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Unravelling the anti-carbamylated protein antibody response in rheumatoid arthritis

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Unravelling the anti-carbamylated protein antibody response in rheumatoid arthritis

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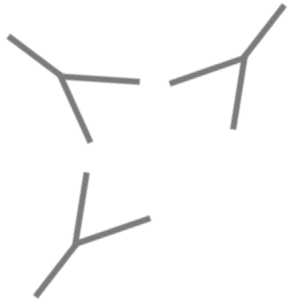

Chapter 1

Introduction

During a lifetime, the human body encounters a large number of immunological challenges, such as infections with viruses and bacteria. If the immune system is sufficiently competent, such a challenge will be met by the activation of one or more immune pathways that will attack and clear the infection. However, there are situations in which complications may arise. In the first situation, a weakened immune system might not be able to control the pathogen, resulting in prolonged infection with accompanying consequences. Often, this situation can be prevented upfront by vaccination or countered during the infection by treatment with antibiotics or passive immunization therapy[1]. In the second situation, the immune system may not attack a foreign pathogen but direct it's action towards (part of) the human body. Diseases resulting from such an unwanted self-reactive immune response have been named autoimmune diseases. Examples of common diseases often categorized as autoimmune diseases are Graves' disease, type I diabetes and rheumatoid arthritis (RA)[2].

Clinical aspects of rheumatoid arthritis

Although RA has been described as an autoimmune disease, it has also been called an inflammatory disease with autoimmune aspects[3]. The fact that multiple of such descriptions are available already indicates that many aspects of the disease, especially with regards to pathogenic processes, are relatively unclear. In the Netherlands, more than 1.3% of the population suffers of a chronic inflammatory disease, belonging to the rheumatology disease category[4]. A large part of this category consists of RA patients. It has been estimated the general prevalence of RA is between 0.5% and 1%[5,6]. The diagnosis of an RA patient is often based on both clinical presentation and serology. The clinical presentation of RA involves inflammation of one or multiple joints. Involvement of larger joints, inflammation in multiple joints and the duration of the complaints each contribute to increased susceptibility of RA. Additionally, two autoantibodies, called rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) are often measured in serum, possibly combined with other markers of inflammation such as CRP and ESR. Positivity or high levels of each of these biomarkers further adds to increased susceptibility for RA. Most of these factors are also part of the most recent classification criteria for RA[7]. An overview of the most recent classification criteria, compared to the 1987 criteria can be found in table 1[8]. The main difference between these two criteria is the increased attention to the presence of autoantibodies. When studying the presence of autoantibodies in clinical cohorts, results may therefore vary depending on the selection criteria.

The 2010 classification criteria are able to capture a large proportion of RA patients[9,10]. These criteria are not diagnostic criteria and may still miss out on the group of (early) RA patients in which few joints are affected and serology is negative[11]. At the moment it is considered important to identify RA patients as early as possible, as early treatment allows the most favourable long-term outcome.

Likewise, during the early phase of disease, there may exist a so-called “window of opportunity” during which treatment is more effective and which may even result in the prevention of chronic disease development[12].

At the moment, there is no cure for RA, although the development of chronic disease may be prevented by effective and early treatment. Therefore, medication prescribed aims to modify the disease or reduce the symptoms. Methotrexate is one of the disease-modifying anti-rheumatic drugs (DMARDs) and is often part of the standard treatment strategy after diagnosis of RA. An important mode of action of methotrexate is to suppress the immune system. This drug is effective in the majority of patients but a substantial proportion of the patients does not respond sufficiently. If the treatment is not effective, methotrexate can be combined with other DMARDs such as TNF inhibitors or rituximab, which both target separate aspects of the immune system. In order to further alleviate symptoms such as pain and inflammation, non-steroidal anti-inflammatory drugs (NSAIDs) can be prescribed as well[13]

Criteria	1987 Criteria	2010 Criteria
Morning Stiffness	Yes	No
Number of joints involved	Yes	Yes
Arthritis of hand joints	Yes	Yes (a subset of joints)
Symmetric arthritis	Yes	No
Rheumatoid nodules	Yes	No
Rheumatoid factor	Yes	Yes
Radiographic changes	Yes	No
Anti-Citrulline antibodies	No	Yes
Duration of symptoms	Yes	Yes
CRP or ESR	No	Yes

Table 1 – Comparison between the 1987 and 2010 diagnostics criteria for rheumatoid arthritis

Risk factors for rheumatoid arthritis

Known risk factors for diseases can often be divided into inheritable and environmental. In RA, it has been estimated using twin studies that the genetic contribution of the disease is approximately 60%[14]. By now, many studies investigating genetics have been carried out for RA. By far the largest genetic components in RA is the presence of certain HLA-DR alleles which can either predispose for the development of RA, in the case of the “shared epitope” alleles, but can also be protective in the case of HLA-DRB1*13[15,16]. Also, PTPN22 is a commonly discussed gene in which SNPs have been associated with the development of RA, although many more genes, which each seem to contribute minimally to RA development have been identified[17].

The main environmental risk factor thought to induce RA is smoking, which is interestingly enough a risk factor for many other (autoimmune) diseases as well[18,19]. Other risk factors described include infections, for example during periodontitis or changes in the microbiome, but these are less evident, or were investigated less when compared with smoking[20].

Pathogenesis of Rheumatoid arthritis

At the moment, little formal evidence is present for the pathogenesis of RA. Also, a large proportion of data at this point is derived from mouse models which may not resemble the human disease process. However, most agree that the interaction between large numbers of different genetic and environmental factors result in a complex interplay of the immune system, possibly inducing a positive feedback loop and eventually chronic inflammation. Although many hypotheses and theories have been developed regarding the pathogenesis of RA, there still remains much to be elucidated.

One of the more general theories for the development of autoimmune diseases is based on molecular mimicry[21]. Initially, an immune response will develop towards a foreign (pathogen-derived) epitope. However, (part of) this epitope may be similar or equal to epitopes already present in the human body, resulting in a break of tolerance against these particular epitopes. A specific example of possible molecular mimicry in RA focuses on the DERAA-sequence which are sequences that fit well in the HLA-DR shared epitope molecules. This sequence is present in multiple pathogens, including influenza A, but can also be found in human self-proteins, such as vinculin. Eventually reactivity towards these pathogens may result in autoimmunity towards (citrullinated) vinculin[22]. This particular theory can be well combined with the genetic risk factors for RA.

A second model focusses on mucosal sites, mainly the lung, as an initiation site for the break of tolerance in RA patients[23]. An initial indication for the involvement of the lung in RA comes from studies that show that early, untreated RA patients seem to have increased inflammation in the lungs[24]. This theory also incorporates the increased risk on RA when smoking. It has been described that smoking may increase inflammation (and citrullination) in the lung, resulting in a break of tolerance and eventually autoantibody production. Why the joint would be targeted in such a situation is currently unclear.
Besides these models of initiation, there are also several cells that are known to play a role in the pathogenesis of RA, although their exact role and whether their presence is cause or consequence are not known. Cells that are very common in the synovial fluid of RA patients, namely neutrophils may be involved in disease in several manners. One of the aspects may be through their general immune functions such as degranulation or the formation of neutrophil extracellular traps (NETs)[25,26], which may contribute to the general immune reaction within the synovium.

Also, neutrophils may provide several antigens in the form of post-translational modifications such as carbamylation, citrullination or malondialdehyde-acetaldehyde modifications, by providing components that promote these modifications [27,28].

Furthermore, T cells are often thought to contribute or even cause autoimmune diseases. Also for RA, the genetic associations seem to indicate that a role for T cells seems likely. However, directly targeting the T cells by depleting them in RA, does generally not result in resolution of the disease, and it has been suggested that a the correct balance of T cell subsets, such as the right amount Tregs, Th1 and Th2 cells is important[29,30]. Another role for T cells may come in the form of T-B cell interactions during which B cells undergo class switching and somatic hypermutation. This may also occur within the joint of RA patients, since tertiary lymphoid structures have been detected in some patients[31].

A final cell type that is thought to be involved in RA is the B cell. Targeting this cell type, for example with rituximab, can be an effective treatment strategy in RA[32], indicating that there is indeed a role for B cells as well. What this role is, is at the moment unclear, but at this point, most research with regards to B cells in RA seems to focus on antibody production and their respective antigens in RA patients.

Autoantibodies in rheumatoid arthritis

The production of autoantibodies in RA has become an important disease characteristic, also indicated by their incorporation into the diagnostic criteria. One of the first autoantibodies discovered in RA is RF, which targets the Fc tail of other antibodies and therefore has the potential to form large immune complexes[33]. However, the specificity of RF for RA is relatively low when compared to ACPA, antibodies that recognize citrullinated proteins or peptides[34,35]. Citrullination is a process of post-translational modification that is carried out by PAD enzymes, which are able to convert arginine residues into citrulline residues.

ACPAs were shown to associate with joint damage and can often be identified before disease onset[36,37]. Furthermore, epitope spreading before disease onset has been observed for these autoantibodies[38,39]. Importantly, most of the genetic association discussed are mainly found for the ACPA+ RA subset. Besides the presence of RF and ACPA, many other autoantibodies have been described in RA. Just as ACPA, some of these antibodies also target a posttranslational modification. Previously described modifications that can be recognized by autoantibodies in RA include acetylation, malondialdehyde modifications and carbamylation[40-42]. Of these, autoantibodies targeting carbamylation proteins have been described most extensively[43].

Carbamylation

Carbamylation is a post-translational modification mainly affecting lysine residues.

The carbamylation of a lysine residue is a chemical process carried out in the presence of cyanate and resulting in a homocitrulline (figure 1)[44]. Besides lysine modification, some other amino acids and the n-terminus can also be modified by cyanate, although these are thought to be less common than lysine modification. The homocitrulline, as the name implies, is rather similar to a citrulline, the last being only a Ch2 group shorter.

At the moment, there are two pathways known that can result in cyanate in the human body. It is not clear how much each of the pathways contributes to carbamylation, although one pathway is constantly present while the other pathway seems to require inflammatory conditions. The more constant production of cyanate is derived from urea, which is in equilibrium with cyanate[45]. This is a pathway that is increasingly involved in renal disease, since an increase in urea can be observed in patients with renal failure. The second method to generate cyanate involves the conversion of thiocyanate in a reaction with myeloperoxidase (MPO) and H2O2[46]. This pathway may be driven by inflammation, which increases the presence of MPO. Smoking may also contribute by increasing thiocyanate levels[47,48].

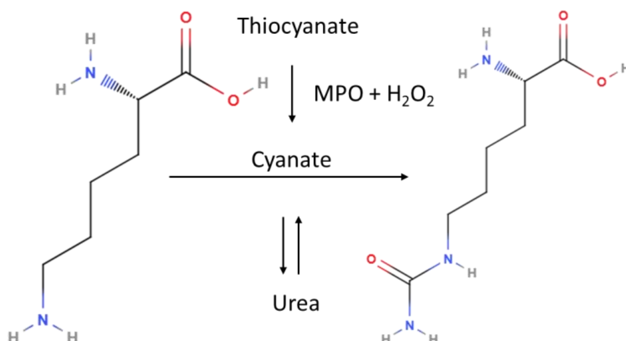

Figure 1 – A lysine is converted into a homocitrulline in the presence of cyanate. Cyanate is generally in equilibrium with urea but can also be formed from thiocyanate by myeloperoxidase (MPO). Structures were made with MolView.

The knowledge on carbamylation in health and disease at this moment is limited. Most studies investigating carbamylation focus on renal disease. Here the amount of carbamylation in serum may help to predict mortality[49], and to distinguish between acute or chronic disease[50]. Upon hemodialysis, levels of carbamylation in the blood of renal disease patients decrease again[51]. Furthermore, the levels of carbamylation may also associate with heart failure in renal disease patients[52], or with the severity of coronary artery disease in the general population[53]. Interesingly, in mice the total amount of carbamylation seems to increase with age[54].

With regards to RA, only one study investigating carbamylation in the foot of one RA patient has been carried out[55]. Homocitrulline residues were indeed detectable in several joint biopsies, but no obvious differences were observed between inflamed and non-affected biopsies.

The direct effect of the carbamylation process on the function of a protein is difficult to study, since the modified lysines of the protein in vivo might be different from the modied lysines when in vitro carbamylation is carried out. However, studies have shown that carbamylation of IgG may prevent complement activation[56] and carbamylation of low-density lipoproteins may increase cell death of endothelial cells[57]. However, the question remains whether the carbamylation in vivo is enough to actually establish such effects.

Anti-CarP antibodies

The existence of anti-CarP antibodies was described by Shi et al, showing that anti-CarP antibodies are increased in RA [43]. The presence of anti-CarP antibodies has also been described for other conditions, including juvenile arthritis, psoriatic arthritis, sjögren syndrome and other non-RA arthritic diseases[58-61]. However, levels and percentage of people positive for anti-CarP antibodies are much higher in RA patients than in the other conditions in which these antibodies have been described.

Besides an increase of autoantibodies in RA, many other clinical aspects have been investigated. For example, the presence of anti-CarP antibodies associates with joint damage over time, also when patients are ACPA-negative[43]. This indicates that there may be a role for the immune response against carbamylated proteins in the pathogenesis of RA. Supporting this hypothesis are the data that show that anti-CarP antibodies are present years before disease onset[62], which also indicates that anti-CarP antibodies alone are not sufficient to induce RA. In arthralgia patients, the presence of anti-CarP antibodies does however associate with the development of RA, indicating that anti-CarP antibodies may also be an interesting biomarker in the early phase of RA development[63]. Another aspect that has been investigated with regards to anti-CarP antibodies are genetics. Interestingly, at the moment, no such associations are known. One study found no independent association with well-known RA genes such as the HLA shared epitope alleles and *PTPN22*[64]]. This study also showed no correlation between anti-CarP antibodies and smoking.

Outline of this thesis

Although quite some data is available on anti-CarP antibodies, several questions remain unanswered, including the reproducibility of the clinical data on anti-CarP antibodies, such as their presence before disease onset and association with joint damage. It is also unknown whether these findings can be expanded to non-caucasian populations. Furthermore, it is unclear how anti-CarP antibodies are induced, what proteins they recognize, whether they are able to recognize multiple carbamylated proteins and what the characteristics of these autoantibodies are. Here, some of these questions will be answered.
In this thesis, I will first discuss the clinical implications of the presence of anti-CarP antibodies compared to RA-specific autoantibodies in both RA patients other relevant conditions (chapters 2 till 7). This is followed by more detailed investigations into the characteristics of anti-CarP antibodies and their antigens (chapters 8 till 12).

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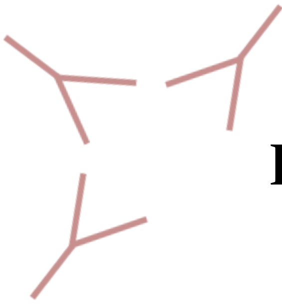
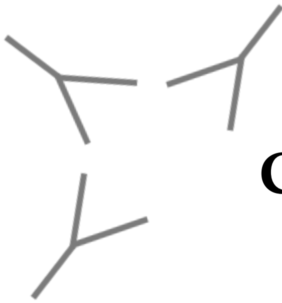


Part I

Clinical characteristics of anti-CarP antibodies



Chapter 2

Biomarkers for rheumatoid and psoriatic arthritis

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Rheumatic diseases, such as rheumatoid and psoriatic arthritis are systemic inflammatory conditions characterized by a chronic form of arthritis, often leading to irreversible joint damage. Early treatment for patients with rheumatic diseases is required to reduce or prevent joint injury. However, early diagnosis can be difficult and currently it is not possible to predict which individual patient will develop progressive erosive disease or who may benefit from a specific treatment according to their clinical features at presentation. Biomarkers are therefore required to enable earlier diagnosis and predict prognosis in both rheumatoid arthritis and psoriatic arthritis. In this review we will examine the evidence and current status of established and experimental biomarkers in rheumatoid and psoriatic arthritis for three important purposes; disease diagnosis, prognosis and prediction of response to therapy.

Introduction

The term rheumatic musculoskeletal diseases (RMD) encompasses a large and varied group of diseases, that share a number of features such as the involvement of connective tissues, muscles and the joints. In addition to similarities, there is also significant variety across the RMD spectrum including inflammatory and non-inflammatory diseases. Rheumatoid arthritis (RA) and psoriatic arthritis (PsA) are two of the most prevalent inflammatory RMD while diseases such as osteoarthritis and fibromyalgia represent the main non-inflammatory conditions. RMD can also be classified according to duration of symptoms or impact on function. The duration may be acute, remitting or chronic persistent and the impact on the subject may vary from mild to severe, often depending on the level of inflammation or tissue damage. The level of inflammation is often quite different in patients with RA and PsA even though both may result in joint damage while fibromyalgia, which is painful, is not associated with inflammation or tissue damage. The signs and symptoms of RA and PsA may be quite similar especially at the earlier phases of disease, so it may be difficult to distinguish between them on clinical grounds, although early treatment may prevent the development of disability in both conditions if introduced appropriately[1,2].

