same plate. Because sera from several individual subjects also displayed reactivity toward nonmodified FCS, we subtracted the "FCS-reactivity" from the reactivity toward Ca-FCS using arbitrary units per milliliter as defined by the standard curve. We subsequently calculated the cut-off as the mean plus two times SD and applied the cut-off to the data of the RA patients following a similar strategy. The disadvantage of this method is that a standard is used on Ca-FCS for the determination of arbitrary units per milliliter toward FCS, another antigenic entity. However, this method did allow the calculation of a specific response to the posttranslational modification.

Every method of establishing a cut-off has advantages and limitations. Therefore, we subsequently confirmed our observations using another strategy as well by calculating the cut-off as the mean plus two times SD of the anti-Ca- FCS response in controls. This cut-off was applied to the data of the RA patients as was also used before (29). The association with radiological progression of anti-CarP IgG in ACPA-negative RA remains significant, albeit with a lower level of significance ($p = 0{,}001$).

From a clinical perspective, the detection of anti-CarP antibodies in early arthritis could be highly rewarding because they predict a more severe disease course. Because early aggressive treatment in RA has been shown to prevent future damage (30, 31), the detection of anti-CarP antibodies might be beneficial to identify anti-CCP2–negative patients at risk

to develop severe disease. The identification of such patients might be important to guide treatment decisions early after onset of symptoms, especially in early arthritis patients that are difficult to classify.

In conclusion, in addition to the autoantibody system that recognizes citrullinated proteins (ACPA), an autoantibody system against carbamylated proteins (anti-CarP) is present in sera of RA patients. Detection of anti-CarP antibodies could offer new possibilities to identify patients at risk for a severe disease course.

Materials and methods

Generation of antigens

As we did not know whether antibodies against carbamylated proteins (anti-CarP) would be present in sera of rheumatoid arthritis (RA) patients, or which proteins they would recognize, we set out to study a diverse set of carbamylated proteins, to maximize the chances to detect as many of the anti-CarP reactivities. For this purpose we have used FCS (Bodinco) that was carbamylated, citrullinated, or left untreated. For generating carbamylated FCS (Ca-FCS), FCS was diluted in H2O to 4 mg/mL and potassium cyanate (Sigma) was added to a concentration of 1 M. Following incubation at 37 °C for 12 h, the sample was extensively dialyzed against H2O. Carbamylated fibrinogen (Ca-Fib) was generated by incubating 5 mg/mL fibrinogen (Fib) with 0.5 M potassium cyanate at 4 °C for 3 d followed by being extensively dialyzed against PBS. Citrullinated FCS (Ci-FCS) and citrullinated fibrinogen (Ci-Fib) was generated by incubation of 10 mg FCS or Fib in a volume of 1 mL containing 0.1M Tris-HCl pH 7.6, 0.15 M CaCl2, and 40 U PAD4 (Sigma) for 24 h at 37 °C. We have confirmed the presence of citrulline and homocitrulline residues using mass-spectrometry analysis. For Fib we observed, in the protein segments that we analyzed, extensive citrullination and complete carbamylation.

Detection of anti-CarP antibodies by ELISA

Nonmodified FCS and modified-FCS were coated at 10 µg/mL in 50 µL [diluted in pH 9.6 0.1 M carbonate-bicarbonate buffer (CB)] on Nunc Maxisorp plates (Thermo Scientific), overnight. Following washing in PBS containing 0.05% tween (Sigma) (PT), the plates were blocked by incubating 100 µL PBS/1% BSA (Sigma) for 6 h at 4 °C. Following additional washing, the wells were incubated with 50

µL serum at a 1/50 dilution in PBS/0.05% tween/1% BSA buffer (PTB) on ice overnight. All subsequent incubations are performed in PTB. As a standard, serial dilutions of a pool

of positive sera were used. Human IgG or IgA was detected using rabbit anti-human IgG antibody (Dako) or rabbit anti-human IgA antibody (Dako) incubated on ice for 3.5 h. Following washing, wells were incubated on ice for 3.5 h with HRP-labeled goat anti-rabbit IgG antibody (Dako). Following the last washings HRP enzyme activity was visualized using ABTS, as described previously (1). Sera of healthy subjects (n = 305) were used as controls. We transformed the absorbance on both Ca-FCS and FCS to arbitrary units per milliliter (aU/mL) and subtracted the background signal (aU/mL) of FCS from the signal (aU/mL) of Ca-FCS as to analyze the specific anti-CarP reactivity (Fig. 2). We established the cut-off for a positive response as the mean plus two times the SD of the specific anti-CarP reactivity of the healthy controls.

ELISA for fibrinogen

Nonmodified Fib Ci-Fib and Ca-Fib were coated at 20 μg/mL in 50 μL (diluted in pH 9.0 PBS) on Nunc

Maxisorp plates overnight. Following washing in PT, the plates were blocked by incubating 200 μL pH 9.0 PBS/2% BSA for 2 h at 4 °C. Following additional washing, the wells were incubated with 50 μL serum at a 1/50 dilution in RIA buffer (10 mM Tris pH 7.6; 350 mM NaCl; 1% TritonX; 0.5% Na-deoxycholate; 0.1% SDS) (Sigma) on ice for 3 h. All subsequent incubations are performed in RIA buffer. As a standard, serial dilutions of a pool of positive sera were used. Human IgG was detected using HRP-labeled rabbit anti human IgG antibody (Dako) incubated on ice for 2 h. Following the last washings, HRP enzyme activity was visualized using ABTS. We analyzed sera of 214 RA patients and 54 healthy subjects as controls. We transformed the absorbance on Fib Ci-Fib and Ca-Fib to arbitrary units per milliliter. We established the cut-off for a positive response as the mean plus two times the SD of the specific anti-CarP reactivity of the healthy controls. These assays were repeated three times showing the same data.

F(ab′)2 preparation

Total IgG from two anti-CarP–positive and two control sera were isolated via a HiTrap protein A HP column (GE Healthcare) following the protocol for the column provided by the manufacturer. F(ab′)2 fragments were generated from purified IgG samples using a F(ab′)2 Preparation Kit (Thermo Scientific) following the protocol provided by the manufacturer. We have verified the molecular nature of the intact IgG and the F(ab′)2 using Coomassie-stained SDS-PAGE gels. These F(ab′)2 were used in ELISA, as described above, now using either HRP-labeled rabbit anti human IgG, IgA, IgMκ, λ-antibody (antilight chain) (Dako), or HRP-labeled rabbit antihuman IgG (Dako).

Detection of ACPA by ELISA

Antibodies against citrullinated protein antigens (ACPA) were measured by the CCP2 ELISA (Immunoscan RA Mark 2; Eurodiagnostica). Samples with a value above 25 U/mL were considered positive according to the manufacturer's instructions. A small percentage of ACPA-positive RA patients may be outside the anti-CCP2 reactivity, and therefore both terms will be used to explicitly indicate what has been used in our analyses. ACPA reactivity toward Ci-FCS was detected using ELISA plates that were coated with Ci-FCS (50 µL per well 10 µg/mL) diluted with CB in the Nunc Maxisorp plates overnight at 4 °C. The plates were washed in PT followed by blocking with 100 µL PBS/1%BSA solution at 37 °C for 1 h. Following washing, sera were incubated at a 1/50 dilution in 50 µL PTB and incubated at 37 °C for 1 h. After washing, human IgA and IgG were detected as described above.

Detection of anti-CarP antibodies by western blot

FCS, Ca-FCS, and Ci-FCS were loaded onto 10% SDS-polyacrylamide gels and transferred onto Hybond-C Extra membranes (Amersham). Blots were incubated in blocking buffer (3% ELK Milk/PBS/0.05% Tween) for 1 h at room temperature, following washing with PT. The blots were subsequently incubated with 2.5 mL 1:500 diluted serum in blocking buffer for 1.5 h at room temperature. The sera were either ACPA-positive anti-CarP–negative or ACPA-negative anti-CarP–positive as determined by

ELISA. After three washes with PT, blots were incubated with 5 mL HRP-labeled rabbit anti-human IgG diluted in blocking buffer for 1 h at room temperature. Next, blots were washed and bound antibodies were visualized using enhanced chemiluminescence (Amersham).

Statistics of radiological progression

Association between anti-CarP antibodies positivity and radiographic progression was analyzed using the Statistical Package for the Social Sciences 17.0 as described earlier. P values below 0.05 were considered statistically significant. A multivariate normal regression analysis for longitudinal data were used with radiological score as response variable. This method analyses repeated measurements at once and takes advantage of the correlation between these measurements, which results in a more precise SE. Radiological scores were log-transformed to obtain a normal distribution. The rate of joint destruction over time was tested by an interaction of time with anti-CarP. The effect of time was assumed to be linear in the interaction term. The effect of time was entered as a factor in the model as well, allowing a mean response profile over time. Age, sex, and inclusion period as proxy for treatment

were included as correction variables in all analyses. In a separate analysis, the effect of anti-CarP antibodies was corrected for the effect of anti-CCP and rheumatoid factor.

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Recognition of citrullinated and carbamylated proteins by human antibodies: specificity, cross-reactivity and the "AMC-Senshu" method

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3

Abstract

Objectives

Anti-citrullinated protein antibodies (ACPA) play an important role in the diagnosis and prognosis of rheumatoid arthritis (RA). The anti-modified citrulline (AMC) ('Senshu') method is the most frequently used method to detect citrullinated proteins. Recently, we identified antibodies against carbamylated proteins (anti-CarP antibodies) and studied whether the 'AMC-Senshu' method and human antibodies could discriminate citrullinated and carbamylated proteins.

Methods

We analyzed the reactivity of the 'AMC-Senshu' method and human antibodies on western blots targeting citrullinated, carbamylated or non-modified fetal calf serum (FCS) and fibrinogen (Fib). The cross-reactivity of ACPA and anti-CarP antibodies in double positive sera were also examined via the inhibition assays and ACPA depletion columns.

Results

The 'AMC-Senshu' method strongly stained both citrullinated and carbamylated FCS and Fib but not the non-modified counterparts. There are sera which stained both citrullinated and carbamylated forms of Fib and sera stained only one form of modified Fib. In the inhibition assays, sera binding to Ca-Fib can be inhibited by Ci-Fib to various degrees whereas binding to Ci-Fib could only be inhibited by Ca-Fib to approximately 30%. After ACPA depletion, more than half of anti-CarP antibodies remained in the flow through in 5 out of 7 samples, confirming that also in double positive individuals two separate antibody families exist.

Conclusions

The 'AMC-Senshu' method can not differentiate citrullinated and carbamylated epitopes. However, human antibodies can partially differentiate between them. In light of the recently identified anti-CarP antibodies, the extent and nature of citrullination and carbamylation in the joint should be re-evaluated.

Introduction

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease mainly affecting synovial joints. The disease process is characterized by joint damage and bone destruction and may lead to severe disability and increased mortality. Around 20 years ago, anti-citrullinated protein antibodies (ACPA) were discovered in RA patients and are now established as an important diagnostic and prognostic marker.[1] Although its role in the pathogenesis of RA is not yet fully elucidated, evidence on a possible pathologic role is accumulating.[2-7] Identifying citrullinated antigens recognized by ACPA is not only essential for a better understanding of RA but may also open a new window for early intervention. The assay used most frequently to identify citrullinated proteins is the anti-modified citrulline (AMC) assay. This assay is based on chemically adding ureido group adducts to citrulline residues followed by detection with a specific antibody developed by Dr. Senshu.[8] This assay has brought insight into the understanding of RA since it was used to show the presence of citrullinated proteins in the target tissues.[9-17] An advantage of the "AMC-Senshu" antibody is that it recognizes citrullinated epitopes irrespective of the neighboring amino acids. Recently, we and others described a novel family of autoantibodies in RA patients, anti-carbamylated protein (Anti-CarP) antibodies, which target carbamylated epitopes instead of citrullinated epitopes.[18,19] Since citrulline and homocitrulline are very similar, we wished to verify whether this method could distinguish between citrulline and homocitrulline as well as to determine to which extent human autoantibodies can differentiate between these two antigenic entities. To address this question, we studied whether the "AMC-Senshu" antibody and selected human sera can differentiate citrullinated and carbamylated proteins.

Methods

Generation of antigens

In brief, citrullinated fetal calf serum (Ci-FCS) and citrullinated fibrinogen (Ci-Fib) as well as carbamylated FCS (Ca-FCS) and carbamylated fibrinogen (Ca-Fib) were generated via incubation with either peptidylarginine deiminase 4 (Sigma) or potassium cyanate (Sigma) and confirmed by mass-spectrometry as described before.[18]

Autoantibody assays and inhibition assays

ELISA for the detection of anti-CarP Fib and anti-CarP FCS was performed as previously described.[18] Anti-CCP2 reactivity was detected using commercial CCP2 assays (Eurodiagnostica).[18] For the inhibition assys, four sera were incubated with Fib, Ci-Fib or Ca-Fib at 4°C overnight before detecting binding to Ci/Ca-Fib.

Coomassie blue staining and western blot (WB)

FCS, Ci-FCS, Ca-FCS, Fib, Ci-Fib and Ca-Fib were loaded with equal amounts onto 10% SDS-polyacrylamide gels and stained by SimplyBlue™ SafeStain staining (Life technologies) following the protocol from the manufacturer. The same gel was prepared and transferred onto Hybond-C Extra membranes (Amersham).

Selected human sera and the "AMC-Senshu" antibody were used to stain the blots. Ci-Fib positive/Ca-Fib negative, Ca-Fib positive/Ci-Fib negative and double positive sera were selected from previously performed anti-CarP Fib ELISA's. The protocol using these 3 sera to stain the Fib, Ci-Fib and Ca-Fib blots is the same as previously described.[18] Staining using the "AMC-Senshu" antibody (Millipore) followed the protocol in the kit. In brief, before applying the AMC antibody and its detection antibody, the blot was incubated with 2,3-butanedione monoxime and antipyrine in strong acidic environment to form ureido group adducts.

ACPA depletion/purification column

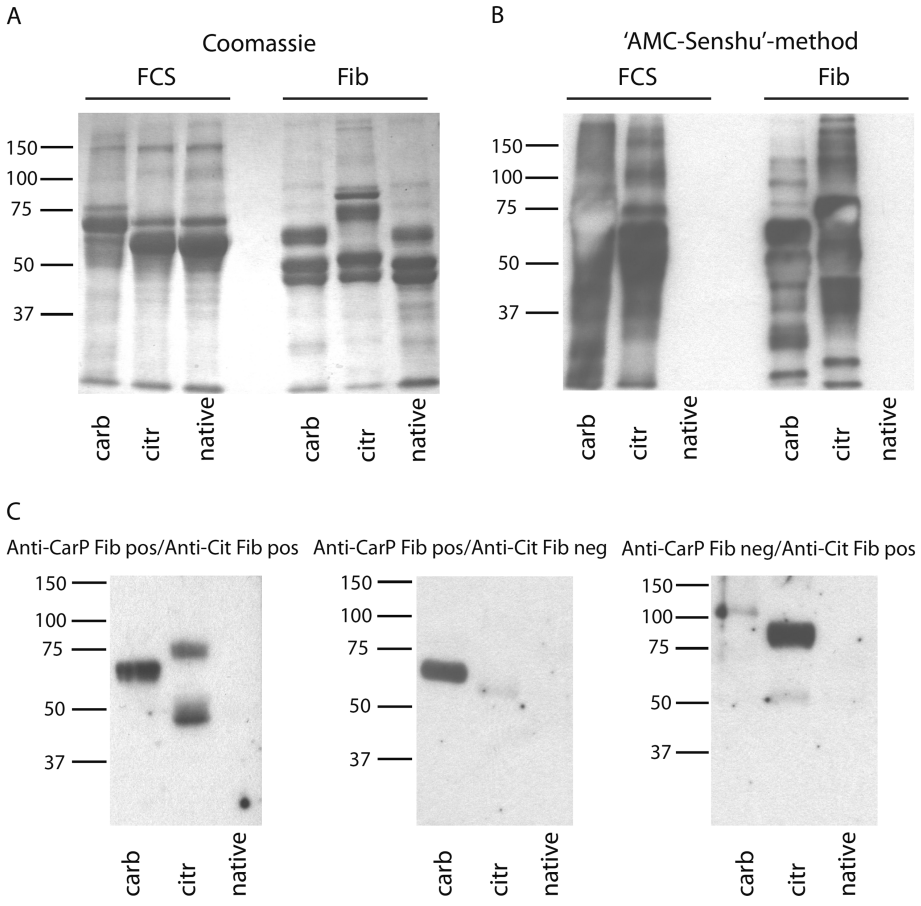
Biotinlylated CCP2 peptides or the arginine control were loaded onto 1ml HiTrap Streptavidin HP Columns (GE healthcare). One column containing the CCP2 arginine peptide was placed on top of two columns containing CCP2 peptides. After washing, ACPA/anti-CarP double positive sera were applied to the columns. Following washing the column was eluted by pH 2.5, 0.1 M glycine-HCl. The eluted antibodies were neutralized by 1M Tris, pH 8. The starting material, flow through and elution were tested on anti-CarP FCS and CCP2 ELISA.