RA occurs in 0.5-1% of the adult population globally [3]. The main characteristics of RA are stiffness and swelling of the joints as a result of inflammation of the synovium, which normally is a thin translucent membrane lining the non-articular surfaces of the joint. The synovium may proliferate and invade surrounding structures leading to damage of the articular cartilage and erosions of the periarticular bone. The cause of RA is not clear, although both genetic and environmental factors have been identified to play a role in disease initiation and progression. RA patients exhibit an increased frequency of cardiovascular disease, a higher susceptibility to infections and have an increased risk for certain malignancies [3].

PsA occurs in 10-40% of psoriasis patients [4,5]. Psoriasis is characterized by red, thickened and inflamed skin lesions and affects up to 3% of the general population.

In addition to the skin lesions, patients may develop a chronic arthritis of the peripheral and/or axial joints, characterized by inflammation of the synovium and erosions similar to but distinct from RA. Classified as one of the spondyloarthropathies, due to axial joint involvement similar to ankylosing spondylitis, patients may also exhibit enthesitis, uveitis and nail disease [4,5]. PsA patients, similar to RA, have an increased mortality due to cardiovascular disease, however there is no evidence of increased susceptibility to infections or lymphoma when compared to the general population[6].

The signs and symptoms of RA and PsA patients, including systemic features such as skin and eye manifestations, appear to respond well to anti-inflammatory drugs (corticosteroids and non-steroidal anti-inflammatories (NSAIDs)) and disease-modifying anti-rheumatic drugs (DMARDS) such as tumour necrosis factor inhibitors (TNFi) . For some other biological agents there may be a differential response when comparing RA and PsA patients[7,8].

Biomarkers

Biomarkers may be defined in several ways. A simple definition proposed by the US Food and Drugs Administration (FDA) is; 'Any measurable diagnostic indicator that is used to assess the risk or presence of disease'. However the US National Institutes of Health (NIH) has suggested a more comprehensive definition of a biomarker - 'A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic intervention'. The NIH definition encompasses the concept of response to therapy, which is becoming more relevant and therefore more important in the context of RA and PsA.

Therapies for RA and PsA patients have developed rapidly in the past decade such that great improvements in signs and symptoms, but also in quality of life and function, have been realised. However, many patients do not respond to the first treatment that is offered, leaving room for substantial improvements [7,8]. Also, in both RA and PsA, early treatment is important in order to prevent irreversible joint damage [1,2]. In order to treat patients in an early stage of the disease, it is essential to determine which of the patients that visit the doctor with psoriasis or joint pain will eventually develop PsA or RA respectively. Only the patients that do acquire PsA or RA will benefit from the treatment, while people who do not develop severe disease might suffer from unnecessary side effects. Furthermore, not all treatments are effective in each patient and treatments are often given on basis of trial and error [7,8]. It would therefore be useful to predict which RA and PsA patients will benefit from a specific treatment.

In this review, we describe the biomarkers that are generally accepted for PsA and RA, after which we will discuss a selection of interesting biomarkers that are still under investigation.

This will include biomarkers that are used to improve diagnosis, to predict prognosis and to identify response to treatment.

Autoantibodies

For RA patients, one of the most important type of biomarkers at the moment are autoantibodies. The most recent criteria for the diagnosis of RA were described in 2010 [9]. Besides joint pain and inflammation, several serological biomarkers are used to classify RA patients. Serological biomarkers, described in the new criteria, include autoantibodies such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA).
Currently there is little evidence for a role of autoantibodies in PsA, as rheumatoid factor is mainly absent in PsA, so that the CASPAR classification of PsA includes rheumatoid factor negativity as an independent diagnostic criterion [10]. Indeed, also the autoantibodies to citrullinated proteins are mostly absent in 90% of the PsA patients [11,12]. There is one recent report of autoantibodies, against fibrillin 3 and desmocollin 3, crossreacting with a shared epitope common to both the skin and the joints that may suggest some as yet unidentified autoantibodies may be associated with PsA [13]. Since the role of autoantibodies as a biomarker in PsA is almost non-existent, we will now discuss the different autoantibodies that have been discovered in RA and shortly explain the relevance of these antibodies as a biomarker.

ACPA and RF

The first autoantibody that was discovered in RA patients is RF, which is present in 60-80% of the RA patients. The antigen that RF binds to is the Fc-region of an IgG molecule [14]. RF has a low specificity, since it can also be found in healthy controls and patients with other rheumatic diseases [15]. But even though RF has a low specificity, it has been used extensively to diagnose RA for a long time, since no better alternatives were available. At least, not until ACPA were discovered. ACPA bind to a different type of antigen than RF; proteins that contain the amino acid citrulline. These citrullines are formed postranslationally by the deamination of arginine, an enzymatic process facilitated by PAD enzymes. In diagnostics, ACPA have a higher specificity and sensitivity than RF [15,16]. The current diagnostic tests to identify ACPAs are mostly based on assays that use cyclic citrullinated peptides (CCP) as antigen [17].

Furthermore, ACPA have been described as a factor that is associated with disease prognosis, since RA patients that are ACPA+ develop a more severe disease compared to ACPA- patients [18,19]. Also with regards to treatment responses, ACPA+ patients seem to respond better to treatment than ACPA- RA patients in an early phase of the disease, but achieve drug-free remission less frequently [20]. Research has also been carried out with regards to the fine specificity of ACPA [21,22]. Several analytical platforms such as ELISA, chips and SPR have been developed to investigate this subject, but so far there is no clear evidence that one of the ACPA fine specificities is a superior biomarker as compared to testing for CCP [23-25].

However, the number of different epitopes recognized may provide information on the process of disease development [26,27]. Another important aspect of disease progression, involves the increased chance to develop cardiovascular diseases that has been observed in RA patients. Both ACPA and RF have been found to associate with cardiovascular disease and mortality in RA patients [28,29].

Anti-carbamylated protein antibodies

Besides RF and ACPA other autoantibodies that may function as useful biomarkers, were identified in RA patients. A first example of these autoantibodies, are anti-Carbamylated Protein (anti-CarP) antibodies, which were recently described by Shi et. al [30]. These antibodies can be found in 45% of early RA patients and are also present in 16% of the ACPA-negative RA patients. This observation, initially made in Dutch patients, was later confirmed in a large Swedish cohort [31]. Anti-CarP antibodies are present more than 10 years before disease onset [32-34] and are associated with development of RA in arthralgia patients [35]. This combination makes anti-CarP antibodies an interesting biomarker for early diagnosis of RA. However, the specificity of anti-CarP antibodies has to be determined by testing the presence of these antibodies in other rheumatic diseases.
As a biomarker associated with disease severity, anti-CarP antibodies seem to be quite promising. The antibodies were found to be associated with a more severe clinical picture, including an increase in joint damage in ACPA-negative RA patients [30]. Similar observations are also made independently in other cohorts [34,36]. Since the presence of anti-CarP antibodies can also be detected in early disease stages [33-35], it may be interesting to adjust treatment of RA patients depending on their anti-CarP status. However, research investigating the response to treatment in both anti-CarP-positive and –negative patient groups is still in progress.

Anti-malondialdehyde and anti-malondialdehyde acetaldehyde antibodies

Besides citrullination and carbamylation, other autoantibodies against posttranslational modifications were identified. In this case, the posttranslational modifications are due to lipid peroxidation, This can result in the presence of malondialdehyde (MDA) and malondialdehyde-acetaldehyde (MAA) – adducts. Antibodies against MDA-adducts, especially MDA-LDL, were identified quite some time ago and were found to associate with cardiovascular problems in RA patients [37], but very little follow-up work has been carried out on this subject. Interestingly, a recent study also investigated the presence of autoantibodies against MAA adducts, which are more stable than MDA-adducts alone and are therefore more likely to be present in vivo. It was found that both MAA adducts and antibodies directed against these adducts were increased in RA patients [38]. However, MAA-antibodies are not very specific, since they have also been detected in people with liver disease and type 2 diabetes [39,40]. Therefore, these antibodies are most likely not very suitable for diagnostic purposes. Furthermore, a positive correlation between the presence of ACPA and anti-MAA antibodies was observed [38].

However, to what extent the presence of anti-MAA adds clinically relevant information on top of ACPA in ACPA-positive patients or in their own right in ACPA-negative patients remains to be established.

Anti-PAD4 antibodies
Autoantibodies directed against PAD4 have also been identified [41,42]. PAD4 is a peptidylarginine deiminidase, one of the proteins that is responsible for the conversion from arginine to citrulline. Anti-PAD4 antibodies were increased in RA when compared to disease-controls and present in 22% - 45% of the measured RA patients [41,42]. Anti-PAD4 antibodies can be detected in 14% of SLE patients [42], but seem to be absent in spondyloarthritis patients [43]. The diagnostic value of anti-PAD4 antibodies, may not be very high since the specificity seems to be lower than 50% [41]. Furthermore, a specific group of anti-PAD4 antibodies that cross-reacts with anti-PAD3 antibodies has also been described [44]. These antibodies can be found in 12%-18% of the RA patients. Anti-PAD3 antibodies can only be found in anti-PAD4 positive RA patients. Interestingly, the cross-reactive antibodies were associated with the severity of radiographic damage [44], so these anti-PAD3/4 antibodies may serve as biomarker for disease prognosis, although they can only be detected at low frequency in RA patients.

BRAF
PAD4 was one of the proteins identified in a proteomic approach aimed at the identification of autoantigens [43]. In the same study, a second antigen, BRAF, a serine-threonine kinase involved in the MAPK pathway, was identified [43]. Of the RA patients, 21 - 32% have been found positive for anti-BRAF antibodies [43,45]. But again, specificity seems to be a limitation, since anti-BRAF antibodies were also present in SLE and primary Sjogren's syndrome in almost similar percentages. The use of these autoantibodies for other purposes has been suggested, but requires more research as well.

RA-33
Another type of autoantibody targeting an intracellular molecule is RA-33, which binds to heterogeneous nuclear protein (hnRNP) A_2, a part of the splicosome. RA-33 is found in one third of RA patients and does not seem to correlate with ACPA or RF, but the frequency of RA-33 in patients negative for ACPA or RF is relatively low. [46,47]. In the RF and ACPA-negative patients, the amount of RA patients that could be identified was 13%, while 9% of the non-RA patients were also positive for RA-33. Since the window between RA and non-RA is very small, it may not be useful as a clinical biomarker for diagnosing RA. Interestingly, patients that are positive for RA-33 do often show a less severe disease development than RA-33-negative patients, so RA-33 might serve as a good marker for prognostic purposes [46].

Other autoantibodies

There are many other autoantibodies that have been suggested as potential diagnostic biomarkers. However, many of these studies lack power, due to small patient numbers. For example, autoantibodies against transthyretin, a hormone carrier, were found to be slightly increased in RA patients when compared to healthy controls, but this was only tested with samples derived from 60 patients [48]. Similar numbers were used in a study that proposed that tryptase was another autoantigen recognised in RA patients [49]. A larger cohort on the other hand, was used to measure anti-agalactosyl IgG antibodies in RA patients [50]. These types of studies are very interesting with regards to the identification of new autoantibodies in RA patients, but should be expanded or require follow-up in order to gain enough power for solid conclusions.

Combining several antibodies, may potentially be a better method to identify those patients most at risk for the development of RA. Therefore, another method that has been used to identify autoantibodies is the use of a cDNA phage display library of RA synovium, which was screened for antigen reactivity, using pooled RA plasma samples [51] Eventually, 11 RA-specific sequences were identified, the combination of which, resulted in reactivity of 50 – 58% of the RA patients plasma samples, from RA patients. Also, increased positivity for these autoantibodies was also associated with higher CRP levels, indicating an increase of inflammation [51]. The most widely used autoantibody combination in the clinic consists of IgM-RF and anti-CCP as these are part of the 2010-RA criteria [9] but currently significant efforts are ongoing to look into combinations of the above described antibodies in providing optimal clinical utility regarding the diagnosis, prognosis and prediction of medication efficacy.

Other serum biomarkers

Besides autoantibodies, there are of course many other factors that have been investigated in RA patients. Cytokine levels for example were measured in order to investigate the pathogenesis of RA, although these levels appear to vary widely at different time points with little relationship to disease activity, but some may be useful as a possible biomarker. Also, many different proteins have been found increased or down regulated in RA patients, which might also make these interesting biomarkers. Therefore, in this part we will describe some examples of other proteins besides antibodies that may be relevant biomarkers for the clinical management of RA patients.

Type 1 IFN-signature

Besides the direct measurement of cytokines, the effects of these cytokines on gene expression can also be measured. In RA patients, peripheral blood was isolated in order to measure gene expression levels. A subset of RA patients with a specific phenotype could be identified based on the expression levels of interferon (IFN) type I response genes [52]. This so-called Type 1 IFN-signature can also be adjusted to predict which patients will not respond to rituximab treatment [53].

14-3-3η

The 14-3-3 protein family consists of 7 isoforms that are intracellular chaperonin, present in eukaryotic cells. The levels of one of these isoforms, 14-3-3η is increased in RA patients and may therefore serve as an additional diagnostic marker for RA patients. It has a sensitivity of 73% and a specificity of 93%, which are quite promising numbers [54]. Futhermore, the presence of these proteins appear to be associated with increased joint damage [54,55].

Combinations of serum biomarkers

Different combinations of biomarkers have been commercially developed as a diagnostic test, for example the multi-biomarker disease activity (MBDA) test developed by Crescendo. This test measures 12 serum components using a multiplex format [56]. An algorithm is used to calculate the MBDA score from the acquired data. This score shows a correlation with DAS28 levels [57]. Furthermore, it has been suggested that these biomarkers may predict joint damage in RA patients [58].

In addition, serum proteins have been identified as possible markers for diagnostic purposes. Some proteins or posttranslational modifications thereof have been reported together with their respective autoantibodies. There are many more proteins that are increased in RA patients, but most of these are only slightly elevated when compared to healthy controls, which leaves them unsuitable for diagnostic purposes. L-Ficolin, for example was increased in RA patients, however there was a large overlap between healthy controls and RA patients [59]. Interestingly, M-ficolin has been reported to associate with disease activity and may predict remission in RA patients. The overlap between healthy controls and RA patients seems to be smaller for this ficolin [60]. It would certainly be interesting to measure both ficolins in the same cohort in order to compare the two.

Synovial tissue biomarkers

The antibodies and proteins measured above are mostly measurable by serological tests and do not require invasive methods for analysis. There are, however, other potential biomarkers, such as biomarkers that can be found in the synovial tissue at biopsy. Biomarkers that are derived from the inflamed tissue may be more reliable in predicting the local disease status and response to therapy, since the biomarkers are derived from the target tissue of the disease.

Cellular infiltrates

Extensive studies of synovial tissue markers have been performed over the last 2 decades that have revealed some specificity in relation to PsA and RA patients [61]. The first observations focused on differences in cellular infiltrate and a marked increase in vascularity [62,63] and vascular growth factors expressed in PsA synovial tissue, generally at significantly higher levels than in RA [64]. In RA synovial tissue several multi-centre studies have identified the monocyte/macrophage cells to be a significant and validated biomarker of disease activity and of response to therapy[65].

In PsA there are significantly less macrophages and the lining layer appears to be normal in depth compared to the hyperplastic features in RA [62]. The exact reasons for these differences have not yet been defined, however the changes first noted at a cellular level have been supported with studies of molecular pathways suggesting that molecular expression of vascular growth factors is significantly higher in PsA synovium [64].

In RA the most well-defined and validated tissue biomarker is the CD68 molecule expressed on the surface of activated macrophage cells in the synovial lining layer and in the sub-lining stroma [66]. Expression of CD68 has been validated in several proof-of-concept studies and applied in clinical trial cohorts also demonstrating a significant association with disease activity and response to therapy [67]. In addition, the B cell lineage marker CD20 has been identified in synovium as a potential cellular biomarker, that may complement circulating autoantibodies in predicting a patients response to therapy with Rituximab [68]. There is at least one study that suggests CD68 may not be a useful biomarker in PsA [69] while in the same study there is some evidence to suggest that CD3 positive T cells may be a useful biomarker of disease activity and response to therapy in the synovial tissue of PsA patients. In the synovia of individuals with arthralgia, the presence of CD3 positive cells was weakly positively associated with future development of RA . Surprisingly in the same study the authors noted an association between the presence of CD8 positive cells and ACPA positivity [70].

<u>New methods for biomarker discovery</u>

The general utility of synovial tissue biomarkers is questioned as it does involve an invasive procedure to obtain tissue by either needle biopsy, increasingly undertaken with ultrasound guidance or under direct visualisation at arthroscopy [71]. It is currently hoped that using patient stratification models it may be possible to discover an association between a relevant synovial tissue biomarker and one that can be easily measured in peripheral blood samples. There have been rapid advances in technology in the last number of years, not least of all, an '-omic' approach to biomarker discovery including genomics, proteomics and transcriptomics. In a recent publication Villanova and colleagues have suggested a 'new approach' that will employ multiple 'omic' technologies to discover new biomarkers, that can subsequently be validated, at an analytical and a clinical level, and only then qualify and be commercialized into a standardized assay for clinical usage [72]. These techniques have also been applied to synovial tissue biopsies and to serum samples from RA and PsA patients yielding some preliminary but interesting findings. There does appear to be increased expression of metalloproteinase enzymes in synovium around the cartilage-pannus junction [73]. Furthermore, the circulating levels of MMP, in particular MMP-3, does appear to predict the prognosis with respect to joint damage in early RA [74,75]. Circulating MMP3 levels have also been shown to be independently associated with the response to TNFi therapy in PsA patients [76].

At least one study has also shown that changes in circulating cartilage biomarkers in patients with RA and PsA correlate with disease activity, radiological progression and the response to therapy with TNF inhibitors [77].

There is a large international consortium effort underway to analyse circulating biomarkers in over 1000 PsA patients, as part of an Omeract/GRAPPA programme, examining C-reactive protein, serum amyloid-A; the collagen biomarkers C2C, C1,2C and CPII; MMP3, as mentioned above; markers of bone turnover including Dickkopf-1, sclerostin, bone alkaline phosphatase, C-telopeptide fragments of type II collagen (CTX-II), CTX-1, receptor activator of nuclear factor-κB ligand, and osteoprotegerin [78]. Independently, a group from New Zealand has recently published evidence of biomarkers of bone remodelling such as Dkk-1 and M-CSE are associated with bone erosion and underlying osteoclastogenesis in PsA patients [79].

Genetic markers may be useful in PsA patients, however presently do not have a role to play as biomarkers of diagnosis – HLAB27 is present in up to 70% of cases, and there are associations with HLACw6, PSORS1 and the IL23 receptor among others [72]. The utility of genetic markers of PsA in clinical practice is limited and to date is confined to the research agenda. As outlined above they may be incorporated into a multi-'omic' approach to biomarker development. However, it should be noted that the detailed studies in RA patients have demonstrated a clinical utility of combined biomarkers including genotype, circulating autoantibodies with environmental risk factors, especially cigarette smoking to identify prognosis [80], such studies have not yet been performed in PsA patients.

Conclusion

When comparing the biomarkers between RA and PsA, a clear difference is the presence of autoantibodies, which are observed in RA patients, but not in PsA patients. In RA patients, ACPA and RF are currently in use for clinical diagnosis. It seems that additional autoantibodies identified in RA patients, as yet, may not result in additional value as diagnostic biomarkers, however they may add prognostic information. As many patients are positive for several autoantibodies it is interesting to combine different autoantibodies to investigate whether combining this information will provide more insight regarding the diagnosis, prognosis and prediction of response to therapy. Many apparently healthy people present with one or more autoantibodies, even years before disease onset, and it remains a focus of current research whether healthy subjects in the general population with circulating antibodies are predetermined to develop RA.

Interestingly, even though PsA and RA have many clinical and pathological similarities, regarding biomarkers there do appear to be significant differences, suggesting differences in disease process and mechanisms. In PsA patients the key biomarkers , especially in early phase disease appear to be related to the vascular factors of the inflammatory response.

Biomarkers in the skin and the synovium including angiogenic growth factors do appear to be relevant. It remains to be seen if the measurement of these factors in the peripheral circulation are useful either in the diagnosis or the determination of prognosis in patients with PsA. In addition, the new approach of biomarker discovery in psoriasis and PsA using gene signature data to identify key molecules and their links with the clinical phenotype may yield interesting results in the near future.

Many different biomarkers have been identified in RA and PsA some shared, although most differ between diseases. In many studies, only one biomarker is investigated at a specific timepoint. In order to acquire a clear overview with regards to the usefulness of all different biomarkers, an important aspect of future research would be to measure large numbers of biomarkers longitudinally in the same cohort. In this manner, data can be analysed using a systems biology approach to discover the important associations with disease. This would also allow the identification of the best set of biomarkers to predict prognosis and possibly the response to specific treatments. The first major challenge for the future of biomarker research in RA and PsA is to identify specific circulating biomarkers for the diagnosis of PsA and prognostic markers useful for both RA and PsA. The next major challenge is to develop specific biomarkers to identify RA and PsA individuals at high risk of progressive damage and to predict response to specific therapies.

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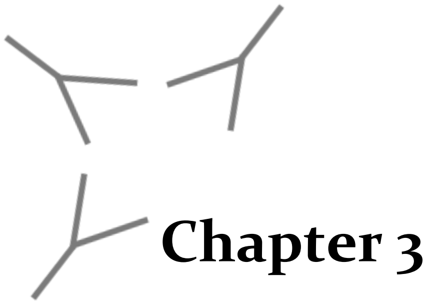

Chapter 3

Anti-Carbamylated protein antibodies precede disease onset in monkeys with collagen-induced arthritis

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Rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA) and anti-carbamylated protein (anti-CarP) antibodies are rheumatoid arthritis (RA)-associated autoantibodies. Besides their presence in human sera, anti-CarP antibodies have also been described in rodent models of arthritis, while ACPA are not consistently detectable. Data on these RA-associated autoantibodies in primates is still incomplete. Therefore, we investigated the presence of RF, anti-CarP antibodies and ACPA in rhesus monkeys before and after collagen-induced arthritis immunizations.

Arthritis was induced in groups of Rhesus monkeys by immunisation with collagen following pre-treatment with either placebo, abatacept or roactemra. Autoantibodies were measured by ELISA, detecting anti-CarP antibodies, RF-IgM and antibodies against CCP2, citrullinated myelin basic protein and citrullinated fibrinogen.

Out of the three autoantibodies, only anti-CarP antibodies were detectable in resus monkeys with arthritis. RF-IgM and ACPA were undetectable and below the detection limit of the ELISAs. The level of anti-CarP antibodies increases over time and, similar to human and mice, these autoantibodies were detectable already before clinical disease onset. Furthermore, preventive treatment with abatacept (CTLA4/IgG1-Fc fusion protein) inhibited the development of anti-CarP antibodies after immunization, while this was less evident for preventive roactemra (anti-IL6-receptor) treatment. Moreover, disease progression was only reduced following abatacept treatment.

In conclusion, rhesus monkeys develop anti-CarP antibodies upon induction of collagen-induced arthritis, while we were unable to detect RF of ACPA. Also, the development of anti-CarP antibodies could be inhibited by preventive abatacept treatment.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disorder characterized, among others, by the presence of autoantibodies. Two such autoantibodies, rheumatoid factor (RF) and anti-citrullinated proteins antibodies (ACPA) have been incorporated into the classification criteria for RA[1]. Other autoantibodies have been identified more recently[2], including anti-carbamylated protein antibodies (anti-CarP)[3]. These antibodies target carbamylated proteins in which a lysine has been converted into a homocitrulline under the influence of cyanate. ACPA on the other hand target citrulline, which arise after enzymatic conversion of arginine[4].

Anti-CarP antibodies have been detected in sera of RA patients in several cohorts around the world and were shown to associate with the development of joint damage[3,5-8]. These antibodies could be detected several years before disease onset and associate with development of RA in arthralgia patients[5,9,10]. Furthermore, anti-

CarP antibodies can recognize multiple carbamylated antigens, such as fibrinogen and alpha-1 antitrypsin[3,11]. Anti-CarP antibodies were also detected in different rodent models for arthritis[12,13]. RF(-like) antibodies have also been described in both rodents and human[14]. While the presence of ACPA is evident in RA, they are largely undetectable in rodents[1,3,12,15].

The combination of these autoantibodies has not been measured in primates yet. We now used the collagen-induced arthritis (CIA) model in rhesus monkeys to establish the occurrence of these autoantibodies in arthritis This model was previously established for the investigation of RA and a large amount of clinical parameters has already been established[16]. To investigate RA-associated autoantibodies in the primate CIA model, we first determined the presence of ACPA, RF and anti-CarP antibodies. Second, we investigated the effect of two different treatments, roactemra (anti-IL6-receptor) and abatacept (CTLA4-IgG1 Fc fusion protein) on the clinical score and autoantibody development after immunization.

Materials and Methods

Animals

This study was conducted at the Biomedical Primate Research Centre (BPRC; Rijswijk, the Netherlands) in accordance with the Dutch law on animal experimentation. The study protocol and experimental procedures were approved by the Experimental Animal Care and Use Committee of the BPRC. CIA-susceptible adult, healthy rhesus monkeys (Macaca mulatta; BPRC) were selected based on the absence of the dominant major histocompatibility complex class I resistance marker Mamu-B26[17]. Individual data are shown in table 1.

Treatment conditions

The data was obtained from 2 studies reported elsewhere[18,19]. The first study[18] contained a group that was placebo-treated (N = 5) and a roactemra-treated (10 mg/kg; N = 7) group. The second study[19] contained a group that was placebo-treated (N = 5) and an abatacept-treated (10 mg/kg; N = 7) group. In both studies, CIA was evoked by immunization with 5mg of chicken type II collagen (MD biosciences, St Paul, MN) as before. The test substances were administered as described[18,19] (The ethical permits for these studies were obtained under number #633 and #695).

Clinical evaluation

For the clinical and ethical management signs of clinical arthritis, soft tissue swelling and redness of affected joints, were scored twice weekly using a previously published semi-quantitative clinical score[20]. To ensure objective clinical scoring the investigators performing the physical examination and rating clinical scores were blinded to the different treatments during the *in vivo* part of the study.

Study	Treatment	Animal ID	Gender	Age	Starting weight
1	Group I (Placebo)	95020	M	14.8	10.2
		R05029	M	4.9	6.1
		R05053	M	4.8	7.8
		R05058	M	4.8	7.3
		R05073	M	4.8	6.6
	Group II (TCZ)	95031	M	14.8	12.7
		BB226	M	6.8	9.9
		R04042	M	5.9	7.5
		R05059	M	4.8	6.9
		R05061	M	4.8	7.2
		R05089	F	4.8	5.1
		R05090	M	4.7	8.0
2	Group III (Placebo)	R00062	F	12.2	5.2
		R02049	F	10.3	7.7
		R05068	F	7.3	5.9
		R06045	M	6.3	7.2
		R07111	M	5.2	5.6
	Group IV (Abatacept)	96089	F	16.3	8.5
		R01091	F	11.1	5.0
		R05084	F	7.3	6.9
		R07003	M	5.5	6.5
		R07031	F	5.4	4.6
		R07068	M	5.3	7.6
		R07075	F	5.3	5.2

Table 1 - Animal identification, gender, age and starting weight at day of stratification. TCZ: tocilizumab

Measurement of anti-CarP antibodies

Anti-CarP-IgG antibodies were measured as described previously, using carbamylated fetal calf serum as antigenic target[3]. ACPA were measured using an in-house assay with CCP2 peptide, citrullinated fibrinogen(cit-fib, Sigma) or myelin basic protein(cit-MBP, Sigma) as antigen. For each antigen, both the modified and non-modified version were taken along.

For CCP2, streptavidin (Invitrogen) was coated on a plate at 5μg/ml in a carbonate buffer with a pH of 9.6 and incubated at 4 ˚C overnight. Peptides were coated at 1μg/ml in PBS with 1% BSA (Sigma) and allowed to bind for 1 hour at room temperature. Serum samples were diluted 50x in PBS with 1%BSA and 0.05% Tween (Sigma) and incubated for 1 hour at 37˚C.

For Cit-Fib and Cit-MBP, the proteins were coated at 10μg/ml in a 9.6 pH carbonate buffer and incubated overnight at 4˚C. Plates were blocked for 1 hour at 37 ˚C with PBS 2% BSA, pH 9.0. Samples were diluted 50x in RIA buffer and incubated for 1 hour at 37˚C.

For the anti-CarP and anti-citrulline ELISAs, antibody binding to the target was detected using rabbit anti-human IgG-HRP(DAKO, P0214), which is also cross-reactive for the IgG antibodies from rhesus monkeys.

RF-IgM antibodies were also measured with an in-house ELISA, using rabbit IgG (Sigma) as antigen which was diluted to 10μg/ml in carbonate buffer, pH9.6 and incubated overnight at room temperature. Plates were blocked for 1 hour at 37°C with PBS, 1% BSA. Serum samples were diluted 100x in PBS, 1% BSA and 0.05% Tween and incubated for 1 hour at 37°C. Antibody binding was detected with goat-anti-human-IgM-HRP(Life technologies, 627520). Cross-reactivity of this antibody was confirmed by measuring anti-collagen IgM antibodies in a selection of the monkey serum samples.

All washing steps were carried out with PBS, 0.05% Tween. ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) was used for final detection.

<u>Statistics</u>
Statistical analyses were carried out in SPSS statistics version 23 (IBM) or with Graphpad Prism version 7. To compare antibody levels over time within the same animals, a Wilcoxon rank test was carried out. To compare antibody levels between two groups, a Mann-Whitney U test was carried out. A spearman correlation was calculated to investigate a possible association between autoantibodies and clinical parameters.

Results

The presence of RA-associated autoantibodies was measured in 10 rhesus monkeys at the last two available time points after CIA-induction. Anti-CarP antibodies were readily detectable (figure 1A), while rheumatoid factor was not observed (figure 1B). ACPA were measured using 3 different antigens; CCP2 peptide, Cit-fib and cit-MBP, but no specific citrulline-directed signal could be detected (figure 1C). In summary, of the three investigated RA-associated autoantibodies, only anti-CarP antibodies but not ACPA or RF could be detected in CIA monkeys. Therefore, we further investigated the development of anti-CarP antibodies. A comparison of the anti-CarP antibodies between the time of immunization and the last available timepoint shows that the levels of anti-CarP antibodies increase over time (figure 1D). Furthermore, we observed that the anti-CarP antibodies are present before disease onset (Figure 1E). A weak correlation was observed between the presence of anti-CarP antibodies and the clinical score. A similar weak correlation could be identified between anti-CarP IgG antibodies and anti-Collagen IgG antibodies (figure F).

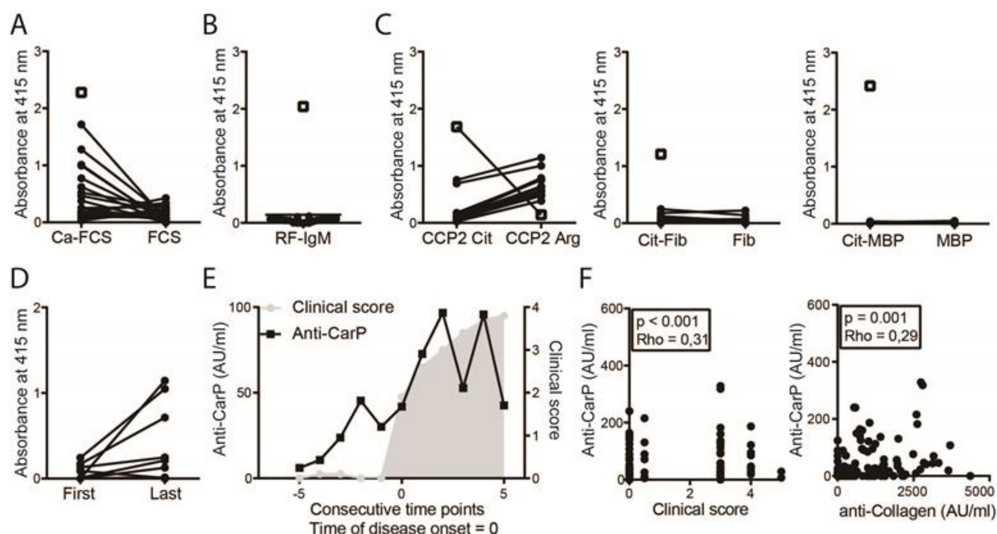

Figure 1 – Anti-CarP antibodies can be detected in rhesus monkeys, while ACPA and RF cannot.
(A) Anti-CarP IgG antibodies were measured by ELISA. Ca-FCS and the non-modified FCS served as the (control) antigen. The open square indicates a human positive control, while the last two available primate samples for each monkey are indicated in closed circles. (B) RF-IgM antibodies were measured by ELISA, using rabbit IgG as binding antigen. The open square indicates a human positive control, while the last two available primate samples for each monkey are indicated in closed circles. (C) ACPA IgG antibodies were measured by ELISA, using several citrullinated and control peptides or proteins as antigens. The open square indicates a human positive control, while the last two available primate samples for each monkey are indicated in closed circles. (D) Anti-CarP IgG antibodies are shown for both the first available sample, taken at the timepoint of immunization and the last available sample are compared to each other. Antibodies were measured as in A. The absorbance values for the FCS were subtracted from the Ca-FCS absorbance values. All samples were measured on one plate. ELISAs were carried out using the 10 control monkeys who did not receive any (preventive) treatment) (E) Anti-CarP IgG antibodies and clinical score are compared over time. The point of disease onset was set at 0. Disease onset was defined as the point at which the clinical score was higher than 0.5 and increased at the next time point.
Therefore, 3 monkeys had to be excluded from this analysis. The grey area indicates the average clinical score, while the black circles show the average levels of anti-CarP antibodies at that particular time point. (F) The presence of a correlation between anti-CarP antibodies and clinical score or anti-Collagen IgG antibodies was investigated. All time points of all 10 monkeys were included for this analysis. The presence of possible associations was tested with the spearman correlation.
Anti-CarP = anti-carbamylated protein antibodies, Ca-FCS = carbamylated fetal calf serum, RF = rheumatoid factor, CCP2 = cyclic citrullinated peptide 2, cit = citrullinated, arg = arginine control, Fib = fibrinogen, MBP = myelin basic protein, AU/ml = arbitrary units per millilitre, ELISA = enzyme-linked immunosorbent assay.