Results

The "AMC-Senshu" method does not discriminate between citrullinated and carbamylated proteins

One gel was used to visualize equal loading of citrullinated, carbamylated or non-modified FCS or Fib (Figure 1A). The other equally loaded gel was used for Western-blotting and the resulting blot was used for the "AMC-Senshu" staining. Development of this blot revealed that both the citrullinated and the carbamylated forms of both FCS and Fib were strongly stained whereas the non-modified proteins did not reveal any staining (Figure 1B). These data indicate that the "AMC-Senshu" method identifies both citrulline and homocitrulline containing proteins and that it does not discriminate between citrullination and carbamylation.

Figure. 1 The "AMC-Senshu" method does not discriminate citrullinated and carbamylated antigens while human autoantibodies do. (A) Coomassie blue staining showed equal loading of FCS, Ci-FCS, Ca-FCS, Fib, Ci-Fib and Ca-Fib. (B) The "AMC-Senshu" antibody used according to the protocol of the manufacturer did not recognize FCS and Fib, but strongly recognized Ci-FCS, Ca-FCS, Ci-Fib and Ca-Fib. (C) Three selected RA sera can recognize both Ci-Fib and Ca-Fib or only one of the modifications specifically.



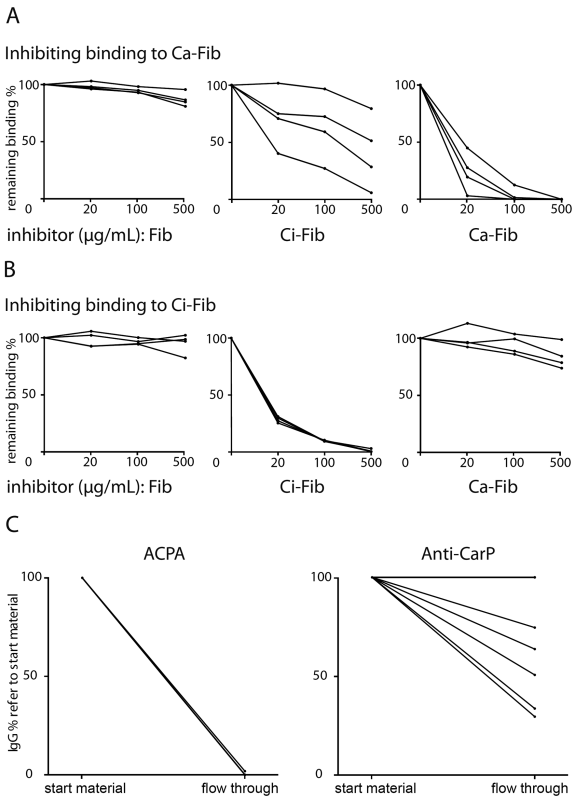
Human autoantibodies can discriminate between citrullinated and carbamylated antigens

To analyze whether human antibodies can actually discriminate between citrullinated and carbamylated proteins we applied sera of selected RA patients to stain blots containing Ci-Fib, Ca-Fib and Fib. Staining similar western blots with selected human sera revealed that sera positive for 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 (Fig 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 distinguish.

In the sera analyzed in the inhibition assays, 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% (Fig 2A, 2B). These data indicate that part, but not all ACPA and anti-CarP antibodies are cross-reactive.

Figure 2 Anti-CarP antibodies and ACPA represent two families of autoantibodies (A) Inhibition studies on sera double positive for ACPA and anti-CarP antibodies. 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 were depleted from the sera while more than 50% of anti-CarP antibodies remained in 5 out of 7 samples.



We have shown previously that anti-CarP antibodies can be found in a subgroup of ACPA negative individuals but that the majority of anti-CarP positive individuals are also ACPA positive. We have shown above that in double positive individuals two separate reactivities exist by performing pre-incubation experiments. We have now verified this aspect further by studying whether in double positive individuals anti-CarP reactivity would remain after removal of ACPA using CCP2 columns. After ACPA depletion, more than 98% of ACPA in the sera were depleted (Figure 2C) while more than half of the anti-CarP antibodies remained in the flow through in 5 out of 7 samples (Figure 2C). Together, these data confirm that two separate autoantibody systems exist directed against citrullinated or carbamylated antigens. Nonetheless, in double positive individuals, there appears to be a cross-reactive portion as well as two mono-specific portions.

Discussion

To answer the question whether the "AMC-Senshu" method and human autoantibodies can distinguish citrullination from carbamylation, we performed western blots using the "AMC-Senshu" system and human sera. We found the "AMC-Senshu" method can not differentiate citrullination and carbamylation. Our finding is in line with a previous report suggesting that the AMC-Senshu method also detects homocitrulline.[20,21]

Importantly, our experiments showed that certain human sera can recognize either citrullinated or carbamylated proteins but not both. Since anti-CarP antibodies and ACPA are often found together we analyzed whether in double positive individuals two separate reactivities co-exist or that this only reflects cross-reactivity. By depletion of ACPA, we showed that double positive samples harbor anti-citrullinated epitope-specific antibodies, anti-carbamylated epitopes specific antibodies as well as cross-reactive antibodies.

In this study, we found that the "AMC-Senshu" method can recognize both citrullinated and carbamylated proteins. This finding does not argue against the notion that citrullinated proteins are present in the synovial fluid and tissues. Especially since in a number of studies, citrullinated proteins were first detected by the "AMC-Senshu" method and then further confirmed by mass-spectrometry fingerprinting.[9-12] However, our findings highlight that in these studies next to citrullinated proteins also carbamylated proteins may have been detected. In light of the recently identified anti-CarP antibodies, the extent and nature of citrullination and carbamylation in the joint should be re-evaluated.

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Anti–carbamylated protein antibodies are present in arthralgia patients and predict the development of rheumatoid arthritis

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4

Abstract

Objective

Recently, we discovered a new autoantibody system in rheumatoid arthritis (RA): anti–carbamylated protein (anti-CarP) antibodies. These antibodies have value in predicting joint destruction; however, it is not clear whether they are present before the diagnosis of RA and whether they have value as predictors of RA development. Therefore, we studied whether anti-CarP antibodies are present in patients with arthralgia and whether their presence is associated with the development of RA.

Methods

Sera from 340 arthralgia patients who did not have clinical signs of arthritis but who were positive for IgM rheumatoid factor (IgM-RF) and/or anti–cyclic citrullinated peptide 2 (anti–CCP-2) and 32 healthy controls were tested for anti-CarP IgG antibodies. Of the patients with arthralgia, 111 were IgM-RF positive/anti–CCP-2 antibody negative and 229 were anti–CCP-2 antibody positive. Patients were observed for the development of RA (based on the 2010 American College of Rheumatology/European League Against Rheumatism classification criteria) during a median followup period of 36 months. Cox proportional hazards regression analysis was performed to compare the risk of developing RA between arthralgia patients who were positive for anti-CarP antibodies and those who were negative for anti-CarP antibodies during followup.

Results

Anti-CarP antibodies were present in the sera of 39% of the patients. One hundred twenty patients developed RA, after a median of 12 months (interquartile range [IQR] 6–24). The presence of anti-CarP antibodies was associated with the development of RA in the entire arthralgia cohort after correction for RF and anti–CCP-2 antibody status (hazard ratio 1.56 [95% confidence interval 1.06–2.29], p = 0.023), as well as in the anti–CCP-2 antibody–positive subgroup (odds ratio 2.231 [95% confidence interval 1.31–3.79], p = 0.003).

Conclusion

Anti-CarP antibodies are present in patients with arthralgia, and their presence predicts the development of RA independent of anti–CCP-2 antibodies.

Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disorder. The disease process often causes the destruction of joints, which can lead to considerable disability. Autoantibodies such as rheumatoid factor (RF) and anti–citrullinated protein antibodies (ACPAs) are important diagnostic markers for RA and may also contribute to pathogenesis (1). ACPA-positive patients with RA have more radiologic damage and a lower chance of attaining disease remission without disease-modifying antirheumatic drugs (DMARDs) than ACPA-negative patients with RA (2–5). In pre-RA states such as arthralgia and undifferentiated arthritis, ACPA and RF are predictive factors for future progression to RA (6, 7).

Recently, we discovered another autoantibody system present in RA patients, which we designated as anti–carbamylated protein antibodies (anti-CarP) (8). These antibodies target carbamylated proteins rather than citrullinated proteins. Carbamylation is a process in which lysines are converted into homocitrullines under the influence of cyanate. Homocitrulline is an amino acid that highly resembles citrulline. Cyanate can be formed in low concentrations from urea under physiologic conditions or it can originate from the environment, e.g., from car fumes. In inflammatory conditions, it can be formed from thiocyanate that has been catalyzed by myeloperoxidase released by, e.g., activated neutrophils. Whether anti-CarP antibodies are directly involved in the pathogenesis of RA is currently unknown.

In a previous article, we reported that anti-CarP antibodies are present in both ACPA-positive patients (74%) and ACPA-negative patients (16%) with RA (8). In RA patients, they are a prognostic factor for a higher likelihood of joint destruction independent of ACPA status. However, at present it is unknown whether they exist in patients who have arthralgia but do not meet criteria for RA and whether they could have predictive value in those patients. Therefore, we tested for the presence of anti-CarP antibodies and studied the association between anti-CarP antibody status and levels and the risk of developing RA in a cohort of patients with arthralgia who were positive for ACPA (determined based on levels of anti–cyclic citrullinated peptide 2 [anti–CCP-2] and/or RF).

Patients and methods

Study population

The inclusion procedure was as previously described (6). Briefly, 340 Caucasian patients from the Amsterdam area, who did not have arthritis but who were positive for anti–CCP-2 antibody and/or IgM-RF and had a history of arthralgia, were included. Absence of arthritis

was confirmed by physical examination of 44 joints by a trained physician and a senior rheumatologist (DvS) (9). Medical history, details of joint symptoms, and the number of tender joints were recorded (10). Patients who had arthritis as determined by chart review or baseline physical examination, were negative for anti-CCP antibodies and IgM-RF on second analysis, had previously been treated with DMARDs, or had recently been treated with glucocorticoids (within the last 3 months) were excluded. Patients were followed up semiannually in the first year and yearly thereafter for the development of RA according to the 2010 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification criteria (1). Additional visits were scheduled if RA developed. Healthy control sera were collected from 32 Caucasian residents of the Leiden area. Protocols were approved by the local ethics committee, and informed consent was obtained from all subjects.

Anti-CarP IgG antibody enzyme-linked immunosorbent assay (ELISA)

Anti-CarP IgG antibodies in the sera from patients and controls were detected by ELISA as previously described (8). Briefly, Nunc MaxiSorp plates (Thermo Scientific) were coated with 10 μg/ml fetal calf serum (FCS; Bodinco) and carbamylated (Ca)–FCS at 4°C overnight. The plates were blocked with 1% bovine serum albumin (Sigma) at 4°C for 6 hours, followed by incubation overnight with 1:50 diluted sera on ice. Bound antibodies were detected by incubation for 4 hours with horseradish peroxidase–conjugated rabbit anti-human IgG (Dako) on ice and subsequently visualized with ABTS. Absorbance was measured at 415 nm and transformed to arbitrary units (AU) per milliliter using the titration curve of a serum pool from 3 anti-CarP antibody–positive samples. The background signal of FCS was subtracted from the signal of Ca-FCS to analyze the specific anti-CarP antibody reactivity. Sera with a level of >202 AU/ml were considered positive for anti-CarP antibodies. This cutoff was equivalent to 2 SD above the mean in the healthy controls.

Statistical analysis

Statistical analysis was performed using SPSS version 17.0 software. Chi-square test, t-test for independent samples, binary logistic regression, and Cox proportional hazards regression analysis were used to compare anti-CarP antibody–positive and anti-CarP antibody–negative groups in the whole population and in the anti–CCP-2 antibody–positive and –negative populations. Binary logistic regression analysis was performed to analyze the association between the anti-CarP IgG antibody level and the risk of developing RA in the anti-CarP IgG antibody–positive subgroup. Hazard ratios (HRs), odds ratios (ORs), and their 95% confidence intervals (95% CIs) were calculated. P values less than 0.05 were considered significant.

Results

The 340 patients with arthralgia included in this study were followed up for a median of 36 months (interquartile range [IQR] 20–52). Baseline characteristics are listed in Table 1. After a median of 12 months (IQR 6–24), 120 patients (35%) developed RA according to the 2010 ACR/EULAR criteria. At the time of diagnosis of RA, these patients had a median swollen joint count of 3 (IQR 2–5). Of the remaining 220 patients, 9 patients developed undifferentiated arthritis.

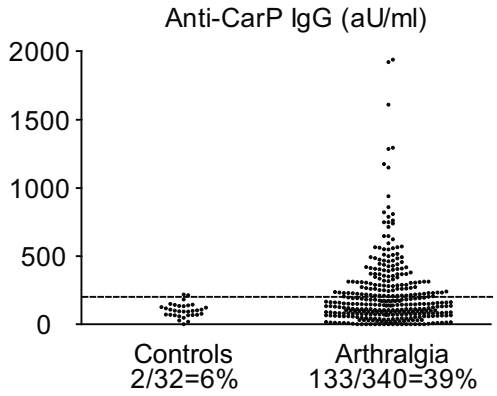
Table 1. Baseline characteristics of the 340 patients with arthralgia*

Age, mean ± SD years	49 ± 11
Sex, no. (%) female	256 (75)
Duration of symptoms, median (IQR) months	12 (8–36)
No. of reported painful joints, median (IQR)	3 (1–8)
Tender joint count, median (IQR)	0 (0–2)
IgM-RF positive, no (%)	209 (62)
Anti–CCP-2 positive, no (%)	229 (67)
Followup time, median (IQR) months	36 (20–52)

*IQR = interquartile range; IgM-RF = IgM rheumatoid factor; anti–CCP-2 = anti–cyclic citrullinated peptide 2.

One hundred thirty-three patients (39%) were positive for anti-CarP antibodies (Figure 1). Of these patients, 68 (51%) developed RA, whereas 52 patients (25%) who were negative for anti-CarP antibodies developed RA. Anti-CarP antibody positivity was significantly associated with RA development ($p < 0.001$). In the group of arthralgia patients positive for anti-CarP antibodies, the levels of anti-CarP IgG antibodies were not associated with the risk of developing RA ($p = 0.215$).

Figure 1. Presence of anti–carbamylated protein (anti-CarP) antibodies in patients with arthralgia and in controls. Anti-CarP IgG antibody positivity was found in 6% of healthy controls and 39% of patients with arthralgia. Each data point represents a single subject. The horizontal dashed line shows the cutoff for positivity.



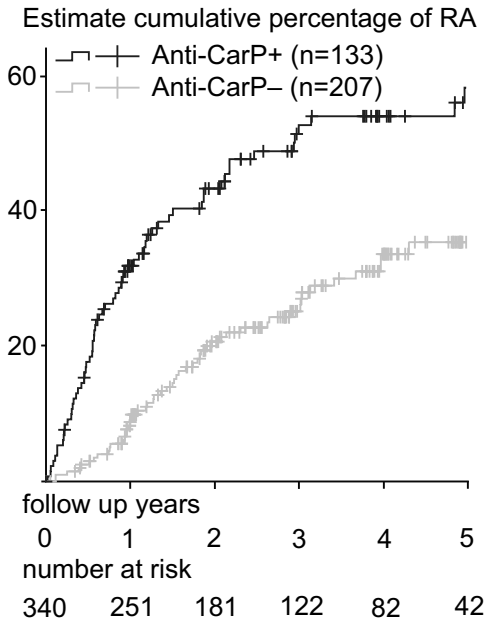
Among 111 anti–CCP-2 antibody–negative patients, 17 (15%) were positive for anti-CarP antibodies, while among 229 anti–CCP-2 antibody–positive patients, 116 (51%) were positive for anti-CarP antibodies ($p < 0.001$). Given this association, we next studied whether anti-CarP antibody positivity is also an independent predictor of RA development in the anti–CCP-2 antibody–positive and anti–CCP-2 antibody–negative subgroups. In the anti–CCP-2 antibody–positive subgroup, 68 patients (58%) who were positive for anti-CarP antibodies developed RA, while only 44 patients (40%) who were negative for anti-CarP antibodies developed RA. The association between anti-CarP antibodies and RA remained significant (OR 2.231 [95% CI 1.31–3.79], $p = 0.003$), while this was not the case in the anti–CCP-2 antibody–negative subgroup (OR 1.12 [95% CI 0.22–5.63], $p = 0.891$).

Anti-CarP antibody–positive patients also displayed higher anti-CCP antibody levels as compared to anti-CarP antibody–negative patients ($p < 0.001$). Similarly, after correction for anti-CCP antibody levels, anti-CarP antibody positivity still increased the risk of developing RA in anti-CCP antibody–positive arthralgia patients ($p = 0.032$). Unlike anti–CCP-2 antibodies, the presence of anti-CarP antibodies was not correlated with IgM-RF ($p = 0.391$).

Taking into account the differences in followup time, Cox proportional hazards regression analysis revealed a statistically significant association between anti-CarP antibody status and the risk of developing RA. This indicates that anti-CarP antibody–positive patients not only were more likely to develop RA, but also were more likely to develop RA within a shorter time frame, with an HR of 2.53 (95% CI 1.76–3.63, $p < 0.001$). This association

remained significant after correction for anti–CCP-2 antibody status and IgM-RF status (HR 1.56 [95% CI 1.06–2.29], p = 0.023) (Figure 2) or after correction for anti–CCP-2 antibody levels and IgM-RF status (p < 0,001).

Figure 2 Association of presence of anti–carbamylated protein (anti-CarP) antibodies in arthralgia patients with future development of rheumatoid arthritis (RA). Anti-CarP IgG antibodies are associated with a higher risk of developing RA after correction for anti–cyclic citrullinated peptide 2 antibody and rheumatoid factor status (hazard ratio 1.56 [95% confidence interval 1,06–2,29], p = 0,023).



Discussion

Although arthralgia patients often have a benign disease course, in a certain subset of these patients the condition may progress to RA. Identifying this subset at an early stage may be beneficial because intervention at this stage might prevent the development of RA. As an established biomarker, the presence of ACPA increases the risk of developing arthritis in patients with arthralgia. Still, the condition progresses to arthritis in only 27% of all ACPA-positive patients with arthralgia after 1 year of followup (6). In the present study we investigated whether anti-CarP antibodies are present in arthralgia patients and whether they are an additional risk factor for RA in these patients. We demonstrated that anti-CarP antibodies are present in arthralgia patients and that they are associated

with a higher risk of developing RA independent of ACPA and IgM-RF status. Within the anti–CCP-2 antibody–negative subgroup, we did not observe a significant association between anti-CarP antibodies and RA, possibly due to the low number of RA cases in this group.

Limited by the nature of the cohort, we were unable to address the question of whether anti-CarP antibodies can predict the development of RA in arthralgia patients who are negative for anti–CCP-2 antibodies and RF. Another limitation was the 3-year median followup time, which is relatively short and may have affected the percentage of patients developing RA. However, we observed that with increasing followup time the percentage of patients with arthralgia who develop RA decreases. Therefore, we believe that this effect will be limited. A further concern could be that these patients might have subclinical arthritis at baseline, undetected by physical examination. However, we have previously seen that the frequency of pathology as determined on ultrasound was very low in this population and moreover, that ultrasound was not superior to physical examination in the prediction of RA (11).

Our findings suggest that not only ACPA positivity, but also the presence of anti-CarP antibodies, can have clinical value in the prediction of RA in patients with arthralgia. Additionally, the presence of anti-CarP antibodies in persons at risk of developing RA provides a rationale for further studies on their potential pathogenetic properties. Although the presence of anti-CarP antibodies is associated with the risk of developing RA in ACPA-positive arthralgia patients, we previously did not obtain evidence that their presence is associated with radiologic progression in ACPA-positive patients with RA; such an association was only found in ACPA-negative RA patients (8). The reasons for these findings are not yet known and further replication is required; however, these results do resemble observations made in studies on ACPA fine specificity (12–14). The ACPA recognition profile does not correlate with radiologic progression in ACPA-positive RA (12), but the number of citrullinated epitopes recognized by ACPA is associated with RA development in patients who have arthralgia or undifferentiated arthritis (13, 14). Apparently, in the first stage of disease, the number of epitopes recognized and isotypes used by ACPA (5), as well as the number of autoantibodies present, are determining factors for disease progression. They matter less, however, when a certain threshold has been passed, possibly explaining the lack of association in established RA.

Despite the similarity between the presence of anti-CarP antibodies and the broadening of ACPA fine specificities with respect to prediction of RA, anti-CarP antibodies are not a fine specificity of ACPA, since they are largely non–cross-reactive with defined (homo) citrullinated antigens (15). Indeed, the effect of anti-CarP antibodies in arthralgia patients

as described herein is still present after correction for the effect of anti–CCP-2 antibodies, as would be expected for two independent autoantibody systems.

Taken together, our data reveal that anti-CarP antibodies are present before RA becomes clinically apparent, since they can be found in patients who have arthralgia without signs of arthritis. Furthermore, their presence in this population is associated with the development of RA.

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Anti-carbamylated protein (anti-CarP) antibodies precede the onset of rheumatoid arthritis

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Abstract

Objective

The presence of anti-citrullinated protein antibodies (ACPA) and IgM-rheumatoid factor (IgM-RF) years before the clinical diagnosis of rheumatoid arthritis (RA) suggests they are possibly involved in the pathogenic process underlying RA. In this study, we analysed whether anti-carbamylated protein (anti-CarP) antibodies, a novel autoantibody system against carbamylated proteins, can also be detected in healthy individuals before they developed RA.

Methods

Multiple sera from asymptomatic blood donors prior to the onset of their RA symptoms and sera from age-matched and sex-matched controls were tested for the presence of antibodies directed against carbamylated-fetal calf serum (Ca-FCS), carbamylated-fibrinogen (Ca-Fib), cyclic citrullinated-peptide 2 and IgM-RF.

Results

Anti-Ca-FCS and anti-Ca-Fib antibodies were each present in 27% and 38% of the last serum samples of blood donors prior to the diagnosis of RA. Both anti-Ca-FCS and anti-Ca-Fib antibodies could be detected many years before the onset of RA. Anti-CarP antibodies as well as ACPA are, on average, detected earlier than IgM-RF.

Conclusions

In addition to ACPA and IgM-RF, also the newly identified anti-CarP antibodies appear many years before the diagnosis of RA.

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease primarily affecting the synovial joints.[1] Early and aggressive intervention in individuals who are developing RA may prevent irreversible bone loss and induce early remission.[2] Identification of individuals who could benefit most from such an intervention is a challenge, but the presence of autoantibodies could be a useful marker. Besides IgM-rheumatoid factor (IgM-RF) also anti-citrullinated protein antibodies (ACPA) are implicated in the disease progression of RA[3] and are now included in the 2010 American College of Rheumatology (ACR)/ The European League Against Rheumatism criteria for RA.[4] Both ACPA and RF can be detected in serum many years prior to the onset of symptoms.[5–7] Recently, we identified a novel autoantibody family consisting of anti-carbamylated protein (anti-CarP) antibodies. [8] Carbamylation is a post-translational modification in which lysines are chemically converted to homocitrullines.[9] The presence of anti-CarP antibodies is associated with more radiological progression over time in ACPA-negative RA patients [8] and with the conversion from arthralgia to RA.[10]

In this study, we have analysed whether anti-CarP antibodies are already present in healthy individuals before the diagnosis of RA and how the appearance of anti-CarP antibodies relates to the appearance of ACPA and IgM-RF.

Methods

Study population

Sera from 79 asymptomatic blood donors (48 women), with a mean age at diagnosis of 51 years, collected before they developed RA as well as from 141 age-matched and sex-matched controls were obtained as described before.[6,11]

In brief, sera of patients who fulfilled the ACR 1987 criteria for RA12 obtained before the diagnosis of RA were collected from the Sanquin Blood Bank. A median of 5 (IQR 4–6) sequential sera from blood donors obtained at 1–6-year intervals were available for analysis. The clinical information of patients was retrieved from medical records. Sera of 141 sex-matched and age-matched healthy blood donors collected in the same period as the samples of the individuals who developed RA were also collected from the Sanquin Blood Bank. All patients and controls are Caucasian. Informed consent was obtained from the participating RA patients. The study was approved by the Slotervaartziekenhuis and Reade ethical review board, Amsterdam, The Netherlands, with the condition, however, that we were not allowed to contact the control donors for reasons of privacy.

Antibody detection

Sera collected on the last visit prior to the diagnosis of RA (median time before diagnosis: 1,4 years, IQR: 0,8–1,8 years) were measured for the presence of anti-carbamylated-fetal calf serum (anti-Ca-FCS), anti-carbamylated-fibrinogen (anti-Ca-Fib), anti-cyclic citrullinated-peptide 2 (anti-CCP2) antibodies and IgM-RF. Next, of individuals positive for at least one autoantibody, all consecutive samples were analysed.

Anti-Ca-FCS and anti-Ca-Fib antibodies were detected by ELISA as described before.8 The anti-CCP2 ELISA (Euro-Diagnostica) was performed following the manufacturer's instructions. The IgM-RF ELISA was performed as previously described.[6,11] The cut-off for the anti-Ca-FCS and anti-Ca-Fib ELISA was set as the mean plus two times the SD of healthy controls.

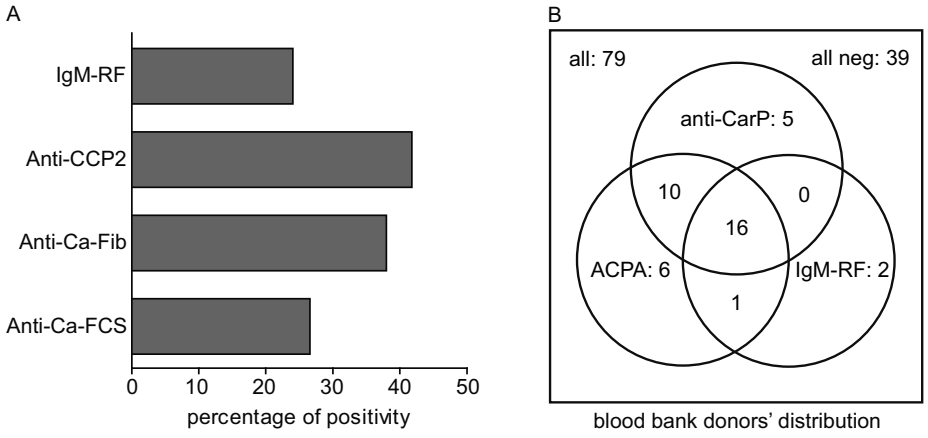
Statistics

Mann–Whitney U test (one-tailed) was performed to compare the time points before the diagnosis of RA when the different autoantibodies were detectable for the first time.

Results

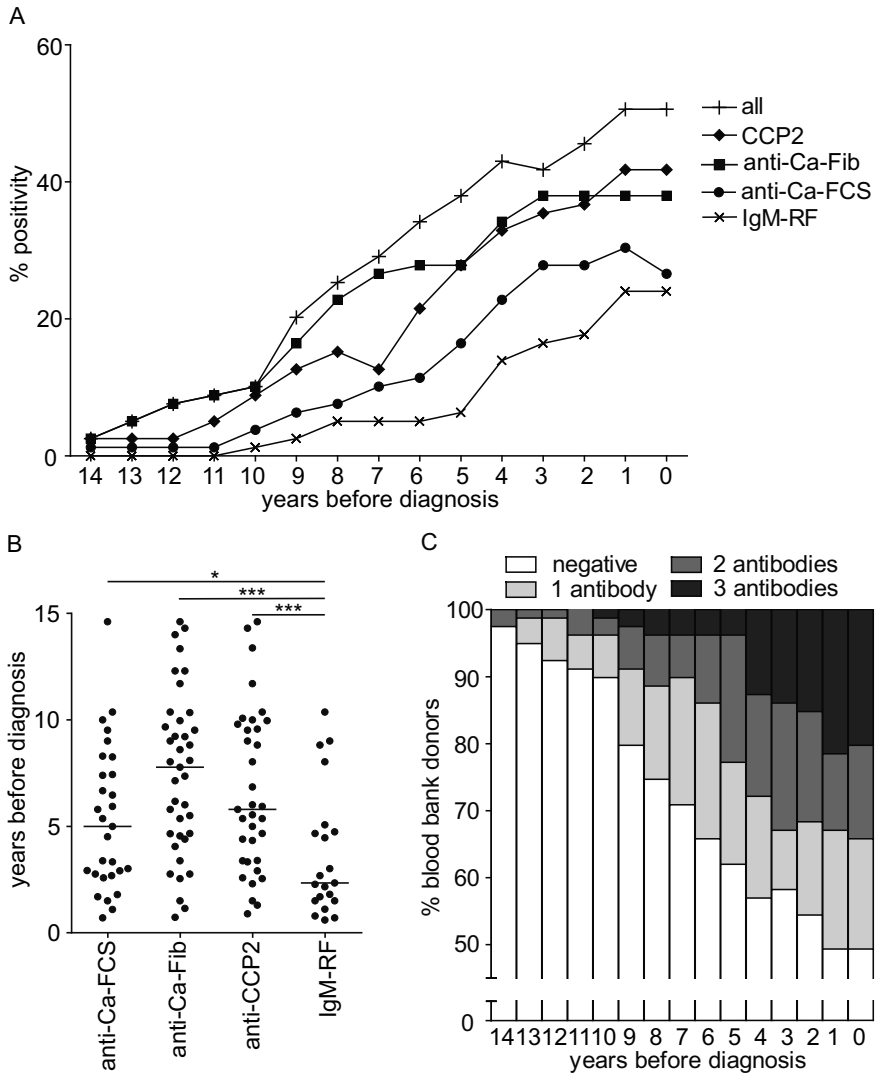
The characteristics of the 79 asymptomatic blood donors who developed RA later used in this study have been described previously.[6,11] Sera collected on the last visit prior to the diagnosis of RA were analysed for the presence of anti-Ca-FCS, anti-Ca-Fib, anti-CCP2 antibodies and IgM-RF. At the time of diagnosis, the patients reported a 0.9-year median duration of joint symptoms. Twenty-one (26.6%) donors were positive for anti-Ca-FCS antibodies, 30 (38,0%) for anti-Ca-Fib antibodies, 33 (41.8%) for anti-CCP2 antibodies and 19 (24,0%) for IgM-RF (figure 1A). The numbers of single-positive, double–positive and triple-positive donors are listed in figure 1B.

Figure 1 Anti-carbamylated protein (Anti-CarP) antibodies are present in asymptomatic blood donors before they developed rheumatoid arthritis (RA) (A) Anti-carbamylated-fetal calf serum (anti-Ca-FCS), anti-carbamylated-fibrinogen (anti-Ca-Fib), anti-cyclic citrullinated-peptide 2 (anti-CCP2) antibodies and IgM-rheumatoid factor (IgM-RF) are each present in 27%, 38%, 42% and 24% of the serum samples taken from donors most recently before their diagnosis of RA. (B) The absolute numbers of single-positive, double-positive, triple-positive and autoantibody-negative donors are listed. ACPA, anti-citrullinated protein antibodies.