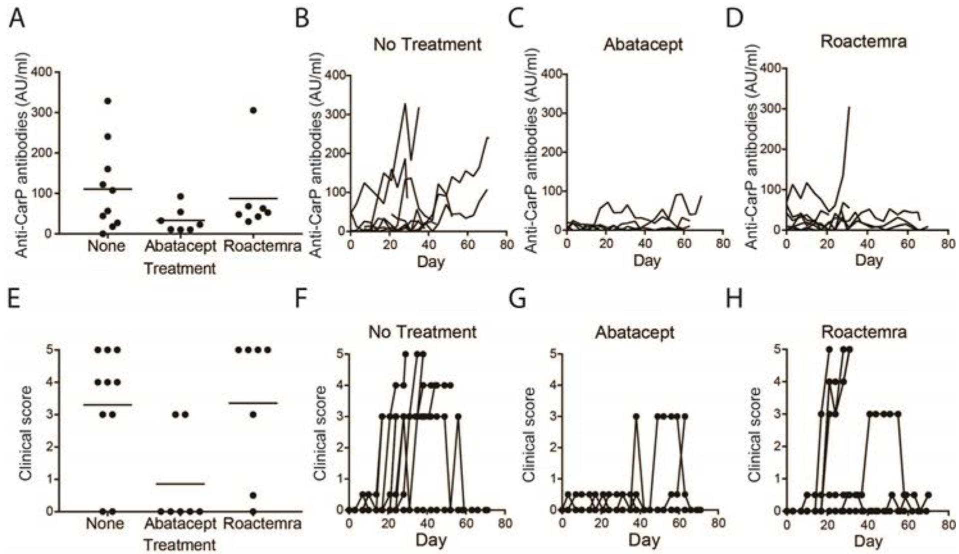

Figure 2 – Abatacept treatment reduces the development of anti-CarP antibodies and affects the severity of collagen-induced arthritis in rhesus monkeys.
(A) Anti-CarP IgG antibodies were measured by ELISA in rhesus monkeys treated with no medication (n=10), abatacept or (n=7) roactemra (n=7). The highest anti-CarP antibody level for each of the monkeys is shown. Timelines for the anti-CarP antibodies over time are shown for no treatment (B), abatacept (C) and roactemra (D). (E) The clinical score is shown in rhesus monkeys treated with no medication (n=10), abatacept or (n=7) roactemra (n=7). The highest clinical score for each of the monkeys is shown. Timelines for the clinical score over time are shown for no treatment (F), abatacept (G) and roactemra (H). Day 0 is the time point of immunization with collagen. The data in figure E-H were presented before in two separate studes [18,19] and are shown here as comparison. Anti-CarP = anti-carbamylated protein, AU/ml = arbitrary units per millilitre, ELISA = enzyme-linked immunosorbent assay.

After the detection and characterization of anti-CarP antibodies in rhesus monkeys, the effect of several (preventive) treatments on these autoantibody levels was investigated as well, using abatacept and roactemra as model treatments. Lower levels of anti-CarP antibodies are observed in the group treated with abatacept when compared to the control group without treatment (figure 2A). Although differences between the abatacept and the no treatment-group are not significant at these small group sizes, a clear trend is visible (Mann-whitney U test, p=0,087). These differences also seem to be consistent over time, showing an increase in anti-CarP antibodies in the control group, while low levels of anti-CarP antibodies were detected in the abatacept treated group (Figure 2B-D). For Roactemra, an increase in anti-CarP antibodies is only seen for one of the animals, but no statistical differences were observed between roactemra and the control group (Mann-whitney U test, p=0,89).

Furthermore, the effect of the treatments on the clinical score was investigated. Out of the three treatment groups, abatacept-treated monkeys show a reduced clinical score (Mann-whitney U test, p=0,02) (Figure 2E). The timelines show that the abatacept-treated animals do not develop full-blown arthritis, while this is not the case for the animals without treatment or for animals treated with roactemra (Figure 2F-H). No differences in clinical score were observed between the no treatment group or treatment with roactemra (mann-whitney U test, p=0,67).

Discussion

Here we have shown that anti-CarP antibodies can be present in rhesus monkeys and show that these autoantibodies are especially increased after the induction of collagen-induced arthritis. Interestingly, ACPA, which target a very similar post-translational modification as anti-CarP antibodies could not be detected in this animal model. In RA patients, there is a large overlap in the positivity for ACPA and anti-CarP antibodies and detailed studies have been performed to confirm that ACPA and anti-CarP antibody positive sera contain both cross-reactive and non-cross-reactive antibodies[3]. Now in the context of both mice[12,15] and monkeys, only anti-CarP reactivity can be observed, indicating that in these animals, anti-CarP antibodies are not cross-reactive to citrullinated proteins. In mice, ACPA have also been difficult to detect, while they are prominently present in human RA patients. The notion that ACPA are difficult to detect in both rodents and rhesus monkeys, indicates that there is a clear difference between the two autoantibodies. Furthermore, RF could not be detected in rhesus monkeys after CIA-induction conform previous data using the same model[16].

We observed a clear effect on anti-CarP antibody levels by preventive treatment with abatacept, which is a fusion protein consisting of the CTLA4-domain that can bind to CD80 or CD86 and the IgG1 Fc region[21]. This treatment is currently used in RA patients when they have failed one or more DMARDs (disease-modifying anti-rheumatic drug). In previous CIA-experiments using abatacept treatment, a general reduction of IgM and IgG antibodies was observed[19], which is in line with the reduction in anti-CarP antibodies.

Roactemra, the other intervention used, is also known under the name tocilizumab and is a monoclonal antibody that targets the IL6-receptor[22]. This is also a receptor that is involved in plasma cell development and might therefore be important for antibody production. As in RA patients, the monkeys which developed collagen-induced arthritis also showed an increase in IL-6 levels (data not shown), indicating that this disease mechanism might be similar. It is therefore not clear, why the treatment with roactemra did not have an effect on clinical score in this model.

It should also be noted that the presence of anti-CarP antibodies and clinical score did not always correlate. Some of the monkeys, did show a large increase in the levels of anti-CarP antibodies, while no arthritic symptoms were observed. Also, some animals with a higher clinical score did not develop high levels of anti-CarP antibodies. These data indicate that anti-CarP antibody levels are not directly correlated with clinical score.

All together, we conclude that abatacept treatment had a dampening effect of the antibody response in collagen-induced arthritis while also preventing disease development. Furthermore, anti-CarP antibodies, but not ACPA or RF could be detected in this animal model. Also, as observed in both mice and human, the anti-CarP antibodies presented before the actual disease onset.

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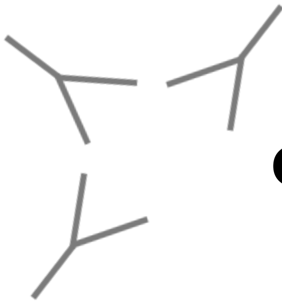

Chapter 4

Anti-Carbamylated Protein antibodies in Rheumatoid Arthritis patients of Asian descent

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Anti-Carbamylated protein (anti-CarP) antibodies were recently described to be present in Rheumatoid Arthritis (RA) patients[1]. The presence of anti-CarP antibodies has been found prior to disease onset, associates with the conversion towards arthralgia and with a more severe disease course in patients negative for anti-citrullinated protein antibodies (ACPA) [2,3]. The presence of anti-CarP antibodies has been described in other cohorts of RA patients as well [4-6]. So far, all of the investigated patient cohorts were Caucasian. However, the development of autoantibodies in RA patients can depend on genetic background. The presence of ACPA, for example, is strongly associated with certain HLA-DRB1 genes[7]. For anti-CarP antibodies, genetic associations have not yet been investigated thoroughly, but no specific association has been found with HLA-DRB1 in previous studies[4]. Since Caucasians and Asians have a different genetic background and positivity for autoantibodies, also for ACPA, can differ between ethnicities[4,8,9], we set out to investigate the levels of anti-CarP antibodies in serum samples from Asian RA patients. We now describe, for the first time, the presence of anti-CarP antibodies in a Japanese cohort.

Samples were obtained from Japanese RA patients on first visit at the Konan Kakogawa Hospital between April 2003 and March 2006 as described previously[9]. Written informed consent was obtained from the patients, according to the declaration of Helsinki and the study was approved by the Kobe Univ Hospital Review Board and Kohnan Kakogawa Hospital Review Board for Ethics. The average disease duration of the RA patients was 3,6 years. The presence of anti-CarP antibodies was determined in 268 RA patients and 324 healthy local controls by an enzyme-linked immunosorbent assay (ELISA) as described before[1]]. For this study, we measured the presence of IgG antibodies directed against carbamylated fetal calf serum (Ca-FCS). The levels were determined in arbitrary units per mL (aU/ml) using a standard curve.

RA patients display increased levels of anti-CarP antibodies when compared to healthy controls (mean ± standard deviation: 449 ± 544 vs 71 ± 133, Mann-Whitney U test: $P < 0,001$)(figure 1A). In order to determine positivity for anti-CarP antibodies, we fixed the cut-off at a specificity of 97%, as was the case in the Leiden early arthritis clinic (EAC) study (Figure 1B)[1]. Using this cut-off detection of anti-CarP antibodies had a sensitivity of 45,2% in RA. Further subdivision of the cohort on the basis of both anti-CarP antibodies and anti-CCP2 antibodies revealed four patient populations (Figure 1C). We observed 42,2% anti-CarP^{+}CCP2^{+}, 31,7% anti-CarP^{-}CCP2^{+}, 23,1% anti-CarP^{-}CCP2^{-} as well as 3.0 % anti-CarP^{+}CCP2^{-} patients (11,4% of all CCP2^{-} patients). Similar percentages were observed when measuring CCP3 instead of CCP2. These proportions differ slightly from the Leiden EAC cohort, but are more similar to the epidemiological investigation of rheumatoid arthritis (EIRA) cohort, likely because of the higher frequency of CCP+ patients (EAC 53%, EIRA 63%, Japan 74%)[1,4].

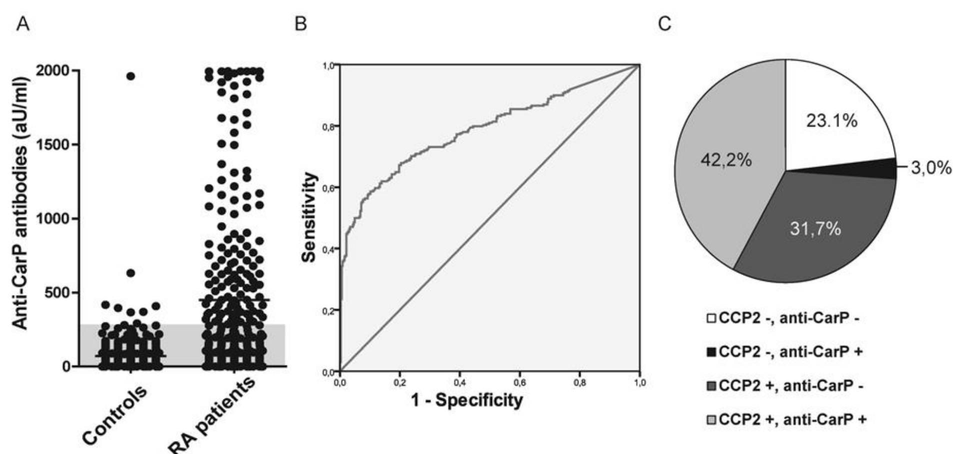

Figure 1 – Anti-CarP antibodies are present in the sera of Japanese RA patients. (A) The levels of anti-CarP antibodies as measured in the serum of RA patients and healthy controls by ELISA. (B) ROC curve using the presence of RA as the positive disease state and the anti-CarP antibody levels as the test variable. The area under the curve is 0.786. (C) The percentage of RA patients that are positive for CCP2 antibodies and / or anti-CarP antibodies. The cut-off for anti-CarP antibodies was set at the 97th percentile, while the cut-off for CCP2 antibodies was set according to the manufacturer's instructions.

Here we show for the first time that anti-CarP antibodies are also present in Asian RA patients. Importantly, also in the Japanese cohort, patients are present that are positive for anti-CarP antibodies, but not for ACPA. Also, some patients are positive for ACPA but not for anti-CarP antibodies. Previously, it has been suggested that anti-CarP antibodies and ACPA may be cross-reactive[6]. This data shows that although cross-reactivity might be present in the patients that show positivity for both autoantibodies, ACPA and anti-CarP antibodies are not always cross-reactive. In the future, it would be interesting to further investigate whether anti-CarP antibodies associate with disease progression in Asian RA patients.

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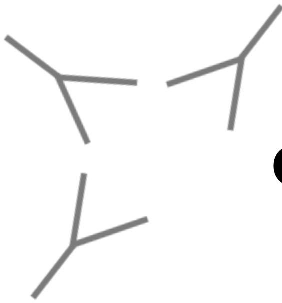

Chapter 5

Anti-carbamylated protein antibodies in the pre-symptomatic phase of rheumatoid arthritis, their relationship with multiple anti-citrulline peptide antibodies and association with radiological damage

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The presence of a new autoantibody system, anti-carbamylated protein (anti-CarP) antibodies, has been identified in rheumatoid arthritis (RA). The presence of anti-CarP antibodies was evaluated in samples taken from individuals who subsequently developed RA, before and after onset of symptoms. Results were related to previously analysed antibodies against citrullinated peptides (ACPA specificities) and anti-CCP2.

A total of 252 individuals, with 423 samples from before onset of symptoms of RA, and 197 population controls were identified as donors to the Medical Biobank of Northern Sweden; 192 of them were also sampled at the time of diagnosis. All samples were analysed for anti-CarP IgG and anti-CCP2 antibodies using ELISAs. Ten different antibody reactivities against citrullinated antigens (ACPA specificities) were analysed using a custom-made microarray based on the ImmunoCAP ISAC system (Phadia).

The concentration of anti-CarP antibodies was significantly increased in the pre-symptomatic individuals compared with controls ($P < 0.001$) and also increased significantly after disease onset ($P < 0.001$). The sensitivity for anti-CarP antibodies in the pre-symptomatic individuals was 13.9% (95% CI: 11 to 17.6) and 42.2% (95% CI: 35.4 to 49.3) following development of RA. Anti-CarP antibody positivity was found in 5.1% to 13.3% of individuals negative for anti-CCP2 or ACPA specificities. Presence of anti-CarP antibodies was significantly related to radiological destruction at baseline, at 24 months and also to radiological change ($P < 0.05$, all).

The results indicate that anti-CarP antibodies are associated with disease development, even after adjusting for the presence of different ACPA fine specificities and also in anti-CCP2 negative individuals. Anti-CarP antbidies further contribute to the identification of a subset of patients with worse radiological progression of the disease independent of ACPA.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by inflammation within the joints which eventually leads to the destruction of cartilage and bone. However, the aetiopathogenesis of this disease is not yet fully understood. A number of autoantibodies have been associated with the disease *e.g.,* rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA) and the recently described antibodies directed against carbamylated proteins (anti-CarP antibodies).[1]

The citrullinated proteins recognized by ACPA arise due to the conversion of an arginine residue into citrulline by an enzyme, peptidyl arginine deaminase (PAD).[2] We and others have shown that the presence of antibodies against citrullinated proteins/peptides (ACPA) measured as anti-CCP antibodies of immunoglobulin (Ig)G, IgA, and IgM isotypes, precedes the development of RA by a number of years.[3-6]

More recently, we have also shown that an increased number of ACPA specificities predates the onset of RA.[7] The ACPA specificities were initially restricted and relatively non-specific but over time expanded and involved more specific responses, especially with regard to antibodies against α-Enolase (CEP-1/Eno5-21), Fibrinogen (Fib)β36-52, and filaggrin (CCP-1/Fil307-324), when approaching the onset of symptoms.[7,8] The presence of ACPA in RA patients has also been shown to predict a more severe disease.[9-11]

In addition to citrullination, another post-translational modification of proteins is carbamylation, where preferentially lysines are converted to homocitrulline by a non-enzymatic process.[12] Anti-CarP antibodies have been identified in patients with RA.[13] Furthermore, these antibodies have been shown to be associated with the development of RA in patients with arthralgia [14] and were associated with a more severe disease course in ACPA-negative patients.[13] Similar to ACPA, anti-CarP antibodies have also been observed in individuals before the onset of clinical symptoms of RA.[15]

5

In this study blood samples donated to the Medical Biobank of Northern Sweden by individuals prior to the onset of RA and of controls derived from the same population were analysed for anti-CarP antibodies. Although the presence of anti-CarP antibodies prior to clinical diagnosis of RA has already been shown in a small Dutch cohort,[15] we were now able to analyse samples from a larger cohort of individuals and the presence of anti-CarP antibodies in relation to antibodies against several ACPA specificities. Furthermore, samples were collected before and after the onset of disease allowing a comparison between anti-CarP antibodies and ACPA fine-specificities with regard to their possible predictive values for development of RA as well as after subsequent disease progression.