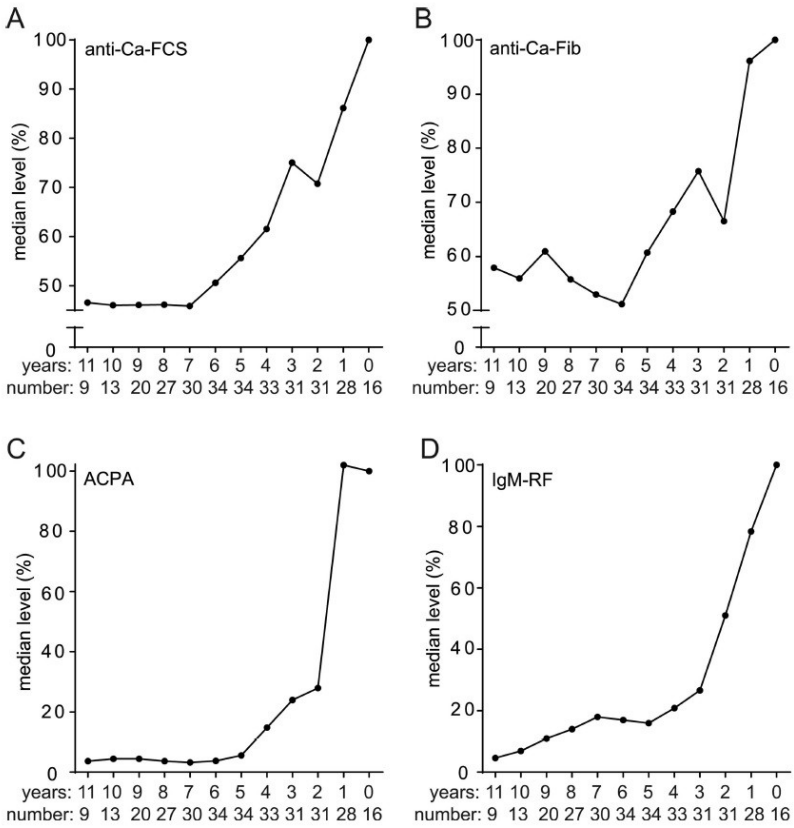
Analysis of the longitudinal samples from the donors who were positive for at least one autoantibody at the last visit prior to the diagnosis of RA revealed that similar to ACPA and IgM-RF also anti-Ca-FCS and anti-Ca-Fib antibodies can be detected years before the diagnosis of RA (figure 2A). There is a steady increase in the percentage of individuals with a positive antibody status for each of the autoantibodies studied over time. The moment when IgM-RF, anti-Ca-FCS, anti-Ca-Fib and anti-CCP2 antibodies were detectable for the first time were 10, 14, 14 and 14 years before diagnosis, respectively (figure 2A,B). These are the time points when the first samples of these donors were collected. The median (IQR) of time points when anti-CCP2, anti-Ca-FCS, anti-Ca-Fib antibodies and IgM-RF were detectable were 6 (3–10), 5 (3–7), 7 (4–10) and 2 (1–5) years before the diagnosis (figure 2B). Comparing the time points when each of these three autoantibody families could be detected for the first time in this cohort revealed that anti-CarP and anti-CCP2 antibodies appeared around the same time, while IgM-RF appeared significantly later (figure 2B). We observed an increase in the number of autoantibody reactivities present in the samples over time. Six years before the diagnosis, more than 50% of the autoantibody-positive donors harboured only one autoantibody family, whereas at least 30% of the autoantibody-positive donors displayed all three autoantibody families within 4 years before diagnosis (figure 2C).

Figure 2 The development of autoantibodies over time (A) The percentage of autoantibody-positive donors increases over time. (B) The first time point that IgM-rheumatoid factor (IgM-RF) appeared is significantly later than anti-cyclic citrullinated-peptide 2 (anti-CCP2) and anti-CarP antibodies reacting with Ca-FCS and with Ca-Fib. (C) The percentages of blood bank donors that harbour 0, 1, 2 or 3 autoantibody families are calculated. The number of autoantibody-positive donors and the number of recognised autoantibodies of autoantibody-positive donors increase over time.



Next to positivity, the levels of autoantibodies increased within the majority of followed blood donors. The levels of anti-CCP2, anti-Ca-FCS, anti-Ca-Fib antibodies and IgM-RF levels each increased in 83% (33/40), 78% (31/40), 53% (21/40) and 85% (34/40) of donors. The median levels of all autoantibodies start to increase around 5–7 years before the diagnosis or RA (figure 3).

Figure 3 The median levels of autoantibodies increase over time Median level of each autoantibody was calculated for every year. The median levels at time point 0 of all autoantibodies were set at 100%. The median levels of other time points were presented as the percentage of the median level of time point 0. The number below the x-axis is the number of all available samples at the given time points. Time points before 10 years were not shown since the total sample number is too low. (A) Anti-Ca-FCS, (B) Anti-Ca-Fib, (C) anti-cyclic citrullinated-peptide 2 (anti-CCP2) and (D) IgM-rheumatoid factor (IgM-RF).



Discussion

ACPA and IgM-RF have been reported to be present many years before the onset of the clinical symptoms of RA.[5–7] Here, we observed that also anti-CarP antibodies are present before the onset of the clinical symptoms of RA. The moment of first appearance of anti-CarP antibodies is comparable with ACPA and earlier than IgM-RF. Levels of each of these autoantibodies appear to increase with similar kinetics. From the current data, it is not possible to conclude on the relative contribution of each of these antibodies on the pathogenesis of RA.

Incidences and levels of anti-CCP2 antibodies and IgM-RF in this report are slightly different from previous reports [6,11] on this cohort, because of a different approach in patient follow-up. In the present report, only donors who had a positive test in the last sample before the diagnosis of RA were followed, while previous reports mentioned cumulative percentages of positivity in all samples.

Homocitrulline is rather similar to citrulline but is one methylene group longer. Nonetheless, anti-CarP antibodies and ACPA appear by and large as two different autoantibody systems as anti-CarP antibodies are present in both ACPA-positive and ACPA-negative RA patients. In addition, inhibition studies, ACPA depletion studies and western blot analyses have revealed that next to a 'cross-reactive' component both antibody families have components recognising only citrulline-containing or homocitrulline-containing proteins/peptides. [8,13] Compared with anti-Ca-FCS antibodies, anti-Ca-Fib antibodies may have more cross-reactivity to citrullinated proteins.[13] In this study, we observed five blood bank donors who were ACPA negative but positive for either anti-Ca-FCS [1] or anti-Ca-Fib antibodies [4]. We also observed six donors who were only positive for ACPA. Furthermore, ACPA and anti-CarP antibodies do not appear at the same moment in individuals as in 26 double-positive donors, 13 donors developed anti-CarP antibodies first, 16 donors developed both at the same time and 6 donors developed ACPA first. Collectively these observations strengthen the concept that next to a cross-reactive component also non-cross-reactive antibodies exist. Because of the limited sample size of 79 individuals, replication studies in comparable cohorts are warranted.

Since autoantibodies can be detected many years before the onset of clinical symptoms, it is tempting to speculate that these antibodies may contribute to the pathogenesis of RA, for instance, by inducing osteoclastogenesis, tumour necrosis factor (TNF)-α secretion by macrophages or complement activation.[14–16] Our finding that anti-CarP antibodies can be present years before diagnosis adds another source of antibodies, which can potentially contribute to RA pathogenesis. Several genetic and environmental factors, such as shared epitope, PTPN22 and smoking, have been reported to associate with ACPA-positive RA

as recently reviewed.[17] It would be interesting to know whether similar risk factors also predispose to anti-CarP antibodies.

The assays used to detect ACPA, anti-CarP antibodies and IgM-RF may differ in definition of cut-off and sensitivity, potentially limiting the comparability. Interestingly, IgM-RF seems to occur pathogenically subsequent to ACPA or anti-CarP antibodies. Observations made in American-Indian RA patients and their first-degree relatives may support this hypothesis.[18] These studies revealed that although either anti-CCP antibodies or IgM-RF can be found in both patients and their healthy first-degree relatives, the combination of both anti-CCP and RF is predominantly found in the RA patients. This supports the notion that ACPA and anti-CarP antibodies may initiate the primary target recognition but that amplification of IgM-RF is important for progression towards clinical RA, for example, by enhancing complement activation.[16,19,20]

In conclusion, we discovered that anti-CarP antibodies can be present in healthy blood donors many years before the development of clinical symptoms of RA.

Acknowledgments

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Anticarbamylated protein (anti-CarP) antibodies are present in sera of juvenile idiopathic arthritis (JIA) patients

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Abstract

Objectives

Anti-citrullinated protein antibodies (ACPA) have a low prevalence in juvenile idiopathic arthritis (JIA) patients. Recently, autoantibodies recognizing carbamylated proteins (anti-CarP) were observed in rheumatoid arthritis (RA) patients and were reported to be associated with a more severe clinical course in ACPA negative RA. We investigated the presence of anti-CarP antibodies in JIA patients and their relation to ACPA and IgM-rheumatoid factor (IgM-RF).

Methods

Cross-sectional samples of 234 JIA patients and 107 age-matched controls were analyzed for the presence of ACPA, IgM-RF and anti-CarP antibodies against carbamylated Fetal Calf Serum (FCS) or carbamylated Fibrinogen (Fib). The samples were obtained from patients in all different categories of JIA. Cut-off for positivity of anti-CarP antibodies was determined as the mean plus two times standard deviation of levels in sera of healthy controls.

Results

Anti-CarP FCS antibodies were present in 8,1% (19/234) of all JIA patients versus 4,7% (5/107) of the controls. Anti-CarP FCS antibodies were predominantly present in IgM-RF positive polyarticular JIA patients (42,1%, $p<0.0001$ vs other JIA categories). A similar observation was made using anti-CarP Fib antibodies.

Conclusions

Anti-CarP antibodies can be detected in sera of JIA-patients, especially in the polyarticular IgM-RF positive patients, the category most similar to RA.

Introduction

Juvenile idiopathic arthritis (JIA) is a heterogeneous group of chronic arthritides that starts before the age of 16. Different categories according to ILAR criteria are discerned [1] and the diagnosis is made clinically after exclusion of other causes of arthritis. Prognosis is currently largely unpredictable, except for rheumatoid factor (RF)-positive individuals. Reaching sustained remission is still scarce [2] although treatment options have improved with biologicals [3], resulting in better outcome.[4] Research to identify markers predicting disease flares is ongoing,[5-7] but markers that predict severe disease are currently lacking.

The discovery of anti-citrullinated protein antibodies (ACPA), often detected using assays based on cyclic-citrullinated peptides (CCP), has contributed substantially to the understanding of rheumatoid arthritis (RA).[8] The presence of ACPA is now part of the 2010 EULAR/ACR criteria for RA. ACPA-positive RA patients have generally more severe disease courses with increased joint destruction.[9] Incidence rates for ACPA-positivity, usually detected by a CCP-assay, in JIA are low (2-5%).[10-13] The presence of ACPA in JIA is merely confined to the polyarticular IgM-RF positive JIA category that resembles RA.

Recently, anti-carbamylated protein antibodies (anti-CarP) were described as a novel serological marker.[14-16] These antibodies are directed against proteins that have been modified by a post-translational modification named carbamylation. The physiological process of carbamylation increases during inflammation. In carbamylated proteins lysines are converted into homocitrullines. Anti-CarP antibodies were detected in sera of approximately 45% of RA patients and importantly in sera of 16-20% ACPA-negative RA patients, in comparison to less than 3% in healthy controls.[15, 17, 18] Within the ACPA-negative patients the presence of anti-CarP antibodies was associated with more severe radiographic progression.[15] Therefore anti-CarP antibodies could serve as a new prognostic marker in ACPA-negative RA patients[19] Since the majority of JIA patients are ACPA-negative we analyzed whether anti-CarP antibodies can be detected in sera of JIA patients and whether their occurrence correlates with the presence of ACPA and/or IgM-RF.

Materials and methods

Sample collection

JIA patients from three Dutch sources were included. The first group (n=33) consisted of patients participating in the BeSt for Kids trial, (NTR 1574) a treatment strategy study enrolling JIA patients. The second group (n=48) contained patients in early years of disease

participating a retrospective study described by Albers et al.[20] The third cohort (n=153) comprised participants of the Arthritis and Biologicals in Children (ABC) Register, an ongoing prospective observational study initiated in 1999, that aims to include all Dutch JIA patients treated with biologicals.[21] Healthy controls (n=107, mean age 11 years, range 2-20 years) were anonymous pediatric donors of allogeneic hematopoietic stem-cell grafts. Written informed consent was obtained from all patients and controls. Patients' disease characteristics and part of laboratory data (IgM-RF, ANA) were collected from patient files. Blood collection and storage are comparable among different cohorts. Median disease duration of the 234 JIA patients at the time of serum collection was 2,3 years (IQR 0,7-6,8) (Table 1). All International League against Rheumatism JIA categories were included with polyarticular JIA over-represented.[1, 22]

Table 1 Disease characteristics of 234 juvenile idiopathic arthritis (JIA) patients

Characteristics	Number
Gender m/f (%f)	76/158 (67,5%)
Median age (years) (IQR)	12.1 (8,4–16,2)
Median disease duration (IQR)	2.3 (0,7–6,8)
Median age at JIA onset (IQR)	8.8 (3,4–12,4)
ANA-positive at disease onset	64 (27,4%)
Systemic JIA	35 (15,0%)
Polyarticular JIA RF-negative	90 (38,5%)
Polyarticular JIA RF-positive	19 (8,1%)
Oligo-articular JIA extended	41 (17,5%)
Oligo-articular JIA persistent	18 (7,7%)
Juvenile psoriatic arthritis	24 (10,3%)
Enthesitis-related arthritis	5 (2,1%)
Undifferentiated	2 (0,8%)

ANA, anti-nuclear antibodies; RF, rheumatoid factor.

The correlation between anti-CarP antibodies and time in active disease as a measure of severity was investigated in the second cohort. The correlation between anti-CarP antibodies and ACRpedi30 response [23] (at least 30% improvement from baseline in 3 of 6 variables in the core set, with no more than 1 of the remaining variables worsening by >30%. Core set variables are 1) physician global assessment of disease activity; 2) parent/patient assessment of overall well-being; 3) functional ability; 4) number of joints with active arthritis; 5) number of joints with limited range of motion; and 6) erythrocyte sedimentation rate) after start of a biological was determined in the group from the ABC register.

Detection of anti-CarP antibodies, anti-CCP antibodies, ANA and IgM-RF

Anti-CarP antibodies were measured by ELISA using carbamylated Fetal Calfs-Serum (Ca-FCS) and human Fibrinogen (Ca-Fib) as antigens as described previously.[15] ACPA were measured using the CCP2 ELISA (Immunoscan RA Mark 2; Eurodiagnostica). Samples with a value above 25 units/ml were considered positive according to the manufacturer's instructions. IgM-RF and ANA levels were determined at disease onset as part of routine patient care. For measuring ANA levels most Dutch hospitals use a standard indirect immunofluorescence technique on ethanol fixed HEp-2 cells and IgM-RF levels are usually determined by ELISA.

Statistical Analysis

Statistical analyses were performed with SPSS 17,0. Fisher's exact test was used for testing the significance of differences between the percentages anti-CarP-positive and -negative patients. Pearson's chi square and student's t-test were used for identifying differences in time–in-active-disease [20] and ACRpedi30 response [23] between anti-CarP-positive and negative patients. Binary logistic regression with sensitivity analysis was used to test the interaction between age and the presence of anti-CarP antibodies. A p-value of less than 0,05 was considered statistically significant.

Results

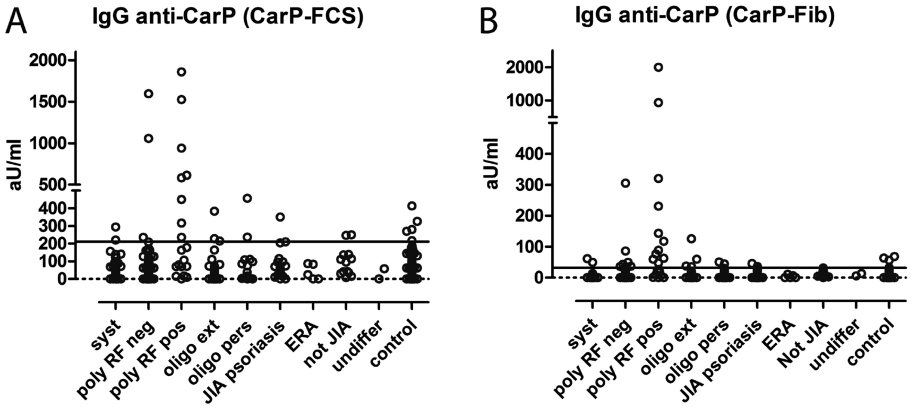
Anti-CarP antibodies are present in JIA patients

All sera were tested for the presence of anti-CarP antibodies using Ca-FCS and Ca-Fib as antigens. In the total JIA cohort 8,1% (19/234) of the patients were positive for antibodies reacting to Ca-FCS versus 4,7% (5/107) of controls (p=0,20). In addition 13,2% (31/234) of patients versus 2,8% (3/107) of controls were positive for antibodies reacting to Ca-Fib (p=0,003). Not all individuals harbored both reactivities and (39/234) 16,7% of patients and 8/107 (7,5%) of controls were positive for at least one anti-CarP antibody (p=0,028) (data not shown) and 11/234(4,7%) vs 0 of the controls (p=0,017) were positive for both anti-CarP reactivities.

Since the cohort of JIA patients consisted of different disease categories these were analyzed separately (Figure 1). Anti-CarP antibodies were predominantly present in polyarticular IgM-RF positive patients (8/19, 421%) as compared to the other JIA categories (p<0.0001). This observation was made for both Ca-FCS and Ca-Fib as detecting antigens (Figure 1).

Together, these data indicate that the presence of anti-CarP-antibodies in JIA is mainly confined to polyarticular IgM-RF positive patient group.

Figure 1 IgG anticarbamylated protein (anti-CarP) antibodies are present in juvenile idiopathic arthritis (JIA) sera. A cut-off for positivity (horizontal line) was determined using the mean plus two times the SD of the healthy controls. Antibodies against Ca-FCS (A) and Ca-Fib (B) in the sera of JIA patients and healthy controls are depicted in aU/mL. (C) Results of anti-CarP antibodies: positivity above cut-off per JIA category in absolute number, percentage and significance (NS, not significant, *p<0,05, **p<0,01). FCS, fetal calfs serum; RF, rheumatoid factor.



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	anti-CarP-FCS		anti-CarP-Fib	
	n/total (%)		n/total (%)	
All JIA in total	19/234 (8.1%)	NS	31/234(13.2%)	**
Systemic onset JIA	2/35 (5.7%)	NS	2/35(5.7%)	NS
Polyarticular RF negative	3/90 (3.3%)	NS	9/90(10%)	*
Polyarticular RF positive	8/19 (42.1%)	**	11/19(57.9%)	**
Oligo articular extended	3/41 (7.3%)	NS	4/41(9.8%)	NS
Oligo articular persistent	2/18 (11.1%)	NS	2/18(11.1%)	NS
Juvenile Psoriatic Arthritis	1/24 (4.2%)	NS	3/24(12.5%)	NS
Enthesitis related Arthritis	0	NS	0	NS
Undifferentiated	0	NS	0	NS
Controls	5/107 (4.7%)		3/107 (2.8%)	

Anti-CCP and IgM-RF in JIA and in relation to anti-CarP

Comparing anti-CarP antibodies to anti-CCP antibodies and IgM-RF revealed that 53% (8/15) of anti-CCP-positive children and 42.1% (8/19) of IgM-RF-positive children were also positive for anti-CarP antibodies. Importantly, anti-CarP antibodies were also found

in ACPA and IgM-RF-negative children as 57,9% (11/19) of anti-CarP-positive children were negative for anti-CCP antibodies and 27,3% (3/11) were negative for IgM-RF. In total 9 JIA patients were positive for IgM-RF, anti-CCP and anti-CarP (Ca-FCS and/or Ca-Fib). All triple positive patients were part of the ABC register.

Correlation with clinical features

Disease duration at sample collection, ANA status or age (at onset or at sample collection) was not associated with the presence of anti-CarP-antibodies. In addition, in the group previously described by Albers[20] we did not find an association of anti-CarP positivity with disease activity measured by time-in-active-disease at the time of sampling. Within the ABC register cohort no association was found between the presence of anti-CarP antibodies and ACR-Pedi 30 response[23] or reaching inactive disease at 15 months after start of anti-TNF treatment.[24] The cross-sectional nature of this study comprising three cohorts did not allow further detailed clinical association studies.

Discussion

Here we report that anti-CarP antibodies, initially identified in samples of adult RA-patients, are also present in (categories of) JIA. The detection of anti-CarP antibodies, especially in the IgM-RF-positive JIA category, reflects the similarity between RA and polyarticular IgM-RF positive JIA. The presence of anti-CarP antibodies in anti-CCP-negative RA is associated with a more severe disease course as expressed by radiological damage.[15] In our analyses information on radiographic damage is currently not available. We did not observe an association between anti-CarP antibodies and time-in–active-disease or clinical response to anti-TNF therapy but this could be due to lack of power. Disease severity differed across the three studies, but in general a severely affected group was collected as represented by the high percentages of polyarticular JIA and the use of biologicals. Although the numbers are too small to draw conclusions we observed that the triple positive sera (IgM-RF, anti-CCP and anti-CarP) were all confined to the ABC group that represent severe cases.

One limitation of this study is the cross-sectional nature of the sera used. Although this contributed to a large sample size detailed analyses of the association between the presence of anti-CarP antibodies and clinical outcome were not possible, as patients were included at different follow-up times and clinical parameters were recorded differently in each study.

In conclusion, anti-CarP antibodies are not only present in RA patients, but are also detectable in patients with JIA. They are present predominantly in the polyarticular RF-positive JIA category in both anti-CCP-positive and negative patients. Studies dedicated to

the diagnostic and prognostic value of anti-CarP-antibodies for (categories of) JIA patients can now be conducted.

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The specificity of anti-carbamylated protein antibodies for rheumatoid arthritis in a setting of early arthritis

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7

Abstract

Introduction

Anti-carbamylated protein (anti-CarP) antibodies have been described in rheumatoid arthritis (RA) and arthralgia patients at risk to develop RA. To what extent these auto-antibodies are specific for RA is unknown. Therefore, we investigated the diagnostic performance of the presence of anti-CarP antibodies for RA in a setting of early arthritis.

Method

Anti-CarP antibodies were detected using carbamylated-fetal calf serum as substrate. Anti-CCP2 antibodies were measured using ELISA and IgM rheumatoid factor (RF) as part of routine care. Sera were derived from patients in the Leiden Early Arthritis Clinic (EAC) cohort obtained at inclusion. Test characteristics were determined using the fulfillment of the 2010 RA criteria after 1 year as outcome.

Results

In total 2,086 early arthritis patients were studied regarding the presence of anti-CarP antibodies. We observed that the sensitivity and specificity of the presence of anti-CarP antibodies for RA were respectively 44% and 89%. As a reference sensitivity and specificity of the presence of anti-CCP2 antibodies were respectively 54% and 96% and of IgM-RF 59% and 91%. Patients harboring anti-CarP antibodies not classified as RA were mainly diagnosed with undifferentiated arthritis and less frequently reactive arthritis and psoriatic arthritis.

Conclusion

Anti-CarP antibodies are predominantly present in RA but can also be detected in other forms of arthritis.

Introduction

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease affecting synovial joints. RA can be classified using the 2010 American College of Rheumatology/European League Against Rheumatism classification criteria (2010 ACR/EULAR criteria) for rheumatoid arthritis [1]. In this quantitative system points can be obtained from: joint involvement, auto-antibodies, acute phase reactants and duration of symptoms [1]. Anti-citrullinated protein antibodies (ACPA) and rheumatoid factor (RF) are included in the 2010 ACR/EULAR criteria because of their high sensitivity and specificity in RA patients [1]. The presence of either ACPA or RF contributes 2 points and a 'high' level of either ACPA or RF contributes 1 extra point. The sensitivity of ACPA (~67%) for RA is comparable to IgM-RF (~69%) while their specificity (~95%) is higher than that of IgM-RF (~85%) [2]. Recently, we identified anti-carbamylated protein (anti-CarP) antibodies in both anti-cyclic citrullinated peptide 2 (anti-CCP2) antibody positive and negative RA patients [3-5]. Anti-CarP antibodies target proteins which are modified through a post-translational modification named carbamylation [6]. Carbamylation is mediated by cyanate which mainly modifies lysine residues. The level of cyanate is in equilibrium with urea and can be increased for example during renal failure, during smoking and during inflammation through a mechanism depending on myeloperoxidase (MPO) [7], the level of which is increased in RA patients [8]. The process of carbamylation is not restricted to RA but the formation of antibodies against these modified proteins is. Since the presence of anti-CCP2 antibodies is strongly associated with the HLA-Shared Epitope (SE) alleles and smoking, there is no association between anti-CarP antibodies and smoking after the correction for anti-CCP2 antibodies [4]. Anti-CarP antibodies are also not associated with HLA-SE following the correction for anti-CCP2 antibodies but possibly with HLA-DR*3 [4]. How anti-CarP antibodies would contribute to arthritis is unknown but may involve immune complex formation between anti-CarP antibodies and carbamylated proteins in the joint.

The presence of anti-CarP antibodies in anti-CCP2 antibody negative RA patients was associated with increased disease activity [5,9] and with a more severe joint damage [3,9,10]. Anti-CarP antibodies were also found in about 40% of RF and/or ACPA positive arthralgia patients, who have joint pain without clinically detectable arthritis [11]. Comparable to ACPA, also anti-CarP antibodies are independently associated with the risk of developing RA in these arthralgia patients [11]. Anti-CarP antibodies can be detected in serum many years before the clinical diagnosis of RA [10,12,13] and are independently associated with increased joint damage at the baseline of RA diagnosis [10].

Since anti-CarP antibodies have prognostic value in RA patients we are interested in their diagnostic performance for RA in comparison to ACPA and RF in a clinically relevant setting of early arthritis.

Patients and methods

Patients

We analyzed baseline sera of patients included in the Leiden Early Arthritis Clinic (EAC) cohort, which contains patients with arthritis of at least one joint and a symptom duration less than 2 years [13]. We measured the presence of anti-CarP, anti-CCP2 antibodies and IgM-RF in the sera of 2,086 unselected consecutive EAC sera that were collected between 1993 and 2011. The outcome was the diagnosis after 1 year of disease, we classified RA by strictly applying the 2010 ACR/EULAR criteria. [1]. Disease categories containing less than 20 patients were merged as "other" rheumatic diseases. The control sera were collected from healthy, non-arthritic, inhabitants of the Leiden area. The protocols were approved by the Leiden University Medical Center ethics committee and informed consent was obtained.

Anti-CarP, anti-CCP2 and IgM-RF measurements

Anti-CarP antibodies were detected using carbamylated Fetal Calf Serum as antigen as described before [3,10,12]. IgM-RF was determined as part of routine care and anti-CCP2 ELISA (Euro-Diagnostica) was performed following the manufacturer's instructions. The cut-offs for the anti-CarP antibody ELISA was set as the mean plus two times the standard deviation (SD) of the healthy controls.

Statistical analysis

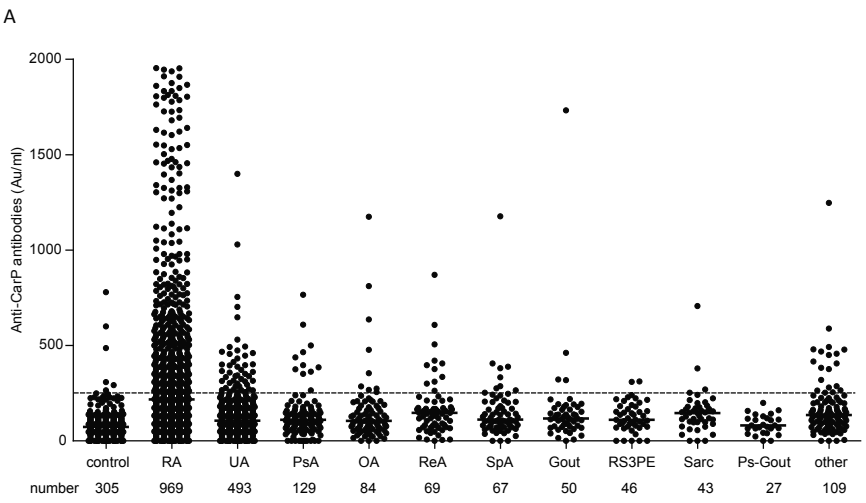
Statistical analysis was performed with SPSS version 20.0 (SPSS Inc., Chicago, USA). Dunn's multiple comparison test were performed to compare the levels of anti-CarP antibodies between diagnoses. Chi-square test with multiple testing correction was performed to compare the percentages of anti-CarP antibodies, anti-CCP2 antibodies and IgM-RF positive patients in different types of arthritis. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (LR+) and negative likelihood ratio (LR-) of anti-CarP antibodies for RA were calculated. The area under the receiver operator characteristic curve (AUC) of anti-CarP antibodies for RA was calculated. P-values below 0.05 were considered statistically significant.

Results

Sensitivity and specificity of anti-CarP antibodies for RA

The Leiden EAC cohort comprises patients with several forms of recent onset arthritis which can be encountered in the setting of an outpatient clinic [14]. Of the 2,086 patients analyzed 969 patients (47%) were classified with RA and 493 (24%) patients as undifferentiated arthritis (UA). A complete overview of the diagnoses is presented in Figure 1. We observed that anti-CarP antibodies were present in 26% of all patients analyzed and in 2% of the healthy controls. The test characteristics were subsequently determined with RA according to the 2010-criteria as outcome. The sensitivity of detection of anti-CarP antibodies in RA patients was 44% and the specificity of anti-CarP antibodies for RA 89%. In the ACPA negative stratum the sensitivity and specificity were respectively 12% and 91%.

Figure 1. Distribution of anti-CarP antibodies in sera of patients suffering from early arhtritis The number of controls and patients in each disease and the levels of anti-CarP antibodies in the serum of each individual are shown. Horizontal dashed line indicates cut-off. Abbreviations used: RA; Rheumatoid Arthritis, UA; Undifferentiated Arthritis, PsA; Psoriatic Arthritis, OA; inflammatory Osteo Arthritis, ReA; Reactive Arthritis (bacterial and viral), SpA; Spondylarthropathy with peripheral arthritis, RS3PE; Remitting Seronegative Symmetrical Synovitis with Pitting Edema, Sarc; Sarcoidosis, Ps-Gout; Pseudo Gout.



Diagnostic performance of anti-CarP antibodies in relation to anti-CCP2 and RF for diagnosing RA

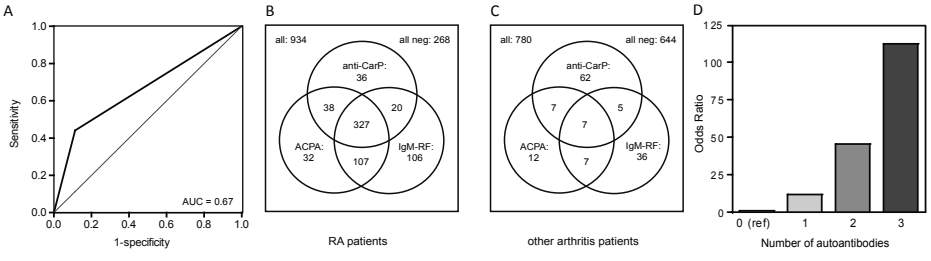
The performance of detecting anti-CarP antibodies for diagnosing RA was compared to that of anti-CCP2 and RF. We observed a sensitivity for RA of respectively 44%, 54% and 59% for anti-CarP, anti-CCP2 and RF, with a specificity of respectively 89%, 96% and 91% (Table 1). The LR+ of anti-CarP antibodies for RA was 4.2 which was lower than the LR+ of anti-CCP2 antibodies (12.9) and IgM-RF (6.9). The LR- for anti-CarP antibodies (0.62) was slightly higher compared to anti-CCP2 antibodies (0.48) and IgM-RF (0.44). Within the total study population, the AUC of anti-CarP positivity was 0.67 (95%CI 0.64-0.69) (Figure 2A). In anti-CCP2 negative early arthritis patients it was 0.52 (95%CI 0.48-0.55) suggesting that knowledge on anti-CarP auto-antibody status added only limited information for diagnosing RA.