Material and methods

Subjects

A case-control study was conducted based on individuals included in population surveys within the Medical Biobank of Northern Sweden and the Maternity cohort. The criteria for recruitment and collection and storage of blood samples have been described previously.[6] Cohorts included in the Medical Biobank are population based, and all adult individuals residing in the county of Västerbotten are continuously invited to participate. The Maternity cohort is a collection of serum samples obtained from pregnant women in northern Sweden who had undergone screening for immunity to rubella. The registers from the Medical Biobank and the Maternity cohort were co-analysed with the registers of patients with RA fulfilling the 1987 American Rheumatism Association classification criteria for RA [16] and attending the Department of Rheumatology, University Hospital in Umeå, with a known date for the onset of symptom to identify individuals who had donated blood samples prior to the onset of symptom of RA. In this study only samples donated less

than 13 years before the onset of symptoms were included. The number of individuals identified and the procedure for excluding any sample has previously been described in detail.[7] Consequently, 252 individuals (58 men and 194 women) were included in this study, who were referred to as "pre-symptomatic individuals" with a total of 423 blood samples at different time points. Forty-six individuals had contributed with 2 samples, 24 three samples, 13 four, seven with five and two with six samples. The median (IQR) time pre-dating onset of symptoms using all samples was 5.2 (6.3) years and for the samples obtained closest to before disease onset it was 3.5 (4.9) years. Controls (N=197) were randomly identified from the same cohorts within the register at the Medical Biobank and matched for age, sex and date of sampling. Of the pre-symptomatic individuals 192 were also sampled when they presented at the early arthritis clinic (Department of Rheumatology, University Hospital, Umeå) and were diagnosed with RA. The median (IQR) duration of symptoms before these patients were diagnosed following the onset of symptom was 7 (5.8) months. Radiographs of the hands and feet of patients with RA (n=181) were available at baseline and after 24 months; these radiographs were graded according to the Larsen score [9] and read in chronological order by two readers, who were blind to the antibody data. Radiological progression was defined as the increase of the Larsen score between baseline and 24 months, with the smallest detectable change less than four (calculated according to ref. [17]). All individuals were classified either as a being "non-smoker" or an "ever-smoker" (past or current). Demographic data of the cases and controls is presented in table 1.
The Regional Ethics Committee at the University Hospital, Umeå, Sweden, approved this study and all participants gave their written informed consent when donating blood samples.

	Controls (n=197)	Pre-symptomatic individuals (n=252)	Patients (n=192)
Female sex, %	84.3	77	75
Median age, years (IQR)	50.1 (20.2)	52 (17,4)[2]	57.5 (14.3)
Ever smoker n/total (%)	83/168 (49.4)	159/246 (64.6)	127/188 (67.6)

Table 1 – Demographic data for 252 individuals with 423 samples at a median (IQR) of 5.2(6.2) years before the onset of symptoms of RA, patients with RA and controls. Median age as calculated for all samples at moment of collection. IQR: interquartile range, RA: rheumatoid arthritis.

<u>Analyses of anti-CarP antibodies and anti-CCP2 antibodies</u>

The anti-CarP antibodies were determined by ELISA as described previously.[13] Briefly, Nunc Maxisorp plates (Thermo Scientific) were coated overnight using carbamylated fetal calf serum (Ca-FCS) or non-modified FCS. After washing and blocking, the samples were added and allowed to bind overnight. Binding was determined using horseradish peroxidase (HRP)-conjugated rabbit-anti-human IgG (DAKO) in combination with ABTS *(*2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid*)*. The investigators who carried out the anti-CarP ELISA tested the samples in a blind fashion.

The cut-off level for anti-CarP antibodies was defined as the antibody reactivity expressed as the sum of optimal sensitivity and specificity, using receiver operating characteristic (ROC) curves, based on the concentrations of the patients with RA included in this study and the controls from the Medical Biobank. The cut-off for positivity was set at 256 arbitrary units/mL (AU/mL) giving a specificity of 97%. Detection of anti-CCP antibodies was successfully performed using ELISAs according to the manufacturer's instructions (Euro-Diagnostica). The cut off for positivity was set at 25 AU/mL.

<u>Multiplex assay</u>
Serum and plasma samples were analysed for levels of ACPA specificities of the IgG isotype using a custom-made microarray based on the ImmunoCAP ISAC system (ThermoFisher/Phadia, Uppsala, Sweden).[18] Briefly, 10 peptides representing the four candidate autoantigens: fibrinogen (Fib) α573, Fibα591, Fibβ62-81a (72), Fibβ62-81 (74), Fibβ36-52, α-Enolase (CEP-1/Eno5-21), Collagen type II (citC1^{III}/CII359-369), filaggrin (CCP-1/Fil307-324), vimentin (Vim) 2-17, and Vim60-75, were analysed as previously described.[7,18] For all reactivities both the citrullinated and the native arginine-containing peptides were analysed and for all except one, citC1^{III}, the delta-value was determined used. The exception was due to the fact that the arginine containing CitC1 is an autoantigen in itself.[19] The cut-off values were basedon ROC curves analysed on the RA patients and controls.

<u>Statistical analysis</u>
For comparing continuous data between two groups the Mann-Whitney U test was used and the Kruskal Wallis test for several groups. Correlation analyses were performed using Spearman's rank correlation test (r_s). Logistic regression analyses were performed to identify associations between antibodies and disease development, presented as odds ratios (ORs) and 95% confidence intervals (95% CIs). Univariate analyses of variance were used to identify associations between factors and continuous data. Relationships between categorical data (positive versus negative) were compared using chi-square analysis or Fisher's exact test as appropriate. Considering the study to be explorative, *P* values less than or equal to 0.05 were considered significant.
Statistical calculations were performed using SPSS for Windows, version 22. Sensitivity, specificity, ORs, and 95% CIs were calculated with the XLSTAT program (version 2014.1.04) in Microsoft Excel 2013 (Addinsoft).

Results

Levels of anti-CarP antibodies in pre-symptomatic individuals, patients and controls

The levels of anti-CarP antibodies in the pre-symptomatic individuals who contributed more than one sample were significantly increased compared with that of controls, namely, a median(IQR) of 44(142) AU/mL and 7(43) AU/mL, respectively ($p<0.0001$). The concentration after the onset of disease (median (IQR) 198(379) AU/mL) was significantly increased compared with that for both the control subjects and pre-symptomatic individuals ($p< 0.0001$, Kruskal-Wallis) (figure 1).

Frequency of anti-CarP antibody positivity in pre-symptomatic individuals, patients and controls

The increased levels of anti-CarP antibodies in the pre-symptomatic individuals are also reflected in an increased frequency of anti-CarP positive individuals. The frequency of antibodies against CarP was 13.9% (11.0-17.6%) when all samples were included in the analysis and the frequency of being ever positive for the antibody 18.3% (14.0-23.5%) and in the RA patients 42.2% (95%CI 35.4-49.3). The frequency of samples with positive anti-CarP antibody detection, was higher the closer in time to onset of symptoms, for example, 21.8%(95% CI: 14% to 32.3%) of the pre-symptomatic individuals had anti-CarP antibodies above the cut-off value less than 2 years before the onset of symptoms and 18.6% (95%CI: 12.8 to 26.3) when analysed at less than 3 years before disease onset, although the gradual increase was not significant during the pre-dating period (Figure 2).

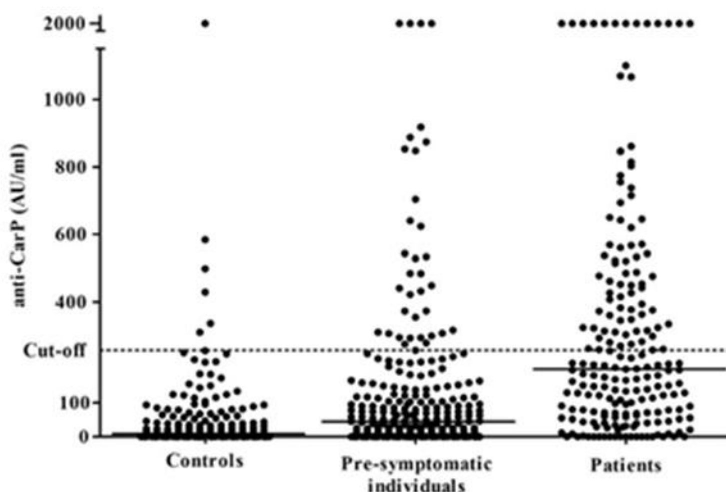

Figure 1 – Concentrations of anti-CarP antibodies in pre-symptomatic individuals closest to symptom onset (median (IQR) 3.5 (4.9) years) and in controls and patients with RA. Anti-CarP: anti-carbamylated protein antibodies, IQR: interquartile range, RA: rheumatoid arthritis.

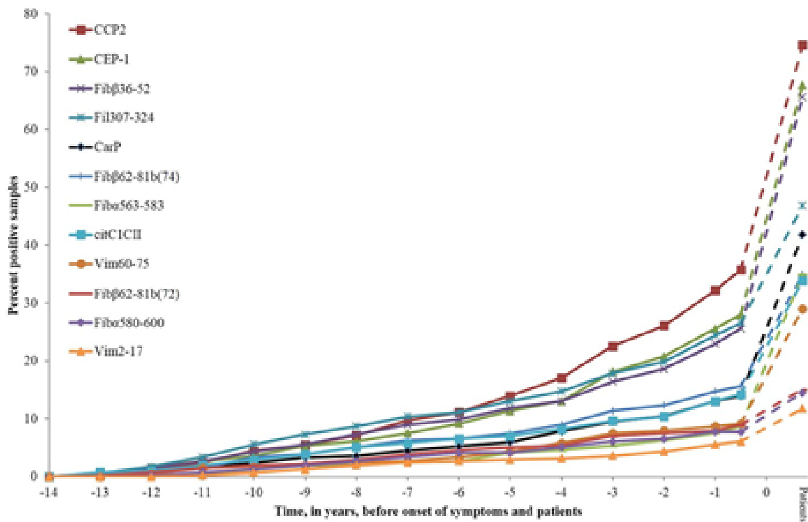

Figure 2 - Accumulated percentage of positivity of anti-CarP antibodies, the different ACPA specificities and anti-CCP2 antibodies. ACPA,anti-citrullinated protein antibodies; anti-CarP, antibodies against carbamylated proteins; anti-CCP2, anti-cyclic citrullinated peptide 2.

Ever being positive for anti-CarP antibodies was associated with the development of RA in the pre-symptomatic individuals (OR=7.1 95%CI: 3.0 to 17.0). Adjustments of age, sex or ever being a smoker did not affect the association between anti-CarP antibodies and disease development (data not shown). Furthermore the association remained after adjusting for of each ACPA specificity with the exception of anti-CCP2 antibodies (OR= 2.4, 95%CI: 0.9 to 6.7) (Table 2). However, after stratification for anti-CCP2 antibodies anti-CarP antibodies were associated with disease development in anti-CCP2 negative individuals (OR= 3.4 95%CI: 1.1 to 9.8). Analysis in patients with RA showed an association between anti-CarP antibodies and RA, also after adjusting for all ACPA specificities including anti-CCP2 antibodies (Table 2).

	Pre-symptomatic individuals		RA patients	
Antibodies against	OR	95% CI	OR	95% CI
CarP	7.1	3.0-17.0	23.2	9.8-55.0
after adjustments for each antibody:				
CarP	2.4	0.9-6.9	3.8	1.2-12.2
CCP2	43.8	13.54-141.9	125.0	37.4-417.7
CarP	3.4	1.35-8.8	8.1	3.02-21.4
CEP-1	10.0	4.67-21.6	31.4	14.3-69.2
CarP	4.9	2.0-12.1	16.2	6.7-39.0
citC1	5.1	2.23-11.8	19.2	7.9-46.8
CarP	4.5	1.81-11.3	9.9	3.9-25.3
Fibß36-52	6.6	3.50-12.3	17.3	8.9-33.6
CarP	6.3	2.60-15.3	20.9	8.8-49.8
Fibß62-81a(72)	6.1	2.32-16.1	4.9	1.7-14.0
CarP	4.6	1.86-11.5	17.3	7.1-42.3
Fibß62-81b(74)	37.6	5.11-277.2	79.1	10.7-586.7
CarP	5.6	2.33-13.7	14.4	6.0-35.0
Fibα563-583	3.1	1.28-7.3	7.4	3.1-17.5
CarP	6.1	2.54-14.8	21.3	8.9-50.5
Fibα580-600	1.9	0.89-4.2	2.1	0.87-4.9
CarP	3.8	1.51-9.7	15.0	6.1-36.9
Fil307-324	12.8	5.40-30.3	19.2	7.9-46.9
CarP	5.9	2.42-14.2	17.3	7.2-41.4
Vim60-75	2.5	1.22-5.2	4.0	1.9-8.5
CarP	6.2	2.58-15.1	22.0	9.26-52.3
Vim2-17	3.1	1.24-8.0	3.6	1.333-9.9
CarP	3	1.2-8.1	12.2	4.9-30.4
CCP2 and all ACPA	7.2	4.7-11.2	9.7	5.7-16.5

Table 2 – Association between the presence of anti-CarP antibodies and the development of RA, adjusted for ACPA fine specificities and anti-CCP, respectively. Calculations are carried out as ever begin positive with n = 253 for the pre-symptomatic individuals and n = 192 for RA patients. ACPA: anti-citrullinated protein antibodies, anti-CarP: anti-carbamylated protein antibodies, anti-CCP2: anti-cyclic citrullinated peptide 2, CI: confidence interval, fib: fibrinogen, OR: odds ratio, RA: rheumatoid arthritis, vim: vimentin

Antibodies against	Pre-symptomatic individuals		RA patients	
	Total n (%)	Anti-CarP + n(%)	Total n (%)	Anti-CarP + n(%)
All individuals	423 (100)	59 (13.9)	188 (100)	81 (42.2)
CCP2 +	151 (35.8)	44 (29.1)	141 (74.6)	74 (52.5)
CCP2-	271 (64.2)	14 (5.2)	48 (25.4)	5 (10.4)
CCP1 +	110 (26.6)	26 (23.6)	88 (46.8)	31 (54.5)
CCP1-	304 (73.4)	29 (9.5)	100 (53.2)	31 (31.0)
CEP-1 +	116 (28.0)	31 (26.7)	128 (68.1)	68(53.1)
CEP-1-	298 (72.0)	24 (8.1)	60 (31.9)	11 (18.3)
Cit CIIC1 +	59 (14.3)	17 (28.8)	63 (33.5)	38 (60.3)
Cit CIIC1-	355 (87.7)	38 (10.7)	125 (66.5)	41 (32.8)
Fib 36-52 +	106 (25.6)	24 (22.6)	124 (66.0)	66 (53.2)
Fib 36-52-	308 (74.4)	31 (10.1)	64 (34.0)	13 (20.3)
Fibß72 +	38 (9.2)	5 (13.2)	28 (14.9)	16 (57.1)
Fibß72 -	376 (90.8)	50 (13.3)	160 (85.1)	63 (39.4)
Fibß74 +	65 (15.7)	20 (30.8)	67 (35.6)	36 (53.7)
Fibß74-	349 (84.3)	35 (10.0)	121 (64.4)	43 (35.5)
Fiba591 +	32 (7.7)	8 (25.0)	27 (14.4)	16 (59.3)
Fiba591-	382 (92.3)	47 (12.3)	161 (85.6)	63 (39.1)
Fiba573 +	36 (8.7)	8 (22.2)	65 (34.5)	43 (66.2)
Fiba573-	378 (91.3)	47 (12.4)	123 (65.4)	36 (29.3)
Vim 2-17 +	25 (6.0)	4 (16.0)	22 (11.7)	10 (45.5)
Vim 2-17-	389 (94.0)	51 (13.1)	166 (88.3)	69 (41.6)
Vim 60-75 +	38 (9.2)	9 (23.7)	56 (29.8)	35 (62.5)
Vim 60-75-	376 (90.8)	46 (12.2)	132 (70.2)	44 (33.3)

Table 3 – Frequency of positivity for anti-CarP antibodies in relation to ACPA fine specificities. ACPA: anti-citrullinated protein antibodies, anti-CarP: anti-carbamylated protein antibodies, anti-CCP2: anti-cyclic citrullinated peptide 2, fib: fibrinogen, n: number, RA: rheumatoid arthritis, vim: vimentin

<u>Relationships between anti-CarP antibodies and ACPA specificities and anti-CCP2 antibodies</u>

The presence of anti-CarP antibodies, anti-CCP2 and several ACPA fine-specificities was investigated in order to describe populations of patients and pre-symptomatic individuals with overlapping and unique antibody specificities (table 3). Among the pre-symptomatic individuals negative for any ACPA specificity 8.1 to 13.3% were positive for anti-CarP antibodies, the lowest frequency being for anti-CEP-1 and the highest for anti-Fibß72. When selecting samples negative for all ACPA specificities irrespective of anti-CCP2 antibodies, the frequencies of positivity for anti-CarP antibodies were 2.8%, 5.8%, 13.3% in controls, pre-symptomatic individuals and RA patients, respectively (for further details on the relationships to sub specificities, see Table 3). The distribution of the combinations of anti-CarP and anti-CCP2 before and after disease onset showed a relative increase of double positivity after onset compared with the distribution before onset, where the number of double negativity dominated (Figure 3).