Table 1. The test characteristics of different autoantibodies in RA

	Sensitivity	Specificity	PPV	NPV	LR+	LR-
anti-CarP	44%	89%	78%	65%	4.2	0.62
anti-CCP2	54%	96%	94%	64%	12.9	0.48
IgM-RF	59%	91%	86%	72%	6.9	0.44
Anti-CarP in anti-CCP–neg pt	12%	91%	37%	71%	1.3	0.97

A comparison is provided on the test characteristics of anti-CarP, anti-CCP and IgM RF for diagnosing RA. In addition the performance of anti-CarP in the CCP2 negative stratum is analyzed. Abbreviations used: PPV; positive predictive value, NPV; negative predictive value, LR+; positive likelihood ratio, LR-; negative likelihood ratio.

Figure 2. Anti-CarP antibodies in relation to ACPA and IgM-RF in RA and other forms of early arthritis (A) Receiver Operator Curves and AUC analyses of dichotomous data of anti-CarP antibodies in the whole cohort. (B) Distribution of positivity for anti-CarP antibodies, anti-CCP2 antibodies and IgM-RF in the patients diagnosed with RA. (C) Distribution of these autoantibodies in the patients diagnosed with the non-RA forms of early arthritis. Both for all individuals for whom data on anti-CarP and CCP2 and RF were available (n=934 for RA and n=780 for non-RA). (D) Odds Ratio's for having the diagnosis RA based on the presence of one, two or three autoantibodies relative to having zero autoantibodies.



Occurrence of anti-CarP antibodies in other forms of arthritis

In figure 1 the level of anti-CarP antibodies in the sera of individual types of early arthritis are depicted. Anti-CarP antibodies were most prevalent in RA, but were also detected in other forms of early arthritis (table 2), similar to ACPA and RF. This does not seem to be restricted to certain forms of early arthritis, possibly with the exception of pseudogout (table 2). Analyzing the anti-CarP positive non-RA early arthritis patients (n=120) separately revealed that these patients were mainly diagnosed as undifferentiated arthritis (42%), reactive arthritis (9%), psoriatic arthritis (9%) or peripheral spondyloarthritis (8%).

Comparing the levels of the anti-CarP antibodies in anti-CarP-positive patients across the different forms of early arthritis revealed that the levels were significantly higher in RA compared to the non-RA conditions ($p<0.05$ in all conditions). However, also in other forms of arthritis high levels were detected occasionally (Figure 1).

Table 2. Prevalence of different autoantibodies in early arthritis patients with various diagnoses

	RA	UA	ReA	Gout	Ps-gout	PsA	OA	Sarc	SpA	RS3PE	other
anti-CarP	44%	10%	16%	8%	0%	9%	11%	9%	15%	4%	17%
anti-CCP2	54%	3%	8%	0%	0%	6%	7%	5%	8%	0%	7%
IgM-RF	59%	5%	7%	6%	11%	10%	20%	7%	4%	7%	20%

Abbreviations used: RA; Rheumatoid Arthritis, UA; Undifferentiated Arthritis, PsA; Psoriatic Arthritis, OA; inflammatory Osteo Arthritis, ReA; Reactive Arthritis (bacterial and viral), SpA; Spondylarthropathy with peripheral arthritis, RS3PE; Remitting Seronegative Symmetrical Synovitis with Pitting Edema, Sarc; Sarcoidosis, Ps-Gout; Pseudo Gout. The percentages indicate the proportion of patients positive for one of the three autoantibodies analyzed in all samples available for each of the diagnoses.

Even though anti-CCP2 antibodies and IgM-RF are both part of the 2010 classification criteria, some patients positive for these autoantibodies had diagnoses other than RA (table 2). We did not observe a non-RA condition that harbored significantly more anti-CarP, anti-CCP2 antibodies or IgM-RF positive patients compared to any other non-RA conditions ($p>0.05$ in all conditions). The distribution of anti-CarP, anti-CCP2 antibodies and IgM-RF in RA and other patients is shown in figure 2 B and C. In the RA patients the three antibodies analyzed frequently occur together or as combinations of two autoantibodies, whereas in the non-RA group, there are less patients that display double or triple positivity.

Presence of one, two or three autoantibodies and diagnosis RA

When comparing the presence of one, two or three autoantibodies to the patients with zero autoantibodies we observed that with increasing numbers of autoantibodies the OR of

having the diagnosis RA highly increased (Figure 2D). For the group with one autoantibody the OR is 3.8 (CI 2.9-5.0), for two autoantibodies this is 20.9 (CI 12.7-34.3) and increases for three autoantibodies to 112.2 (CI 52.4 – 240.5), with all the groups significantly different from the zero autoantibodies reference group ($p<0.0001$).

The high OR's observed for the double and triple positives may be expected as ACPA and RF are part of the 2010-criteria. However, it is important to note that comparing the ACPA and RF double positive patients (OR 36.7 (CI 16.9-79.9)) to the ACPA, RF and anti-CarP, triple positive group (112.2 (CI 52.4 – 240.5)), there is an additional effect of OR 3.0 (1.1-8.9) $p= 0.04$.

Discussion

Here we have analyzed the sensitivity and specificity of detecting anti-CarP antibodies for RA in a setting of early arthritis encountered at the rheumatology outpatient clinic. The sensitivity of the presence of anti-CarP antibodies for RA patients is slightly lower than that of anti-CCP2 antibodies and IgM-RF. The specificity of detection of anti-CarP antibodies is similar to IgM-RF and is slightly lower than anti-CCP2 antibodies. Even though anti-CCP2 antibodies and IgM-RF are both part of the 2010 classification criteria, still individuals positive for these autoantibodies are also identified in the non-RA arthritis groups, as has also been reported before [15-17] . Therefore, it may not be surprising that anti-CarP antibodies, not being part of the 2010 classification criteria, can also be present in arthritic conditions other than RA. We have adhered very closely to definition when the 2010-classification criteria may be applied and have not considered patients for the possible diagnosis RA if their synovitis was more likely explained by another diagnosis [1]. Comparing the data on the sensitivity and specificity of detection of anti-CarP antibodies for RA using either the 2010-criteria to the 1987-criteria (data not shown) gave almost identical results. With the only exception that when using the 1987-criteria the presence of anti-CarP antibodies in undifferentiated arthritis (UA) patients was significantly associated with future development of RA independent of ACPA and RF, similar to what we observed before for arthralgia patients [11]. When the 2010-criteria were used this was not the case (data not shown). Patients are frequently double positive for anti-CCP and anti-CarP antibodies and since anti-CCP has a more prominent role in the 2010-criteria this may explain why anti-CarP antibodies are associated with conversion of UA to RA using the 1987-criteria but not when using the 2010-criteria.

So far we have applied a cut-off for positivity defined as the mean plus two times the standard deviation of a set of healthy control sera for anti-CarP antibody ELISA [3,11,13]. At the current cut-off for positivity, the AUC of anti-CarP antibodies in the total group

was 0.67 whereas in the anti-CCP2/RF negative group it was only 0.52. This indicates that for purely diagnostic purposes the presence of anti-CarP antibodies is not discriminating in the anti-CCP2/RF negative stratum. However, we have previously been able to identify a prognostically relevant group of RA patients who were clinically distinct from the double negative patients regarding disease activity and especially joint destruction using our current cut-off [3], an observation now confirmed in other studies [9,10]. In our view the current definition of the cut-off is reasonable and we welcome replication of these findings in other cohorts.

The diagnosis of RA is predominantly based on clinical observations with possible supportive information from serology [18]. However, as we are moving forward to identifying persons in the pre-RA phase where the full clinical picture of RA is not yet apparent, biomarkers will become more important. We have previously shown anti-CarP antibodies can already be present years prior to clinical onset of RA [10,12,13] and that that the presence of anti-CarP antibodies in addition to RF and ACPA provided relevant information on future development of RA [11]. The high OR for RA in triple positive early arthritis patients observed in the current study may suggest that triple positivity may also be used to identify future RA patients among individuals that do not yet display clinical symptoms.

In the current study we observed that anti-CarP antibodies occur in almost all forms of early arthritis. Although at lower frequencies, also anti-CCP antibodies were detected in many other forms of arthritis (Table 2) [15-17]. Whether or not anti-CarP positive patients with diagnoses other than RA have a different disease course than the anti-CarP negative patients the same diagnosis is a subject of future studies.

Conclusions

Anti-CarP antibodies are predominantly present in RA but can also be detected in small subsets of patients suffering from other forms of early arthritis.

Abbreviations

ACPA; anti-citrullinated protein antibodies
Anti-CarP; anti-carbamylated protein
Anti-CCP2; anti-cyclic citrullinated peptide 2
EAC; Early Arthritis Clinic
LR+; positive likelihood ratio
LR-; negative likelihood ratio
NPV; negative predictive value
PPV; positive predictive value
RA; Rheumatoid Arthritis
OA; inflammatory Osteo Arthritis
Ps-Gout; Pseudo Gout
PsA; Psoriatic Arthritis
RA; Rheumatoid arthritis
ReA; Reactive Arthritis (bacterial and viral)
RF; rheumatoid factor
RS3PE; Remitting Seronegative Symmetrical Synovitis with Pitting Edema
Sarc; Sarcoidosis
SE; Shared Epitope
SD; standard deviation
SpA; Spondylarthropathy with peripheral arthritis
UA; Undifferentiated Arthritis

Competing interests

J.S., T.W.J.H, R.E.M.T and L.A.T. are listed as inventors on a patent regarding the testing for anti-CarP antibodies. R.E.M.T and L.A.T. have received a research grant from INOVA diagnostics, San Diego, USA. No non-financial conflict of interest exists for any of the coauthors.

Authors contribution

JS and HvS were involved in designing the study, acquisition of data, analysis and interpretation of the data and drafting the manuscript. JvN and NL were involved in acquisition of data, analysis and interpretation of the data and drafting the manuscript

TH, AvdHvM, RT and LT were involved in designing the study, analysis and interpretation of the data and drafting the manuscript. All authors approved the final version of the manuscript.

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8

Discussion

Discussion

In this thesis we report the presence of anti-carbamylated protein (anti-CarP) antibodies in a subset of patients suffering from established rheumatoid arthritis (RA) (chapter 2),(1) juvenile idiopathic arthritis (JIA) (chapter 5) (2) or one of the conditions preceding RA, such as undifferentiated arthritis (UA) (chapter 7), arthralgia (chapter 4) (3) or asymptomatic period before the onset of clinical symptoms of RA (chapter 6).(4) The time points when anti-CarP antibodies first appear in asymptomatic individuals are comparable to that of anti-citrullinated protein antibodies (ACPA) but significantly earlier than rheumatoid factor (RF) (chapter 6).(4) In arthralgia patients, the presence of anti-CarP antibodies is independently associated with the future development of RA (chapter 4).(3) In ACPA-negative RA patients, the presence of anti-CarP antibodies is associated with more severe joint damage in the disease course (chapter 2).(1) Overall these studies highlight anti-CarP antibodies as a novel and interesting autoantibody system with potentially important implications in the diagnosis and prognosis of RA. Regarding this series of studies, several issues are worthwhile to be discussed.

The specificity and reproducibility of the anti-CarP antibody ELISAs

Since the anti-CarP antibody ELISAs were the major technique which we applied in chapter 2-7,(1-5) its reproducibility is crucial for the validity of our conclusions. Therefore, we performed several internal control experiments to judge their reproducibility. The intra-assay and inter-assay variability of anti-carbamylated fetal calf serum (anti-Ca-FCS) and anti-carbamylated fibrinogen (anti-Ca-Fib) ELISAs were both around 10% and 15%. In repeated measurements, the chances for negative samples becoming positive and vice versa, were generally around 5% in both anti-Ca-FCS and anti-Ca-Fib ELISA's. The samples of which anti-CarP antibody levels are around the cut-off line have relatively high chance to change their status. These data suggests a certain degree of variation in anti-CarP ELISAs. However, we have also observed that integrating data of repeated measurements of anti-CarP antibody ELISAs increased the effect size of all our reported clinical associations (chapter 2,4).(1,3) This observation suggests that random variation might contribute to a great part of all variation of our assays.

Furthermore, recently we saw independent replications of our two findings (chapter 2, 6). These replications include the presence of anti-CarP antibodies in both ACPA positive and ACPA negative RA patients (6) and their association with joint damage after corrected for the presence of ACPA (chapter 2),(7-10) the presence of anti-CarP antibodies before the appearance of RA symptoms in asymptomatic individuals (chapter 6). With the help of these replications, our major conclusions have already been reproduced in at least two

independent cohorts. Therefore we are convinced that the possibility that our previous published conclusions are chance findings is very low.

When generating the first version of the anti-CarP antibody ELISA we choose to use Ca-FCS as the antigen because FCS contains a wide mix of proteins and is readily available and relatively cheap. Carbamylated human serum is a potentially better source of antigens but the interference of human IgG in the ELISA cannot be easily avoided. Later we identified carbamylated human fibrinogen as another antigen for anti-CarP antibodies, which is our first identified human protein antigen of anti-CarP antibodies.

Since homocitrulline structurally highly resembles citrulline, it is possible that anti-CarP antibodies would be cross-reactive to citrullinated antigens. Here we carefully discussed this possibility with respect to our previous results. When analysing the RA patients from the Leiden early arthritis cohort, we observed that 9.4% of the patients are anti-CarP antibody positive (defined as either anti-Ca-FCS or anti-Ca-Fib antibody positive) and ACPA negative (defined as anti-cyclic citrullinated peptide 2 (CCP2) antibody negative), 9.6% are ACPA positive and anti-CarP antibody negative, 37.3% are double negative and 43.7% are double positive. In this double positive group we observed patients whose anti-CarP antibodies and ACPA totally do not cross-react to the other type of antigens and patients whose anti-CarP antibodies and ACPA are cross-reactive to the other type of antigens to a certain degree (chapter 3).(5) In a small double positive RA cohort, we used a CCP2 peptide bound column to deplete anti-CCP2 antibodies in patients' serum samples. After depletion, the median percentage of remaining anti-Ca-FCS antibodies in these samples is 70% (interquartile range, IQR: 47%-87%) (chapter 3).(5) The remaining anti-Ca-Fib antibody in serum samples is lower (median: 27%, IQR: 18%-58%). This higher cross-reactivity of anti-Ca-Fib antibodies might be explained by the hypothesis that some B cell receptors (BCR) and/or T cell receptors (TCR) are able to recognize both citrullinated and carbamylated epitopes/peptides of fibrinogen.

Anti-CarP antibodies in mice

Even though ACPA are considered to be a highly successful serological marker in RA patients, their importance in mouse arthritis models is far less clear. Appearance of ACPA without immunization with citrullinated antigens was reported in the collagen induced arthritis (CIA) model of DBA1 mice (12-14) but this observation is in debate.(15) A recent study suggested that the appearance of ACPA in CIA mice depends on their genetic background.(14) Appearance of ACPA following immunization of citrullinated antigens was reported in several mouse strains in the presence or the absence of arthritis.(16-18) However, whether ACPA can induce or exacerbate symptoms of arthritis on DBA1 and Balb/c mice is also in debate.(13,17,18)

Following our studies on the presence of anti-CarP antibodies in RA patients, our team has studied the presence and the role of anti-CarP antibodies in arthritis mouse models. Using sera of mice with CIA we observed the presence of anti-CarP antibodies, but not anti-CCP2 antibodies, in the majority of DBA1 and some of the C57BL/6 mice. The onset of CIA was preceded by an increase of anti-CarP antibody levels. This observation is in line with the data that in asymptomatic blood bank donors there is a clear rise in the levels of anti-CarP antibodies prior to the clinical onset of RA (chapter 6).(4)

As only part of the C57BL/6 mice developed CIA in this setting this allowed us to analyse the relationship between the presence of anti-CarP antibodies and clinical manifestation of arthritis. The appearance of anti-CarP antibodies in C57BL/6 mice was not limited to the mice which developed arthritis. This observation is in line with the observation that we can detect anti-CarP antibodies in the healthy population as well as in individuals prior to the onset of clinical symptoms (chapter 4,6).(3,4)

As a control DBA mice were injected with CFA only instead of the combination of CFA and CII and some of them also developed anti-CarP antibodies. However, the speed and magnitude of anti-CarP antibody responses were clearly associated with the presence of arthritis. In addition the presence of anti-CarP antibodies in the mice immunized with CFA only was largely limited to mice with significant tail damage and systemic inflammation as a consequence of the immunization procedure.

The immunization of DBA1 mice with carbamylated ovalbumin and CFA also invoked a strong anti-CarP antibody response which is cross-reactive to other carbamylated antigens. Currently our team is generating mouse monoclonal anti-CarP antibodies to study the role of anti-CarP antibodies in arthritis and to identify more antigens of this auto-antibody system.

In summary, the hypothesis that ACPA/autoantibodies play a key role in the pathogenesis of RA still awaits solid and repeatable animal data. The presence of anti-CarP antibodies may offer an alternative and potentially more powerful tool to study the generation and contribution of autoantibodies in murine models of arthritis.

Autoantibodies in the pathogenesis of RA

Despite many years of intensive research, the pathogenesis of RA remains to be elusive. Generation of ACPA is believed to be a hall mark in the pathogenesis.(19) Genetic predisposing and environmental factors, as elaborated in the introduction, are suggested to contribute to the break of tolerance and the generation of autoantibodies. However, asymptomatic individuals can harbor autoantibodies without developing to RA for many years, which suggests that only the presence of autoantibody is insufficient to trigger the onset

of RA. Currently, it is unclear if a second environmental stimulus is compulsory for the onset of symptoms, since the autoantibody-antigen system may develop, as the recognition of fine-specificities, isotype usage and avidity of autoantibodies increase over time. The symptoms of joints may appear after sufficient amount of modified proteins have accumulated on e.g. matrix molecules. Detailed analysis on the presence of citrullinated and carbamylated proteins in the inflamed joints of mice suffering from CIA and in samples from human RA patients is currently underway. Preliminary data clearly indicated that in both mice and human beings, there are carbamylated proteins in the inflamed joints.(20) After binding to ACPA and/or anti-CarP antibodies, the immune complexes can initiate inflammation via complement activation and/or attraction of leucocytes and ultimately lead to chronic inflammation and bone erosion. Based on this hypothesis, ACPA and anti-CarP antibodies may lead to tissue destruction through the same pathways, both in orchestra with RF. As we have shown in chapter 6,(4) either ACPA or anti-CarP antibodies may appear earlier in asymptomatic blood bank donors who developed to RA later. The epitope-spreading across these two types of auto-antigens may occur no matter which autoantibody system appeared first. Whether or not ACPA and anti-CarP antibody responses are initiated in the joints is not known but unlikely. The presence of anti-CarP antibodies implies that the citrullination is not indispensible in the pathogenesis of autoantibody positive RA. Taking the risk factors, such as smoking and chronic inflammation into account, one could speculate that the lungs and or other sites of chronic inflammation may actually be the places where the tolerance against these post-translationally modified proteins is broken. But the elucidation of these processes awaits experimental proof.

We cannot readily detect autoantibodies in RA patients against glycated, carbonylated and tobacco smoke treated FCS (unpublished data). Interestingly, anti carbamylated low density protein (anti-Ca-LDL) antibodies were found cross-reactive to malondialdehyde (MDA)/malondialdehyde acetaldehyde (MAA) modified and carbonylated LDL in healthy individuals.(21,22) MDA and MAA modified proteins belong to advanced oxidation protein products which occur during lipid peroxidation.(18) MDA and MAA modifications occur on lysine, the same as carbamylation.(18) Previously we have also detected the presence of anti-Ca-LDL antibodies (unpublished data) in RA patients. It would be interesting to study if anti MDA/MAA modified/carbonylated LDL antibodies may appear in RA patients independent of anti-Ca-LDL antibodies.

The future of anti-CarP antibodies

The characteristics of anti-CarP antibodies, in comparison with ACPA and IgM-RF, are listed in table 1. In summary, the specificity and sensitivity of anti-Ca-FCS and anti-Ca-Fib antibodies in RA patients are slightly lower than ACPA and RF-IgM (chapter 7). Similar to ACPA, anti-CarP antibodies have prognostic value in RA patients and predictive value in pre-

RA stage patients (chapter 2,4).(1,3) Recently together with a Japanese group we confirmed the presence of anti-CarP antibodies in the Japanese population (not published), suggesting the presence of anti-CarP antibodies is not limited to the Caucasian population. Currently we are collaborating with INOVA diagnostics to develop the second generation anti-CarP ELISA which may have better sensitivity, specificity and reproducibility compared to the assays we are currently using. If the new generation assay can considerably improve these aspects of anti-CarP ELISA, the anti-CarP antibodies might have the value to be included in the next version of RA classification criteria.

Table 1 Comparison of ACPA, anti-CarP antibodies, RF in our studies

	IgM-RF	ACPA[1]	Anti-CarP antibodies[2]
Sensitivity in RA[3,4]	59%	54%	44%
Specificity in RA[3,4]	91%	96%	89%
Isotype switch[3,5]	yes	yes	yes
Sensitivity in UA[3,6]	28%	25%	30%
Presence in Arthralgia[7]	yes	yes	yes
Presence before the onset[8]	yes	yes	yes
Presence in JIA[9]	yes	yes	yes
Predict joint damage[3,5]	no	yes	yes
Predict arthralgia to RA[4,7]	no	yes	yes
Predict UA to RA[3,6]	yes	yes	no
Predict DMARD free remission[3,5,10]	no	yes	no

[1]defined as anti-CCP2 antibodies
[2]defined as positive for either anti-Ca-FCS or anti-Ca-Fib antibodies
[3]in LUMC early arthritis cohort
[4]established RA patients according to 1987 ACR RA criteria
[5]studied in RA population
[6]defined as UA patients according to 1987 ACR RA criteria in early diagnosis
[7]in arthralgia cohort from Reade institute
[8]in asymptomatic blood donor cohort from Reade institute
[9]in a combination of JIA cohorts as described in the paper "Anti-carbamylated protein (anti-CarP) antibodies are present in sera of Juvenile Idiopathic Arthritis (JIA) patients"
[10]not published

It is known that ACPA positive RA patients respond better to B cell depletion therapy and their level is associated with treatment effect.(19) ACPA positive UA patients, but not ACPA negative patients, respond to disease modifying antirheumatic drugs (DMARDs).(25) Yet, the relationship between the anti-CarP antibody status and the responses to treatments in RA/UA patients has not been studied. Since the occurrence of anti-CarP antibodies is

supposed to be the same as ACPA, they may be associated with the treatment effects of B cell depletion therapy in RA patients and DMARDs in UA patients as well.

ACPA positive RA patients have rather different genetic risk loci compared to ACPA negative RA patients.(26) They are also suspected having different pathogenesis. Although anti-CarP positive and ACPA positive RA patients may have different HLA class II susceptibility, we assume anti-CarP antibodies positive/ACPA negative RA patients should share similar non-HLA genetic risk loci as ACPA positive patients. After excluding anti-CarP antibody positive patients from ACPA negative patients, it may be easier to find out the true genetic risk factors of autoantibody negative RA patients and unravel their pathogenesis.

Conclusions

In conclusion, we discovered the presence of a new autoantibody system in RA and pre-RA stage patients. This new autoantibody system, anti-CarP antibodies, may contribute to the prediction and prognosis of RA. The discovery of anti-CarP antibody also expanded the profile of autoantibody systems in RA, changing the concept that ACPA are unique in RA. The presence of anti-CarP antibodies in CIA mice might also offer a new study tool to investigate the role of autoantibody in the pathogenesis of RA.

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9

Summary

Summary

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease that causes chronic inflammation, pain and bone loss in synovial joints. RA patients are a heterogeneous group of patients with pronounced differences in disease activity and outcome. This heterogeneous group can be subdivided by the presence of autoantibodies. Autoantibody positive and negative RA patients were found to have different genetic background, disease development processes and responses to treatments. Anti-citrullinated protein antibodies (ACPA) and rheumatoid factors (RF) are the two major autoantibodies of RA, which were included in the 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for RA. RF mainly recognizes the Fc part of IgG and ACPA bind to proteins that have undergone the post-translational modification, citrullination, mediated by peptidylarginine deiminases. Both ACPA and RF are diagnostic markers for RA patients and predictive markers for future development of RA in pre-RA stage patients. ACPA are also a prognostic marker for increased bone erosions in RA patients. As ACPA are only present in about 67% of the RA patients and some ACPA negative RA patients also have severe joint damage, there is a need for additional biomarkers to identify ACPA negative patients in need of a more aggressive intervention. In an attempt to identify additional biomarkers to be used for such identification we addressed the presence of antibodies against proteins modified by another form of post-translational modification, carbamylation. Carbamylation is a post-translational modification in which cyanate reacts to lysines and modifies them to homocitrullines. The structure of homocitrulline resembles citrulline and is only one methylene group longer than citrulline. It has been shown recently that carbamylated proteins may trigger T cell response and autoantibody formation in rodents. We hypothesized that anti-carbamylated protein (anti-CarP) antibodies may also be present in RA patients as described in this thesis.

To detect anti-CarP antibodies we developed ELISAs using carbamylated FCS (Ca-FCS) and carbamylated human Fibrinogen (Ca-Fib) (Chapter 2). In the Leiden early arthritis cohort (EAC) we observed that 45% of the RA patients are positive for anti-Ca-FCS IgG antibodies and 43% of the RA patients are positive for anti-Ca-FCS IgA antibodies. Similar to the observation for anti-Ca-FCS antibodies, we observed also the presence of anti-Ca-Fib antibodies in RA patients, confirming that these antibodies are auto-reactive. Next, we wished to analyze whether anti-CarP antibodies are cross-reactive to citrullinated proteins and vice versa. To answer this question we performed ELISAs with specially designed peptides, inhibition assays and western blots. All these experiments suggested that anti-CarP antibodies and ACPA are only partially cross-reactive to the other type of post-translationally modified antigens. Besides, 16% of ACPA-negative RA patients are anti-Ca-FCS IgG positive and 30% of patients are anti-Ca-FCS IgA positive in the ACPA negative group. To ensure that reactivity towards carbamylated proteins is mediated by

the antigen-binding-part of the antibodies, we generated F(ab')2. As expected, F(ab')2 but not Fc part displayed anti-CarP reactivity and displayed a similarly restricted cross-reactivity to citrullinated proteins as intact antibodies. The presence of ACPA in RA patients is associated with a more severe clinical disease course as measured by radiological damage. We therefore analyzed whether the presence of anti-Ca-FCS antibodies is also predictive for a more severe disease course. We observed that patients positive for anti-Ca-FCS IgG had more joint destruction over time, also after correction of ACPA and RF. In the stratified analyses, the presence of anti-Ca-FCS IgG is associated with a more severe joint damage in the anti-cyclic citrullinated pepetide 2 (CCP2) antibody negative subgroup but not in the anti-CCP2 antibody positive.

In Chapter 2 we have studied the cross-reactivity of anti-CarP antibodies to citrullinated proteins, in Chapter 3 we quantified this cross-reactivity. The Anti-Modified Citrulline (AMC) antibody developed by Dr. Senshu is able to recognize citrullinated epitopes irrespective of the neighboring amino acids. Thus we also aimed to verify whether the AMC assay could distinguish between citrullinated and carbamylated epitopes. We found the "AMC-Senshu" method cannot differentiate citrullinated and carbamylated epitopes. This finding 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. However, our study suggests that the extent and nature of citrullination and carbamylation in the joint should be re-evaluated.

In addition we performed ACPA depletion in which more than 98% of ACPA in the sera were depleted. The median percentage of remaining anti-Ca-FCS antibodies in the sera is 70% (interquartile range, IQR: 47%-87%) and for anti-Ca-Fib antibodies is 27% (IQR: 18%-58%). Again these data indicate that next to a potentially cross-reactive component also antibodies uniquely specific for carbamylated antigens are present in sera of RA patients.

In pre-RA stages such as arthralgia and undifferentiated arthritis (UA), ACPA and RF are predictive factors for future progression towards RA. However, at present it is unknown whether anti-CarP antibodies exist in arthralgia patients (Chapter 4). We studied whether anti-CarP antibodies are present in arthralgia patients and whether their presence associates with the development of RA. For this purpose we have used an arthralgia cohort from our collaborators in Amsterdam which includes patients who were positive for either anti-CCP2 antibodies and/or RF. In this cohort, we could measure baseline sera obtained during the arthralgia phase and relate it to clinical onset of RA years later. We observed that anti-Ca-FCS antibodies were present in sera of 39% of these arthralgia patients. The presence of anti-Ca-FCS antibodies was associated with the development of RA in the whole arthralgia cohort after correction for RF and anti-CCP2 antibody status

(HR: 1.56; 95%CI: 1.06-2.29; p = 0.023), as well as in the anti-CCP2 antibody positive subgroup (OR: 2.231; 95%CI: 1.31-3.79; p = 0.003) but not in the anti-CCP2 antibody negative subgroup (p=0.891). After correction for anti-CCP2 antibody level, anti-CarP antibody positivity still increased the risk for developing RA in anti-CCP2 antibody positive arthralgia patients. Anti-CarP antibody positive patients not only developed RA more often but also within a shorter time frame. This association remained significant after correction for anti-CCP2 antibody and IgM-RF status or the levels of anti-CCP2 antibodies and IgM-RF status.

In Juvenile Idiopathic Arthritis (JIA) patients there is a lack of markers that predict severe disease. Although ACPA have contributed to the understanding of RA, they are not equally useful in JIA patients as their presence in JIA patients is low and merely confined to the polyarticular IgM-RF positive category resembling RA. Since most JIA patients are ACPA-negativewe investigated whether anti-CarP antibodies are present in sera of JIA patients and how they are related to ACPA and IgM-RF (chapter 5). We observed that 8,1% of the JIA patients were positive for anti-Ca-FCS antibodies and 13.2% of the patients were positive for anti-Ca-Fib antibodies. Both anti-Ca-FCS and anti-Ca-Fib antibodies were predominantly present in polyarticular IgM-RF positive patients. Anti-CarP antibodies (defined as anti-Ca-FCS or anti-Ca-Fib antibodies) were also found in ACPA and IgM-RF-negative patients as 57,9% of anti-CarP positive patients were negative for ACPA and 27.3% were negative for IgM-RF. The cross-sectional nature of the cohorts we used in this study did not allow an in depth analysis of the association between the anti-CarP antibody status and the clinical outcome of JIA patients.

Early and aggressive intervention in individuals that are developing to RA can prevent irreversible bone loss and induce early remission. The presence of autoantibodies could be a useful marker to identify the individuals that could benefit most from such intervention. Both ACPA and RF can be detected in serum many years prior to the onset of symptoms and are thought to be implicated in the disease progression of RA. The studies presented in chapter 6 we analyzed whether anti-CarP antibodies are already present in healthy individuals before the diagnosis of RA and how the appearance of anti-CarP antibodies relates to the appearance of ACPA and IgM-RF. From the sera of RA patients collected prior to the diagnosis we detected the presence of anti-Ca-FCS (26.6%) and anti-Ca-Fib antibodies (38.0%). The moment when IgM-RF, anti-Ca–FCS, anti-Ca-Fib and anti-CCP2 antibodies were detectable for the first time were respectively 10, 14, 14 and 14 years before diagnosis of these patients. These are the time points when the first samples of these patients were collected. The median (IQR) of time points when anti-CCP2, anti-Ca-FCS, anti-Ca-Fib antibodies and IgM-RF first appeared were 6 (3-10), 5 (3-7), 7 (4-10) and 2 (1-5) years before the diagnosis. The levels of autoantibodies increased within the majority of followed blood donors. The levels of anti-CCP2, anti-Ca-FCS, anti-Ca-Fib antibodies and IgM-RF each

increased in 83% (33/40), 78% (31/40), 53% (21/40) and 85% (34/40) of donors. The median levels of all autoantibodies start to increase around 5 to 7 years before the diagnosis or RA.