The relative distribution of the combinations of one antibody negative and the other antibody positive (anti-CarP+/anti-CCP- and anti-CarP-/anti-CCP+) were fairly unchanged.

The concentration of anti-CarP antibodies correlated with those of anti-CCP2 antibodies both in the pre-symptomatic individuals and in RA patients (R_s = 0.31 and 0.52 respectively, P < 0.001 for both). Additionally there were correlations between the concentrations of anti-CarP antibodies and the different ACPA specificities in both pre-symptomatic individuals (R_s = 0.18 to 0.34, P < 0.0001 to 0.04) and RA patients (R_s = 0.15 to 0.46, P < 0.0001 to 0.04) with the exception of Vim2-17 and Fiba591 in both and Fibß72 in the pre-symptomatic individuals.

There were no significant differences between anti-CarP antibodies or anti-CCP2 antibodies or ACPA specificities in predating time when the antibodies were first detected positive for any of the antibodies (data not shown). In 20 of 42 individuals anti-CCP2 antibodies and any ACPA specificity, preceded positivity for anti-CarP antibodies. In five and two individuals anti-CarP antibody positivity preceded the presence of anti-CCP2 antibodies and any ACPA specificity, respectively.

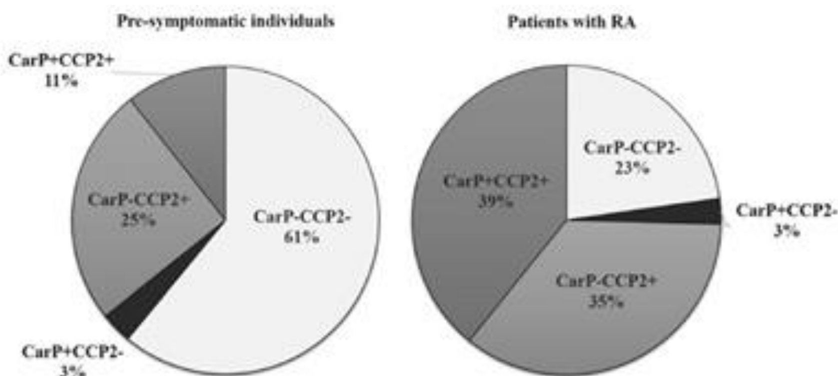

Figure 3 – The relative distribution in percentage of the combinations of positivity and negativity of anti-CarP and anti-CCP2 antibodies illustrated by pie charts. Anti-CarP: anti-carbamylated protein antibodies, anti-CCP2: anti-cyclic citrullinated peptide 2.

<u>The presence of anti-CarP antibodies is associated with radiological damage especially in anti-CCP2 negative individuals.</u>

Positivity for anti-CarP antibodies in the pre-symptomatic individuals was associated with increased radiological damage as detected by the a higher Larsen score at baseline, that is, when RA was diagnosed, the median (IQR) value for the Larsen score was 9.0 (6.5) compared with 5.0 (9.0) (p= 0.003) in anti-CarP antibody negative individuals. This association remained after adjustment for anti-CCP2 antibodies.

Anti-CarP antibodies analysed after disease onset were also associated with the radiological progression and outcome calculated as Larsen score at baseline in the patients with early RA (ß=2.15, 95%CI: 0.40 to 3.90, p=0.017) at 24 months (ß=4.49, 95%CI 1.67-7.32, p=0.002) and also the radiological progression (ß=2.44, 95%CI: 0.53 to 4.35, p=0.013). The outcome at 24 months remained significant after adjustment for the Larsen score at baseline (ß=1.89, 95%CI: 0.02 to 3.75, p=0.048). Stratification for anti-CCP2 antibodies revealed that the association of anti-CarP antibodies was strengthened in anti-CCP2 negative patients at baseline (ß=5.38, 95%CI: 0.94 to 39.82, p=0.019), after 24 months (ß=8.57, 95%CI: 3.36 to 13.79, p=0.002) and the radiological progression (ß=2.95, 95%CI: 0.15 to5.75, p=0.040) and non-significant in anti-CCP2 positive patients. The pattern was similar when stratified for the presence or absence of the triplet of the most frequently occurring ACPA fine specificities (that is, anti-CEP-1, –Fibß36-52 and – Fil307-324) in anti-CarP positive patients (24 months; (ß = 4.25, 95% CI: 0.70 to 7.80, P = 0.019). However, when stratified for these three ACPA specificities separately there were differences be-tween them. The presence of anti-CarP antibodies was associated with a higher Larsen score in anti-Fibß36-52 and anti-CEP-1 antibody positive patients at baseline, and after 24 months, whilst more radiological damage was found in anti-CarP positive and anti-Fil307-324 anti-body negative patients (data not shown). The presence of anti-CCP2 antibodies as well as antibodies against CEP-1, Vim60-75 and CCP1/Fil307-324 was also significantly associated with radiological findings at 24 months and with radiological progression versus being negative, although to a lower extent compared with analysis for anti-CarP antibodies (data not shown). None of the relationships of anti-CCP2, antibodies against CEP-1, Vim60-75 and Fil307-324 remained significant in separ-ate univariate analyses of variance including anti-CarP antibodies that were significantly associated in all analyses (data not shown). Adding the information on positivity for anti-CCP2 antibodies to the presence of anti-CarP anti-bodies further increased the association to radiological findings at 24 months (ß = 5.4, 95% CI: 2.01 to 8.78) and for radiological progression (ß = 3.05. 95% CI: 0.75 to 5.35).

Discussion

In this relatively large collection of samples from individuals before they had any symptoms of a subsequent joint disease increased levels of antibodies against carbamylated proteins were found. The levels were also further increased when these individuals had developed RA. The levels of anti-CarP antibodies correlated with the levels of most of the ACPA specificities and with that of anti-CCP2 both before and after disease development. These findings are consistent with previous findings but now also include several ACPA specificities[15]. The time point for the first antibody to be detectable in samples from the pre-symptomatic individuals was comparable for the anti-CarP antibodies and the different ACPA specificities and to anti-CCP2 antibodies.

There was a gradual increase in the frequency of positivity for the anti-CarP antibodies during the time pre-dating that peaked at the onset of symptoms.

The frequency of these antibodies was lower than the three, most frequently occurring ACPA specificities, (anti-CEP-1, -Fibß36-52 and –CCP1), and also than anti-CCP2 antibodies during the pre-dating period. However, analyses of samples closer to symptom onset showed that the frequency of anti-CarP antibodies almost reached to the level of anti-CCP1 antibodies.

In our previous study we found a clear and significant increase during the predating time of antibodies against CEP-1, Fibß36-52 and CCP1 and also of anti-CCP2 antibodies.[7] Antibodies against Vim60-75 increased shortly before the disease developed. The increasing frequency of anti-CarP antibodies initially appeared to be more similar to antibodies against Vim60-75 but shortly before the onset of symptoms more closely reached the level of antibodies against Fil307-324 peptide.

Even though in our study 3% of the RA patients are singe positive for anti-CarP antibodies and 36% are single positive for anti-CCP, there is also a substantial portion of patients (39%) who are double positive and, therefore, cross-reactivity towards citrullinated and carbamylated proteins must be considered. Previous experiments indicated that, next to cross-reactive antibodies, antibodies only reacting with either citrullinated or carbamylated proteins were also present in RA[20]. In addition, results from a recent study by Jiang et al.[21] on two large cohorts of RA found similar distributions regarding anti-CarP and anti-CCP antibodies and also found completely different HLA associations for anti-CarP and anti-CCP antibodies. In addition, the association between anti-CCP antibodies and smoking was not found for anti-CarP antibodies. Collectively, these findings do not favour the idea that anti-CarP antibodies only represent cross-reactivity to citrullinated antigen. It is likely that both cross-reactive and non-cross-reactive antibodies co-exist.

Our results on the appearance of anti-CarP antibodies in samples collected years before the onset of symptoms confirm previous observations[15], although the number of samples available for this study is larger and not restricted to those individuals identified as being positive in their last sample before being diagnosed with RA. Our cohort is only restricted to samples donated less than 13 years before onset of symptoms. Anti-CarP antibodies were strongly associated with RA both among the pre-symptomatic individuals and among RA-patients (OR = 7.1 and 23.2, respectively). However, when ad-justed for the presence of anti-CCP2, the association was non-significant (OR = 2.4 95% CI 0.9 to 6.7) in the pre-symptomatic individuals but still significant in RA patients (OR = 3.8 95% CI 1.2 to 12.2). However, after stratification for anti-CCP2 antibodies the association of anti-CarP antibodies and disease development remained significant in anti-CCP2 negative individuals irrespective of adjustments for each separate ACPA fine specificities or analysed as one group. Anti-CarP antibodies were as-sociated with disease development when adjusted for all of the ACPA fine specificities, each analysed separately or in combination as one group with anti-CCP2 anti-bodies.

Only 3% of the pre-symptomatic individuals and RA patients, respectively, were anti-CarP positive anti-CCP2 negative when the group of individuals before symptom onset or of RA patients were analysed. How-ever, we found that if we only considered a group of anti-CCP2 negative patients, the frequency of anti-CarP positive antibodies was 10.4%. This information could be useful in a clinical situation to diagnose an arthritis as RA when the anti-CCP2 test is negative.

The present cohort provided the opportunity to evalu-ate the radiological findings when the symptoms had de-veloped and the individuals were diagnosed with RA and at a follow-up after 24 months. We found that the pres-ence of anti-CarP antibodies before onset of symptoms of the disease predicted the radiological findings at base-line. Furthermore, we were able to confirm the findings by Shi and colleagues[13] that the presence of anti-CarP antibodies is associated with the rate of radiological de-struction. In the present study this was already apparent at baseline, after 24 months and also calculated as the change over time. The association of anti-CarP antibody positivity was particularly evident in anti-CCP2 negative or Fil307-324 peptide negative individuals. The associations of anti-CCP2, and some of the ACPA specificities (CEP-1, Vim60-75 and Fil307-324) were also associated with radiological findings at 24 months and with the change over time but they did not remain significant after adjusting for presence of anti-CarP antibodies.

We conclude that the anti-CarP antibodies are present years before the onset of symptoms of RA. These anti-bodies are also associated with disease development after adjusting for the presence of different ACPA fine speci-ficities and in anti-CCP2 negative individuals. We are also able to confirm that the presence of these antibodies is related to radiological destruction at diagnosis and to the radiological progression observed once the disease has developed.

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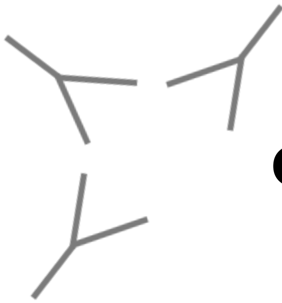

Chapter 6

Anti-Carbamylated Protein antibodies: a specific hallmark for rheumatoid arthritis - comparison to conditions known for enhanced carbamylation; renal failure, smoking and chronic inflammation

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Antibodies that target carbamylated proteins (anti-CarP antibodies) have been described as a disease biomarker in rheumatoid arthritis (RA).[1] However, little is known about the factors that predispose to the production of anti-CarP antibodies, although a likely requirement is the presence of carbamylated antigen(s). Carbamylation is a posttranslational modification resulting from the conversion of a lysine amino acid into a homocitrulline that requires the presence of cyanate. There are several conditions in which the concentration of cyanate (and therefore carbamylation) is increased, such as renal failure, chronic inflammation and heavy smoking.[2-4] We therefore addressed the question whether conditions of enhanced carbamylation could also result in the induction of anti-CarP antibodies.

To investigate this we determined the presence of anti-CarP antibodies in serum samples from patients with renal failure,[5] inflammatory bowel disease (IBD)(expanded from van Erp et. al[6]) and in heavy smokers with or without chronic obstructive pulmonary disease[7] (see table 1). The presence of anti-CarP antibodies in healthy controls and RA patients from the early arthritis clinic (EAC) was used as a comparison.[1,8] The collection of these cohorts was approved by the Leiden University Medical Center ethics committee and informed consents were obtained from all patients. Anti-CarP IgG antibodies were determined by ELISA as described before using carbamylated fetal calf serum as antigen.[1] Positivity for the presence of anti-CarP antibodies was defined as signal higher than the 97th percentile of the healthy controls. Each cohort was tested separately for this study and control samples (between 120-187) were randomly selected from a pool of 209 controls and taken along with each measurement. The anti-CCP2 ELISA (anti-cyclic citrullinated peptide) was also carried out as described before.[9]

The percentage of individuals positive for anti-CarP antibodies was 2.3%, 4.1%, 3.7%, 11.9% and 44.9% for controls, smokers (no differences were observed in anti-CarP antibodies between smokers with or without COPD), patients with IBD, renal failure and RA respectively (Figure 1A). Pearson Chi-square test analyses revealed that next to the highest frequency of anti-CarP antibodies in RA (p =<0.001) also a statistically significant increased frequency was found in patients suffering from renal failure (p=0.004), when compared to controls.

As a comparison to the presence of anti-CarP antibodies, a second autoantibody, another common and RA-specific autoantibody, namely anti-CCP2, was measured. The percentages of individuals positive for anti-CCP2 antibodies was 1.5%, 1.9%, 0.5%, 2.4% and 50.5% for controls, smokers, patients with IBD, renal failure and RA respectively (Figure 1B). These data indicate that anti-CCP2 antibodies are present in RA patients but hardly in any of the other conditions.

In conclusion, especially RA patients, and to a much lesser extent patients suffering from renal disease, are positive for anti-CarP antibodies, while carbamylation is

reported to be increased in each of these conditions. Therefore, these data indicate that enhanced carbamylation is not sufficient for a break of tolerance against carbamylated proteins. Instead, the presence of antibodies against several post-translational modifications is a rather specific hallmark of the immunological abnormalities present in RA.

		Smokers	IBD	Renal failure	Controls	RA
Patients	*Number*	374	433	85	120-187	557
Age	*Average (SD)*	65 (9)	44 (14)	54 (13)	44 (14)	57 (16)
Gender	*Percentage female*	27	58	29	51	67
Smoking	*Percentage current*	42	21	24	8	23
	Pack years (average)	39	n.d.	n.d.	n.d.	n.d.

Table 1 – Patient characteristics. For the controls, the values are given for the total group of 209 controls. For the assays, controls were randomly selected out of the group of 209 controls. Controls were not age or gender – matched. We did not observe a correlation between anti-CarP antibodies and age or gender. IBD; inflammatory bowel disease, RA; Rheumatoid arthritis, stdev; standard deviation, n.d.; not determined. Data from RA patients as in reference[1].

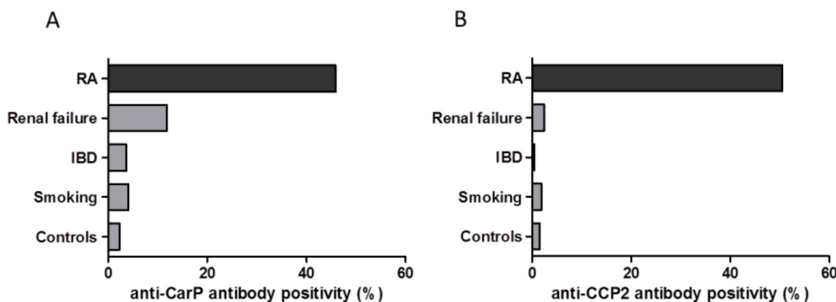

Figure 1 – Anti-CarP and anti-CCP2 antibody positivity. (A) The percentage of anti-CarP antibody positivity in each of the tested cohorts is shown. When compared to controls, significant differences were observed for RA ($p<0.001$) and renal failure ($p=0.004$) (B) The percentage of anti-CCP2 antibody positivity in each of the tested cohorts is shown. When compared to controls, significant differences were observed for RA ($p<0.001$). For both, control percentages are an average of all measurements. IBD; inflammatory bowel disease, RA; rheumatoid arthritis. Percentages of the RA patients are shown as determined in reference[1].

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Chapter 7

The combination of three autoantibodies, ACPA, RF and anti-CarP antibodies is highly specific for rheumatoid arthritis: implications for very early identification of individuals at risk to develop rheumatoid arthritis.

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In rheumatoid arthritis(RA), the autoantibodies anti-citrullinated protein antibodies(ACPA) and rheumatoid factor(RF) are commonly used to aid RA diagnosis. Although these autoantibodies are mainly found in RA, their specificity is not optimal. It is therefore difficult to identify RA patients, especially in very early disease, based on the presence of ACPA and RF alone. Also, anti-carbamylated protein(anti-CarP) antibodies have diagnostic and prognostic value as the presence of anti-CarP antibodies associates with joint damage in RA patients and with future RA development in arthralgia patients. Therefore, we aimed to investigate the value of combined antibody testing in relation to prediction and diagnosis of (early) RA.

A literature search resulted in twelve studies, consisting of RA patients, pre-RA individuals, disease controls, healthy first-degree relatives of RA patients or healthy controls, in which data on RF, ACPA and anti-CarP antibody-status was available. Random effects meta-analyses were carried out for several antibody combinations.

The individual antibodies are highly prevalent in RA(34%-80%) compared to the control groups, but are also present in non-RA controls(0%-23%). To classify most people correctly as RA or non-RA, the combination of ACPA and/or RF often performs well(specificity:65-100, sensitivity:59-88). However, triple positivity for ACPA, RF and anti-CarP antibodies results in a higher specificity(98-100) (accompanied by a lower sensitivity(11-39)).

As the rheumatology field is moving towards very early identification of RA and possible screening for individuals at maximum risk in populations with a low pre-test probability, triple positivity provides interesting information on individuals at risk to develop RA.

Introduction

Rheumatoid arthritis (RA) is a common autoimmune disease, characterized by immune cell infiltration in the joint, joint pain and possibly cartilage and bone degradation. In RA, several antibody systems have been identified based on their target antigens. Two of these autoantibodies, rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA), have also been incorporated into the classification criteria for RA[1]. RFs are antibodies that recognize the Fc tail of other (IgG) antibodies, while ACPA recognize proteins that contain citrulline(s), which arise by a post-translational modification. While ACPA and RF are highly prevalent in RA they can also be identified in a small percentage of healthy controls[2-4]. In a meta-analysis, comparing RA patients to healthy controls for the presence of ACPA (measured by CCP in this meta-analysis (cyclic citrullinated peptide)), a pooled sensitivity of 67% was observed, while this was 69% for IgM-RF. The combined specificity was 95% for ACPA and 85% for IgM-RF[5].

Although more than half of the RA patients are positive for ACPA and/or RF, a substantial part of the patients cannot be identified in this manner. To date, it is unclear whether it will be possible to fill this serological gap[6] with other (antibody) biomarkers.

Importantly, ACPA and RF can both be detected more than 10 years before disease onset[7], which would possibly allow for early identification of individuals at risk to develop RA. However, less than 50% of the ACPA positive patients with non-specific musculoskeletal symptoms develop RA after 1 year[8]. Also, less than 50% of the ACPA- and RF-double-positive arthralgia patients develop RA after up to 2 years of follow-up[9]. The presence of ACPA and/or RF is therefore not sufficient for the prediction of RA development. In RA patients, there seems to be a "window of opportunity" in the early phase of disease. Treatment during this phase may increase the amount of RA patients that reach drug-free remission, effectively reducing the number of individuals with chronic disease[10]. However, since treatment of asymptomatic individuals may not be free from side effects, it is important to identify the individuals at risk to develop RA as accurately as possible and minimize misclassification and unnecessary side effects of treatments.

Besides ACPA and RF, several other autoantibodies, such as anti-CarP antibodies, anti-PAD antibodies and anti-malondialdehyde antibodies have been identified in RA patients[11,12]. Of these autoantibodies, antibodies that target carbamylated proteins (anti-CarP antibodies) have been studied extensively[13]. Carbamylation is a post-translational modification, which can arise via a chemical reaction with cyanate, converting a lysine into a homocitrulline. Anti-CarP antibodies can also be present before disease onset[14-17] and have been measured and analysed in a substantial number of RA patients[13-36] and other conditions[28,29,33-35,37-41]. Importantly, anti-CarP autoantibodies also occur in RA patients that are seronegative for both ACPA and RF and may therefore represent an interesting additional biomarker to aid diagnosis of RA patients[13,25]. Here, we have two aims in relation to ACPA, RF and anti-CarP antibody measurement. First, we aimed to determine whether the combination of these three autoantibodies may assist in improving the diagnosis of RA. Second, we investigated whether this autoantibody combination would provide additive value for the prediction of RA development. To investigate this, we combined newly obtained data from several unique cohorts with a literature search to investigate the value of combining anti-CarP antibodies with ACPA and RF. In this meta-analysis of 12 different studies involving over 5000 unique individuals, we show that the presence of ACPA and/or RF, as often used in the clinical setting, seems to perform well to identify diagnosed RA patients; however, the highest specificity for RA is achieved when the three autoantibodies are present at the same time.

Materials and Methods

Study selection and inclusion

PubMed was searched for "anti-CarP antibodies". Furthermore, a combined search for "carbamylation" and "antibody" was carried out to identify possible missing studies. On the 2nd of January 2017, the first search showed 61 results, while the second search resulted in 52 PubMed results. Studies were selected based on the following criteria: First, antibody data had to be available on ACPA, RF and anti-CarP antibodies for at least two groups, such as RA patients and controls or RA patients and healthy first-degree relatives (HFDR). Studies describing these antibodies in non-RA patients without a comparison to RA were excluded. Second, since the assay to measure anti-CarP antibodies is not yet commercially available, similar antigens, in this case carbamylated fetal calf serum (Ca-FCS), had to be used for the measurement of anti-CarP antibodies. Third, the controls that were included had to be geographically matched controls. The following subgroups were included: RA patients, HFDR, pre-RA and healthy controls.

After the selection, data were extracted from the papers with a standard form, describing the number of patients positive for each of the possible antibody combinations. If data could not be acquired from the published papers, authors were approached for further information.

Data analysis

Informative antibody combinations (Anti-CarP alone, ACPA alone, RF alone, RF and/or ACPA, RF and ACPA, RF and/or ACPA and anti-CarP, at least 1 antibody, at least 2 antibodies, all 3 antibodies), were selected and used for further analysis. Within each group, the percentage of individuals positive for each antibody combination was calculated. Also, specificity, sensitivity, odds ratios(OR), positive likelihood ratios(LR+) and negative likelihood ratios(LR-) were determined. Calculations were carried out in Microsoft Excel version 2010, SPSS statistics version 23(IBM) or R version 3.2.3[42]. The control group did not contain antibody-positive individuals for some antibody combinations, which interferes with the calculation of ORs and LR+s. To estimate these values, a pseudo-frequency modification was used[43]. This modification entails adding a small number to each cell in the contingency table. This number was different for each study and based on the percentage of positives for a certain antibody combination in all of the relevant control samples combined. The replacement values added varied between 0.04 and 1. Meta-analyses were carried out in Stata version 14 using an inverse variance random effects model, resulting in combined ORs as output. The meta-analysis was carried out for the selected antibody combinations, separately for each of the categories(RA vs Healthy controls; RA vs HFDR; RA vs disease controls; Pre-RA vs No RA development). For the RA vs disease controls group, two studies[35,41] were combined before the meta-analysis, since the RA population in both studies was the same.

Results

Study inclusion and exclusion

A total of 12 publications were included in the analysis. Table 1 shows an overview of the included studies and the number of patients included in each of the different groups. The studies that were excluded either investigated less than two groups, making it impossible to compare groups, or none of the groups included were RA patients[32,37-40]. Studies were also excluded because data on the control group was not available for one or more of the three antibodies[18,19,25,36] or because persons negative for ACPA or RF were excluded from the study[17]. Furthermore, some studies used a different antigen than Ca-FCS to measure anti-CarP antibodies[33,44,45] and a study did not use geographically matched controls[24]. Finally, the IMPROVED study was excluded since part of the patients overlap with the patients in the Leiden EAC study which was included[13,20]. All twelve of the studies included were retrospective studies using a case-control setting. However, three studies were nested case-control studies, all investigating serum samples of RA patients before RA development[14-16]. Although prospective studies would have been ideal to include in our study, the only prospective study available had to be excluded due to patient / control group selection based on antibody status[17].

Cohort	RA development	First-degree relatives	Disease controls	RA	Healthy Controls	Ref
Shi 2011, Shi 2015, the Netherlands			780	934	208	13,41
Janssen 2015, the Netherlands			235	86	36	34
Verheul 2015, Japan				268	127	46
Challener 2015, Canada / USA				517	63	23
Koppejan 2016, Canada		105		92	77	26
Allesandri 2015, Italy		141		63		21
Verheul 2016, the Netherlands			759	934		35
Pecani 2016, Italy			298	309		29
Shi 2014 The Netherlands	79				141	16
Gan 2015 USA	76				41	14
Brink 2015 Sweden	224				150	15

Table 1 – An overview of the number of people present in each of the included studies, separated for each category. RA; rheumatoid arthritis, HFDR; healthy first-degree relatives. Shi 2011 and Shi 2015 make use of the same RA patient cohort as a comparison to either healthy controls or disease controls.

In all of the studies that were included, the ACR 1987 criteria were used for the diagnosis of RA patients. Furthermore, ACPA were measured with anti-CCP2 in all studies, except one[26], in which positivity for CCP2 or CCP3 was used. For RF measurement, RF-IgM was measured in each of the included cohorts.

Prevalence of ACPA, RF and anti-CarP antibodies

To acquire more insight into the data that was acquired, initially, simple overviews of the data were made. The different studies could be divided into 4 subgroups, namely RA patients compared to healthy controls, RA patients compared to disease controls, RA patients compared to HFDR and RA patients before disease development (pre-RA) compared to healthy controls (Figure 1A). ACPA, RF and anti-CarP antibody positivity were compared between the different studies within each category (Figure 1B-D). Within, for example RA patients, ACPA-positivity was 50%-78%, RF-positivity 53%-80% and anti-CarP-positivity 34%-53%. This indicates that within each subgroup, there is some variation with regards to antibody positivity. However, the most obvious differences are between the 4 subgroups, indicating that these subgroups should not be pooled in a meta-analysis.

General presence of autoantibody combinations

Since we hypothesize that the combination of three autoantibodies may provide additional insight in diagnosis or prediction of RA, we set out to investigate different autoantibody combinations that may co-occur within one individual. The number of autoantibodies, (0, 1, 2 or 3) present in the samples in the different studies is shown in Figure 2A-D. In the RA patient studies, we observed that a large proportion of the patients is positive for at least one antibody, but also the combination of two and especially three antibodies is common in RA patients (mean number of autoantibodies between 1.4 and 2.1). For the other groups, it was most common to observe positivity for none of the antibodies. However, positivity for one of the three antibodies or a combination of multiple antibodies was not completely absent. The lowest number of antibodies was observed in healthy controls (mean between 0.0 and 0.2) while a larger number could be detected for HFDR (mean 0.4) and disease controls (mean between 0.2 and 0.4).

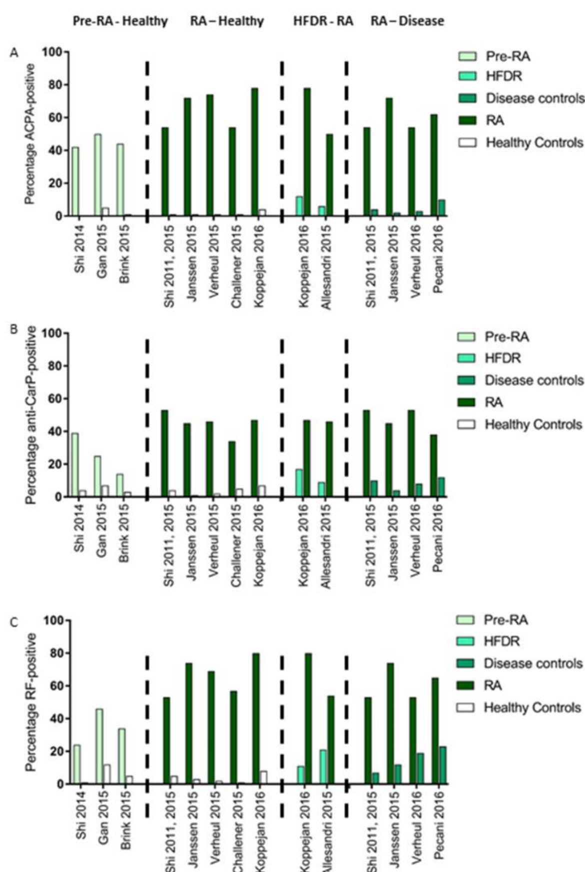

Figure 1 –ACPA, RF and anti-CarP antibody status are similar in studies fulfilling the inclusion criteria. Studies were separated based on the category they were placed in (RA patients compared to healthy controls; People who developed RA after a certain timespan compared to people who do not develop disease; RA patients compared to healthy first-degree relatives and RA patients compared to disease controls). Some studies occur twice, as they fit more than one category. The percentage of antibody-positive people for each of these studies are shown in A) for ACPA, B) for anti-CarP antibodies and C) for RF. The number of patients in each study can be found in table 1. RA; rheumatoid arthritis, ACPA; anti-citrullinated protein antibodies, Anti-CarP; anti-carbamylated protein antibodies, RF; rheumatoid factor.

These data indicate that a large proportion of RA patients has at least one antibody subgroup and more than 40% of the RA patients can be positive for two or three of these antibodies. This pattern is completely different in healthy controls, in which the presence of 1 or 2 antibodies can be observed only in a limited number of people, while the presence of all three of the autoantibodies at the same time is absent in nearly all healthy controls. The other groups, pre-RA, HFDR and disease controls have a slightly higher number of autoantibodies than the healthy controls, but less than the RA patients. The combination of three autoantibodies is also rare in these control groups. Because the combination of three autoantibodies is rare in the control groups, this may be the most interesting antibody combination for further investigation, although some of the other antibody combinations may show surprising results as well. A complete overview of the different autoantibody combinations in the studies are shown in Venn-diagrams in Figure 3.

Sensitivity, Specificity, Odds ratio, LR+ and LR-

To further investigate these observations, several antibody combinations were studied with a focus on the following four: ACPA and/or RF, ACPA and/or RF and anti-CarP antibodies, two out of the three antibodies and three out of the three antibodies. ACPA and/or RF was chosen since this is what has been incorporated into the current guidelines for RA classification[1]. The second combination adds anti-CarP antibodies to the current standard. We hypothesize that the presence of all three autoantibodies at the same time would be the most specific for the diagnosis or prediction of RA. Therefore we also included this combination and as a second option investigated whether the presence of two different autoantibodies out of the three investigated would also result in increased specificity.

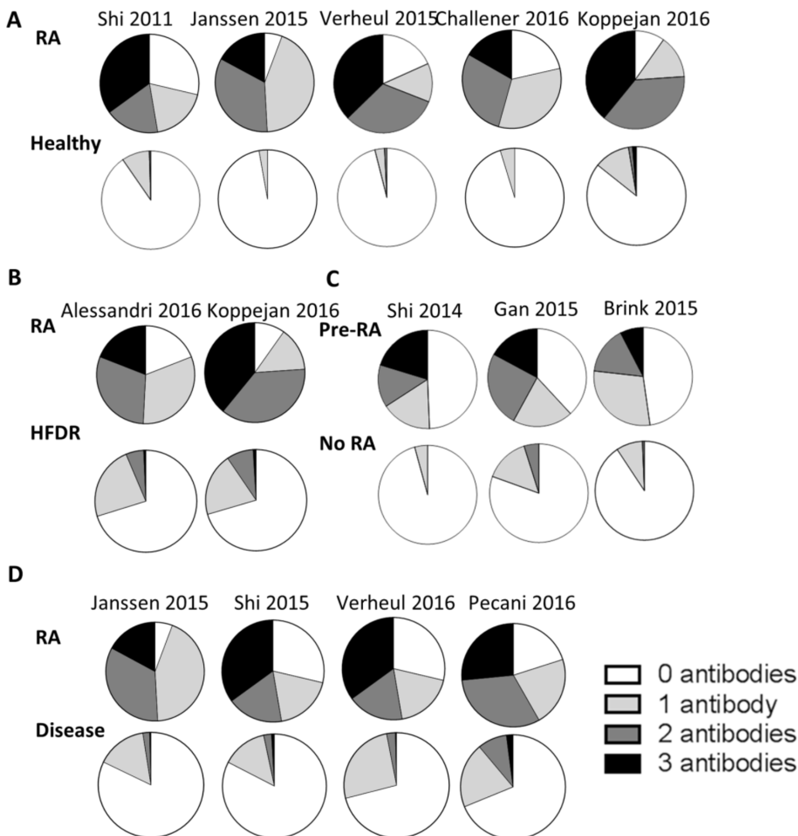

Figure 2 – The number of antibodies is increased in RA patients when compared to non-RA controls. The number of antibodies is shown in pie charts for each of the included studies, showing the comparison for RA vs healthy controls (A), RA vs healthy first-degree relatives (HFDR) (B), Before RA development (Pre-RA)and no RA development (C) and RA vs disease controls (D). For each of the figure, the upper part shows the patients with or who will develop RA, while the lower part shows the respective control group. The number of patients in each study can be found in table 1. RA; rheumatoid arthritis.