In the studies presented in chapter 7 we compared the sensitivity and specificity of anti-CarP antibodies in RA patients with ACPA and RF. We have performed these analyses using 2086 samples from the Leiden EAC, comprising all forms of early arthritis as encountered in the outpatient clinic. The sensitivity and specificity of anti-CarP antibodies for RA are 44% and 89%. As a reference we observed a sensitivity and specificity of anti-CCP for RA of 54% and 96% and for RF 59% and 91%. Although anti-CarP antibodies are predominantly present in RA patients we observed some anti-CarP antibody positive individuals in nearly all other forms of early arthritis. To what extent the presence of anti-CarP antibodies in these conditions is associated with clinical presentation or outcome will be the focus of future research.

In this thesis we report the presence of anti-CarP antibodies in subsets of patients suffering from established RA (chapter 2), JIA (chapter 5) or one of the conditions preceding RA, such as UA (chapter 7), arthralgia (chapter 4) or asymptomatic period before the onset of clinical symptoms of RA (chapter 6). The time points when anti-CarP antibodies first appear in asymptomatic individuals are comparable to that of ACPA but significantly earlier than RF (chapter 6). In arthralgia patients, the presence of anti-CarP antibodies is independently associated with the future development of RA (chapter 4). In ACPA-negative RA patients, the presence of anti-CarP antibodies is associated with more severe joint damage in the disease course (chapter 2). Overall these studies highlight anti-CarP antibodies as a novel and interesting autoantibody system with potentially important implications in the diagnosis and prognosis of RA.

10

Nederlandse Samenvatting

Nederlandse Samenvatting

Reumatoïde artritis (RA) is een chronische, systemische, auto-immuunziekte die leidt tot chronische ontsteking, pijn en botverlies in synoviale gewrichten. De populatie van RA patiënten is heterogeen met nadrukkelijke verschillen in ziekteactiviteit en uitkomst. Deze heterogene groep kan worden onderverdeeld op basis van de aanwezigheid van autoantilichamen. Autoantilichaam positieve patiënten bezitten ten opzichte van autoantilichaam negatieve patiënten andere genetische risicofactoren, ondergaan een ander ziektebeloop en reageren anders op medicatie. Anti-gecitrullineerde eiwit antilichamen (ACPA) en reuma factor (RF) zijn de twee meest bekende autoantilichamen bij RA. Deze twee antilichamen zijn opgenomen in de 2010 American College of Rheumatology / European League Against Rheumatism classificatiecriteria voor RA. ACPA binden aan eiwitten die de post-translationele modificatie citrullinatie hebben ondergaan onder invloed van peptidyl arginine deiminases. RF herkent vooral het Fc gedeelte van IgG. Zowel ACPA als RF zijn diagnostische markers voor RA en de aanwezigheid van deze markers is van voorspellende waarde voor het ontwikkelen van RA vanuit de RA voorloper stadia. De aanwezigheid van ACPA is ook van prognostische waarde voor botafbraak in RA. Omdat ACPA slechts aanwezig zijn in ongeveer 60% van de RA patiënten en ook sommige ACPA negatieve patiënten toch ernstige boterosies ontwikkelen is er vraag naar additionele biomarkers om ACPA negatieve patiënten te identificeren voor wie een agressievere behandeling wenselijk zou zijn.

In een poging biomarkers te ontdekken die een dergelijke identificatie mogelijk zouden maken zijn wij op zoek gegaan naar antilichamen gericht tegen eiwitten die een andere post-translationele modificatie hebben ondergaan, carbamylatie. Carbamylatie is een post-translationele modificatie waarbij cyanaat reageert aan lysines en deze lysines modificeert tot homocitrullines. De structuur van homocitrulline lijkt veel op die van citrulline en is slechts één methyleen groep langer dan citrulline. Het is recent aangetoond in proefdiermodellen dat gecarbamyleerde eiwitten autoantilichamen kunnen induceren. Hierop vormden we de hypothese dat er bij reumapatiënten antilichamen aanwezig kunnen zijn die gecarbamyleerde eiwitten herkennen, anti-gecarbamyleerde eiwit antilichamen (anti-CarP).

Om anti-CarP antilichamen te kunnen detecteren hebben we een ELISA opgezet gebaseerd op gecarbamyleerd Foetaal Kalf Serum (Ca-FCS) en gecarbamyleerd humaan Fibrinogeen (Ca-Fib) (Hoofdstuk 2). In het Leiden Early Arthritis Clinic (EAC) cohort hebben we vastgesteld dat 45% van de RA patiënten positief is voor IgG anti-Ca-FCS antilichamen en 43% voor IgA anti-Ca-FCS antilichamen. Naast deze anti-Ca-FCS antilichamen konden we ook antilichamen meten die aan humaan Ca-Fib bonden, waarmee we auto-reactiviteit aantoonden. Vervolgens vroegen wij ons af of anti-CarP antilichamen kruisreactiviteit

vertonen naar gecitrullineerde eiwitten en of ACPA kruisreactiviteit vertonen naar gecarbamyleerde eiwitten. Om deze vraag te beantwoorden hebben we verschillende experimenten gedaan: ELISA's met verschillende peptides, inhibitie assays en western blots. Telkens tonen deze experimenten aan dat er slechts een beperkte mate van kruisreactiviteit is. Tevens zien we in de RA populatie dat 16% van de ACPA negatieve patiënten positief is voor anti-CarP IgG en 30% voor anti-CarP IgA. Om er zeker van te zijn dat de reactiviteit voor gecarbamyleerde eiwitten daadwerkelijk door het antigeen herkennende gedeelte van de anti-CarP antilichamen gemedieerd wordt hebben we F(ab)2 gegenereerd. Deze F(ab)2, maar niet de Fc gedeeltes, vertoonden anti-CarP reactiviteit en hadden dezelfde beperkte mate van kruisreactiviteit als de intacte anti-CarP antilichamen.

De aanwezigheid van ACPA is geassocieerd met een ernstig ziektebeloop met meer radiologische schade. We hebben daarom geanalyseerd of de aanwezigheid van anti-CarP antilichamen ook geassocieerd is met een ernstig ziektebeloop. We hebben vastgesteld dat patiënten positief voor anti-Ca-FCS antilichamen meer gewrichtsschade hadden in de tijd, zelfs na correctie voor ACPA en RF. In de gestratificeerde analyse is de aanwezigheid van anti-CarP IgG geassocieerd met ernstige gewrichtsschade in de anti-Cyclisch Gecitrulineerd Peptide 2 (CCP2) antilichaam negatieve subgroep maar niet in de anti-CCP2 positieve subgroep.

Hoofdstuk 2 beschrijft het onderzoek naar de kruisreactiviteit van anti-CarP antilichamen voor gecitrullineerde eiwitten en in Hoofdstuk 3 wordt de kwantificatie van deze kruisreactiviteit gepresenteerd. De anti-gemodificeerde citrulline (AMC) test die ontwikkeld is door Dr. Senshu kan citrullines aantonen zonder dat dit beïnvloed wordt door de omringende aminozuren. Vanwege de grote structurele overeenkomst tussen citrulline en homocitrulline wilden we analyseren of deze methode een onderscheid tussen deze twee modificaties zou kunnen maken. We vonden duidelijke aanwijzingen dat de AMC-Senshu methode geen onderscheid maakt tussen deze modificaties. Deze observatie hoeft niet te betekenen dat de eerdere bevindingen gemaakt met deze methode, bijvoorbeeld de aanwezigheid van gecitrullineerde eiwitten in het ontstoken gewricht, niet correct zijn. Studies met massa-spectometrie hebben de aanwezigheid van gecitrullineerde eiwitten bevestigd. Maar onze studie geeft wel aan dat de huidige gedachten over de aanwezigheid en bijdrage van citrullinatie versus carbamylatie in het gewricht wellicht heroverwogen moet worden.

Verder hebben we studies gedaan waarbij we ACPA hebben gedepleteerd uit ACPA/anti-CarP dubbel positieve sera. De depletie van ACPA-reactiviteit was meer dan 98%. In deze samples was de mediaan van het percentage anti-Ca-FCS dat nog aanwezig was 70% (IQR 47%-87%) en van anti-Ca-Fib 27% (IQR 18%-58%). Ook deze data geven weer aan dat er in het serum van RA patiënten naast een potentieel kruisreagerende populatie

antilichamen ook antilichamen aanwezig zijn die uniek reageren met ofwel gecitrullineerde eiwitten ofwel gecarbamyleerde eiwitten.

In pre-RA stadia zoals artralgie en ongedifferentieerde artritis (UA) zijn ACPA en RF beschreven als voorspellende factoren voor het toekomstig ontwikkelen van RA. In hoofdstuk 4 worden studies beschreven waarin geanalyseerd is of er bij artralgie patiënten ook anti-CarP antilichamen voorkomen en of de aanwezigheid van deze antilichamen ook geassocieerd is met het toekomstig ontwikkelen van RA. We hebben gebruik gemaakt van een artralgie cohort uit Amsterdam waarin patiënten zijn geïncludeerd op basis van het hebben van artralgie en positiviteit voor ofwel anti-CCP antilichamen of IgM-RF. In dit cohort konden we anti-CarP antilichamen aantonen in 39% van de artralgie patiënten. De aanwezigheid van anti-CarP antilichamen was geassocieerd met het ontwikkelen van RA, ook na correctie voor anti-CCP antilichamen en RF status. Deze associatie zagen we niet als we de anti-CCP2 negatieve groep apart analyseerden. Ook na correctie voor de levels van anti-CCP antilichamen was de aanwezigheid van anti-CarP antilichamen nog steeds geassocieerd met het ontwikkelen van RA in de anti-CCP positieve populatie. Anti-CarP antilichaam positieve patiënten ontwikkelden RA niet alleen vaker, maar ook eerder. Deze associatie bleef significant ook na correctie voor anti-CCP2 en IgM-RF status of de levels van anti-CCP2 antilichamen en IgM-RF status.

Bij Juveniele Idiopathische Artritis (JIA) is er een gebrek aan markers die een ernstig ziekte verloop kunnen voorspellen. Ondanks het feit dat ACPA een rol spelen bij de diagnose en prognose van RA, hebben deze autoantilichamen geen prominente rol bij JIA. Vooral omdat ACPA vrijwel uitsluitend voorkomen bij de poly-articulaire IgM-RF positieve subgroep van JIA die veel lijkt op RA. Omdat verreweg de meeste JIA patiënten negatief zijn voor ACPA hebben we in het kader van studies beschreven in Hoofdstuk 5 onderzocht of anti-CarP antilichamen aanwezig zijn bij JIA en wat de relatie is met ACPA en IgM-RF. Uit deze analyses bleek dat 8.1% van de JIA patiënten positief was voor anti-Ca-FCS antilichamen en 13.2% voor anti-Ca-Fib antilichamen. Zowel anti-Ca-FCS en anti-Ca-Fib antilichamen zijn voornamelijk aanwezig in de poly-articulaire IgM-RF positieve subgroep van JIA. Anti-CarP antilichamen (anti-Ca-FCS en anti-Ca-Fib samen) waren ook aanwezig in patiënten die negatief waren voor ACPA en of IgM-RF. In totaal waren 57.9% van de anti-CarP positieve patiënten negatief voor ACPA en 27.3% negatief voor IgM-RF. Vanwege het cross-sectionele karakter van de patiënten populatie konden we geen gedetailleerde studies doen naar de associatie tussen de aanwezigheid van anti-CarP antilichamen en klinisch beloop van de JIA patiënten.

Vroege en agressieve interventie kan in individuen die RA ontwikkelen het irreversibele botverlies voorkomen en vroege remissie induceren. De aanwezigheid van autoantilichamen kan een bruikbare biomarker zijn voor het identificeren van individuen die gebaat zouden

zijn bij een dergelijke interventie. Zowel ACPA als IgM-RF kunnen in het serum al worden aangetoond jaren voordat de symptomen merkbaar zijn en deze autoantilichamen worden geïmpliceerd bij te dragen aan het ziekte proces van RA. In studies beschreven in hoofdstuk 6 hebben we geanalyseerd of anti-CarP antilichamen al aanwezig zijn in gezonde individuen voordat de diagnose RA is gesteld en ook hoe de aanwezigheid van anti-CarP antilichamen zich verhoudt tot de aanwezigheid van ACPA en IgM-RF. In sera van gezonde bloedbank donoren, verzameld net voordat bij hen de diagnose RA werd gesteld, konden we anti-CarP antilichamen aantonen in 26,6% van de personen voor anti-Ca-FCS en in 38,0% voor anti-Ca-Fib. Het moment waarop IgM-RF, anti-Ca-FCS, anti-Ca-Fib en anti-CCP2 antilichamen voor het eerst konden worden aangetoond waren respectievelijk 10, 14, 14 en 14 jaar voor de diagnose. Voor veel patiënten was dit het eerste beschikbare monster. De mediaan (IQR) van de tijdstippen (in jaren voor diagnose) waarin de antilichamen voor het eerst te detecteren waren was 6 (3-10) voor anti-CCP2, 5 (3-7) voor anti-Ca-FCS, 7 (4-10) voor anti-Ca-Fib en 2 (1-5) voor IgM-RF. De levels van de autoantilichamen namen toe over de tijd in de meeste donoren. We zagen een toename voor anti-CCP2 in 83% (33/40), anti-Ca-FCS 78% (31/40), anti-Ca-Fib 53% (21/40) en voor IgM-RF in 85% (34/40) van de donoren. De mediane levels nemen voor alle autoantilichamen vooral toe in de periode van 5 tot 7 jaar voor de diagnose.

In de studies beschreven in hoofdstuk 7 vergelijken we de sensitiviteit en de specificiteit van anti-CarP antilichamen voor RA met die van anti-CCP2 en RF. We hebben hiervoor analyses uitgevoerd op 2086 sera van patiënten uit het Leiden Early Arthritis Clinic (EAC) cohort, bestaande uit patiënten met alle vormen van vroege artritis zoals die in de polikliniek voorkomen. De sensitiviteit en specificiteit van de aanwezigheid van anti-CarP antilichamen voor RA is 44% en 89%. Ter vergelijking, voor anti-CCP2 is dit 54% en 96% en voor IgM-RF 59% en 91%. De anti-CarP antilichamen zijn vooral aanwezig bij RA patiënten, maar kunnen ook gevonden worden in enkele patiënten met andere van vroege artritis. In welke mate de aanwezigheid van anti-CarP antilichamen in deze niet-RA artritides is geassocieerd met klinische presentatie of beloop is het onderwerp van toekomstige studies.

In dit proefschrift beschrijven we de aanwezigheid van anti-CarP antilichamen in een gedeelte van de patiënten met RA (hoofdstuk 2), JIA (hoofdstuk 5), of één van de condities die vooraf gaan aan RA, zoals UA (hoofdstuk 7), artralgie (hoofdstuk 4) of de asymptomatische fase voordat de eerste symptomen zichtbaar worden (hoofdstuk 6). Het tijdstip waarop de anti-CarP antilichamen voor het eerst detecteerbaar zijn is vergelijkbaar metde eerste detectie van anti-CCP2 maar significant eerder dan IgM-RF (hoofdstuk 6). In artralgie patiënten is de aanwezigheid van anti-CarP antilichamen, onafhankelijk van ACPA, geassocieerd met het ontwikkelen van RA in de toekomst. In ACPA negatieve RA patiënten is de aanwezigheid van anti-CarP antilichamen geassocieerd met meer gewrichtsschade tijdens het ziekte verloop (hoofdstuk 2).

Deze studies geven gezamelijk aan dat anti-CarP een nieuw en interessant autoantilichaam systeem is met potentieel belangrijke implicaties voor de diagnose en prognose van RA.

Curriculum Vitae

List of Publications

Acknowledgements

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Curriculum Vitae

Jing Shi was born in WuHan, China at 24.04.1985. During 01.09.2003 to 31.06.2007 he achieved the Bachelor of Biotechnology at WuHan University. After that he went to the Netherlands and studied Biomedical Science during 01.08.2007 to 30.09.2009 at Leiden University. He obtained his master degree from this university in September 2009 after which he started hisPhD-study in Leiden. He was trained as a PhD student at the Department of Rheumatology, Leiden University Medical Center by the promotors Prof.Dr. René E.M. Toes, Prof.Dr. Tom W.J. Huizinga and the co-promotor Dr. Leendert A. Trouw during 01.10.2009 to 31.08.2013. Starting from 15.04.2014, he is working as a postdoctoral fellow at the Division of Rheumatology, Johns Hopkins University in the USA under the supervision of Dr. Felipe Andrade.

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