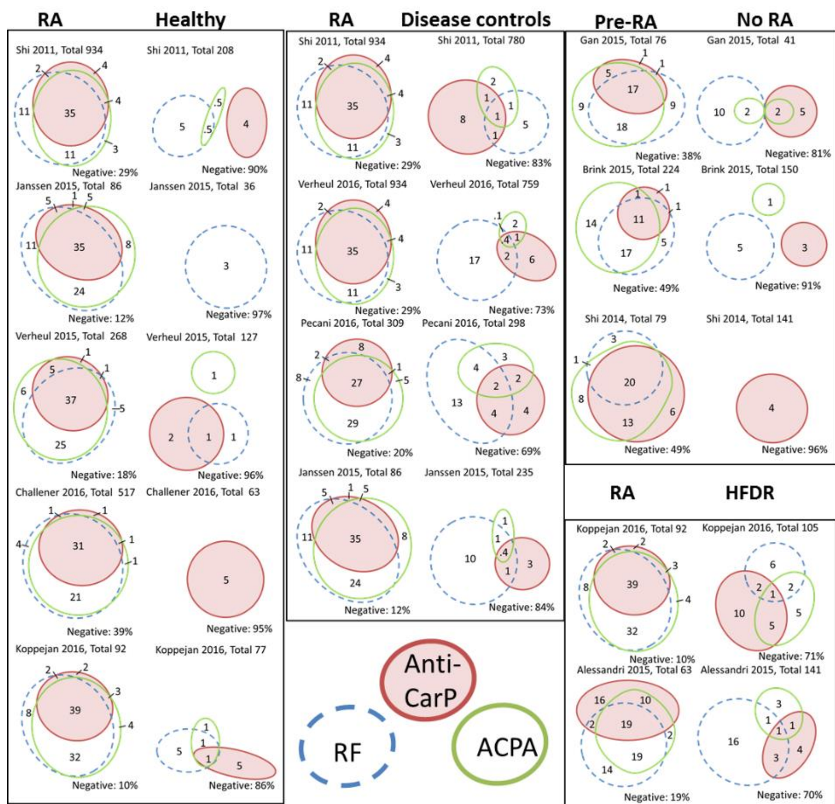

Figure 3 – Detailed overview of autoantibody-status and antibody combinations in the studies included. A detailed overview of the combinations of positivity for ACPA, RF or anti-CarP antibodies is shown in Venn diagrams for RA vs healthy controls, RA vs disease controls, Pre-RA vs RA and RA vs healthy first-degree relatives. The filled red circle represents anti-CarP antibody-positivity. The dashed blue circle shows the RF-positivity and the green solid circle indicates ACPA-positivity. The size of a circle represents the percentage of people with that specific antibody combination when compared to other antibody positive people in the study. Sizes of the circles cannot be compared between groups and are an approximation of the true percentages. The basis for the Venn-diagrams was made in EulerAPE. RA; rheumatoid arthritis, ACPA; anti-citrullinated protein antibodies, anti-CarP; anti-carbamylated protein, RF; rheumatoid factor, HFDR; healthy first-degree relatives.

In general, an increase in the number of antibodies results in a higher specificity and OR, while decreasing the sensitivity. For example, for RA patients compared to healthy controls, the specificity for 1 antibody varies between 85.7 and 97.2, while this is between 98.7 and 100 for the combination of three antibodies. However, the sensitivity for 1 antibody in this same group is between 60.5 and 90.2 and between 30.8 and 39.1 for the combination of three autoantibodies. Interestingly, in many of the studies, a 100% specificity can be achieved with certain antibody combinations.

This occurs most frequently for the combination of all three autoantibodies. This indicates that, in the case-control settings studied, the subjects without RA could be identified perfectly by the absence of the combination of the three autoantibodies. An overview of the specificity, sensitivity, OR, for several antibody combinations is shown in table 2. An overview for other, selected, antibody combinations is shown in supplementary table 1. An overview of the LR+s and LR-s for the same antibody combinations can be seen in supplementary table 2. A complete overview of the number of antibody-positive people for each group and the ORs can be seen in supplementary table 3.

Meta-analysis

A random effect meta-analysis was carried out on the ORs calculated for each of the discussed antibody combinations. These calculations were carried out separately for each of the different categories, since the differences between these categories are too large to combine the data. An overview of the meta-analysis can be found in the supplementary figures, and a summary is provided in Figure 4A-D. When we are interested in the diagnosis of RA, the two most interesting subgroups would be the RA patients compared to disease controls or healthy relatives.

When comparing RA to healthy controls, disease controls or healthy relatives, the combination of ACPA and/or RF seems to perform very well, and might be rather similar to the combination of all three autoantibodies. This indicates that the autoantibodies that are currently in use for the diagnosis of RA may be sufficient and not much improvement may be gained upon the addition of anti-CarP antibodies.

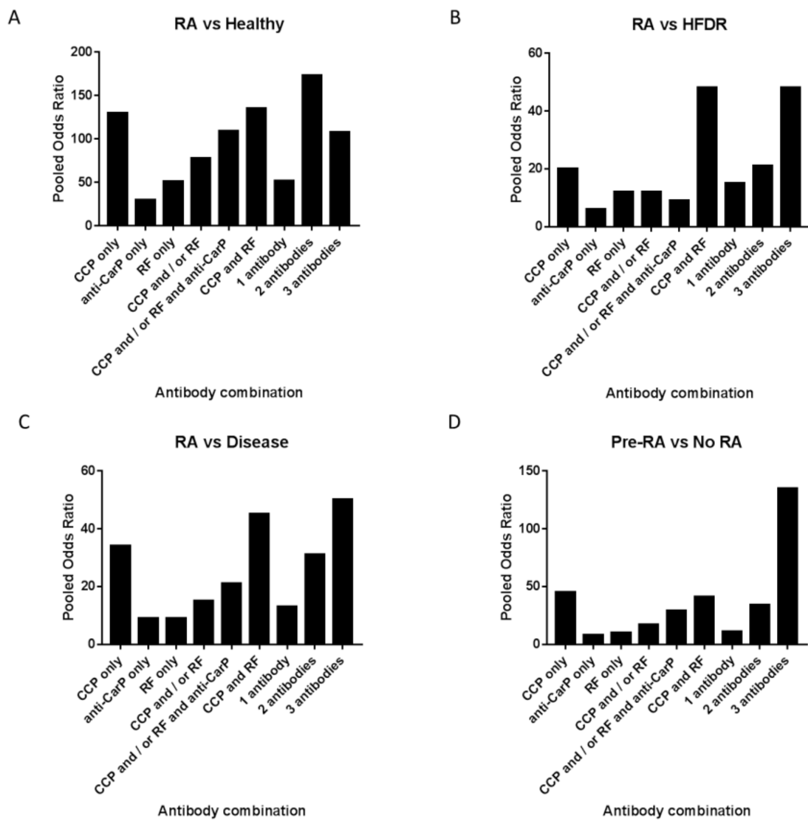

Figure 4 - Overview of pooled odds ratios derived from random effects meta-analyses. Pooled odds ratios are shown separated for the different categories of patients and controls: RA vs healthy controls (A), RA vs healthy first-degree relatives (HFDR) (B), RA vs disease controls (C) and pre-RA vs no RA (D). Random effects meta-analysis was carried out for each of the studies antibody combinations. For the comparison between RA and disease controls two studies were combined before analysis, since the same RA patient cohort was used for comparison [35,41]. An overview of the meta-analyses, with the individual forest plots, can be seen in the supplementary figures. Also, an overview of the individual numbers of antibody-positive patients and ORs for each cohort can be found in supplementary table 3.

		ACPA and / or RF			ACPA / and or RF and anti-CarP			2 antibodies			3 antibodies		
		Spec	Sens	AUC	Spec	Senc	AUC	Spec	Sens	AUC	Spec	Sens	AUC
RA vs Healthy	Shi 2011	94.2	67.5	0.808	100	48.6	0.743	99.5	52.7	0.761	100	35	0.675
	Janssen 2015	97.2	87.2	0.922	100	44.2	0.721	100	68.6	0.843	100	34.9	0.674
	Verheul 2015	97.6	80.2	0.889	99.2	44	0.716	99.2	69	0.841	100	37.3	0.687
	Challener 2016	100	59	0.795	100	32.5	0.662	100	53.4	0.767	100	30.8	0.654
	Koppejan 2016	90.9	88	0.895	98.7	44.6	0.716	97.4	76.1	0.867	98.7	39.1	0.689
RA vs first-degree relatives	Koppejan 2016	80	88	0.84	92.4	44.6	0.685	90.5	76.1	0.833	99	39.1	0.691
	Alessandri 2016	74.5	65.1	0.698	95	30.2	0.626	93.6	49.2	0.714	99.3	19	0.592
RA vs Disease controls	Janssen 2015	87.2	87.2	0.872	98.7	44.2	0.715	97.9	68.6	0.832	99.6	34.9	0.672
	Shi 2015	90.5	67.5	0.79	97.6	48.6	0.731	96.7	52.7	0.747	99.1	35	0.671
	Verheul 2016	78.8	67.5	0.731	97.4	48.6	0.73	97.2	52.7	0.75	99.6	35	0.673
	Pecani 2016	73.2	71.5	0.723	92.6	29.4	0.61	88.9	58.3	0.736	98	26.5	0.623
Before RA vs No RA	Shi 2014	100	44.3	0.722	100	32.9	0.665	100	34.2	0.671	100	20.3	0.601
	Gan 2015	85.4	60.5	0.729	97.6	23.7	0.606	95.1	42.1	0.686	100	17.1	0.586
	Brink 2015	93.3	50	0.717	100	12.9	0.565	100	30.4	0.652	100	10.7	0.554

Table 2 – Sensitivity, specificity and AUCs are shown for 4 different antibody combinations
RA; rheumatoid arthritis, HFDR; healthy first-degree relatives, spec; specificity, sens; sensitivity, OR; odds ratio, ACPA; anti-citrullinated protein antibodies, RF; rheumatoid factor, anti-CarP; anti-Carbamylated protein antibodies, AUC; area under the curve.

When we are interested in the prediction of RA, it is most important to compare the group in which antibodies were measured before RA development and compared to people without RA. Here a clear increase in OR, with an OR over 100, can be observed for 3 autoantibodies when compared to all of the other combinations. This indicates that, especially in a setting of very early RA, the presence of 3 antibodies results in the highest odds for developing RA. Therefore, this combination may help in predicting the development of RA.

Discussion

Here we aimed to investigate the additional value of anti-CarP antibodies compared to ACPA and RF in two different settings, diagnosis and prediction. Therefore, we carried out a literature search and described the studies in which RF, ACPA and anti-CarP antibodies were measured. In a meta-analysis we eventually conclude that measuring all three of these antibodies reduces the chance to misclassify non-RA controls, but may not improve the diagnosis of RA. Therefore the analysis for triple positivity may be especially relevant for populations with a low pre-test probability, although sensitivity will be low with these measurements. These findings are of relevance in view of the efforts towards pre-emptive treatments for people at risk.

A previous meta-analysis has investigated anti-CarP antibodies in RA patients compared to healthy controls[47], resulting in a pooled OR of 17 for anti-CarP antibodies alone. Our pooled OR for anti-CarP antibodies alone when comparing RA patients to healthy controls was 30, which is slightly different, possibly because there were differences in inclusion criteria. The previous study however, did not compare any antibody combinations within the same patient groups and only investigated RA patients compared to healthy controls. In our study, we also compared RA patients to disease controls and HFDR, which are known to have higher autoantibody positivity than healthy controls. The comparison to disease controls is especially important, since the studied antibodies can also be present in non-RA populations[41,48]. Furthermore, we also investigated the number of autoantibodies present in people before RA development (pre-RA) and compared these to healthy controls. One of the studies published after we selected the articles for our meta-analysis, confirms our observations that the use of ACPA and RF might be sufficient after RA diagnosis, although also in this case the addition of anti-CarP antibodies increases the specificity, in exchange for a reduced sensitivity[49].

One of the limitations of this study is that all of the cohorts included were case-control or nested case controls studies and not prospective cohorts. Unfortunately, none of the prospective cohorts available fulfilled the inclusion criteria. Another limitation of this study might be that the assay for anti-CarP antibody measurement is not yet a commercially available, indicating that there might be differences in these measurements. Therefore, rather strict criteria were made with regards to study inclusion, thereby eliminating some interesting studies that could not be

included. Also, some of the studies may have used different methods to determine the cut-off of their assay, however we have used the original data on antibody positivity as described in each individual article. Furthermore, we do not have any info on the stability of the three biomarkers in these patients and whether any seroconversion may occur over time in the patients analysed.

Out of several antibody combinations, measuring all three autoantibodies, ACPA, RF and anti-CarP antibodies or measuring ACPA and RF, often results in the highest specificity and LR+, thereby reducing the sensitivity. Therefore, depending on the context of the investigation, one of the antibody combinations might be more suitable than the other. When aiming to identify RA patients as early as possible, the most relevant group to study would be the group including people before RA, which are currently present in the healthy population. Interestingly, in this group, there is a clearly higher OR for the combination of all three autoantibodies, suggesting that the combination of anti-CarP antibodies, ACPA and RF might result in an improvement of the early identification of people at risk to develop RA.

While the antibody-based biomarkers provide an interesting and robust method to identify persons at risk to develop RA, this will, in the current setting, identify less than 50% of the (future) RA patients as the others are negative for these biomarkers[8,9] . Whether the identification of additional biomarkers will close this "serological gap" remains to be seen[6]. Other biomarkers or early clinical symptoms may serve as additional input into the risk stratification. Furthermore, it has been suggested that the early identification of RA patients, or the identification of arthralgia patients that are at high risk of developing RA is important for effective treatment of RA[50-52]. The combination of these three autoantibodies may help in to identify these high-risk patients. Attractive in the three-autoantibody-approach highlighted here is the low-cost of the assays and equipment, the nature of the sample to be used (serum) and the stability of the antibodies in serum. Moreover, the ease of testing and interpretation of these tests allows for feasible implementation for large-scale testing to identify patients at risk for RA in contrast to other proposed methodologies such as imaging-based tests.

In order to further investigate whether this would be a suitable option and whether the addition of anti-CarP antibodies will result in an increased detection of people at risk for the development of RA, carrying out prospective studies in large healthy populations would be appropriate. In conclusion, the combination of anti-CarP antibodies, ACPA and RF has a very high specificity for the identification of early RA patients compared to different types of controls.

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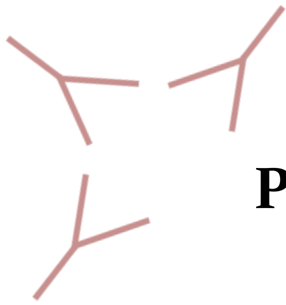

Part II

Anti-CarP antibodies and their antigens

Chapter 8

Pitfalls in the detection of citrullination and carbamylation

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Carbamylation and citrullination are both post-translational modifications against which (auto)antibodies can be detected in sera of rheumatoid arthritis (RA) patients. Carbamylation is the chemical modification of a lysine into a homocitrulline, whereas citrullination is an enzymatic conversion of an arginine into a citrulline. It is difficult to distinguish between the two resulting amino acids due to similarities in structure. However, differentiation between citrulline and homocitrulline is important to understand the antigens that induce antibody production and to determine which modified antigens are present in target tissues.

We have observed in literature that conclusions are frequently drawn regarding the citrullination or carbamylation of proteins based on reagents that are not able to distinguish between these two modifications. Therefore, we have analyzed a wide spectrum of methods and describe here which method we consider most optimal to distinguish between citrulline and homocitrulline.

We have produced several carbamylated and citrullinated proteins and investigated the specificity of (commercial) antibodies by both ELISA and western blot. Furthermore, detection methods based on chemical modifications, such as the anti-modified citrulline-"Senshu" method and also mass spectrometry were investigated for their capacity to distinguish between carbamylation and citrullination.

We observed that some antibodies are able to distinguish between carbamylation and citrullination, but an overlap in reactivity is often present in the commercially available anti-citrulline antibodies. Finally, we conclude that the use of mass spectrometry is currently essential to differentiate between citrullinated and carbamylated proteins present in complex biological samples.

Introduction

The presence of anti-citrullinated protein antibodies (ACPA) is a well-known phenomenon in rheumatoid arthritis (RA). These autoantibodies associate with disease development in RA patients [1,2] and were recently added to the classification criteria for RA[3,4]. ACPA target proteins that have undergone a post-translational modification, citrullination, which is the conversion of an arginine into a citrulline. This is an enzymatic reaction that is facilitated by peptidyl arginine deiminases (PAD)[5]. There are several PAD enzymes with the capacity to citrullinate proteins. These PAD enzymes are often present in the cell, in which they can citrullinate e.g. histones and play a role in gene regulation[6]. Under certain inflammatory conditions, PAD is thought to be released from cells, which may induce local extracellular citrullination[7]. Furthermore, protein citrullination occurs in different physiological processes as evidenced by the presence of several citrullinated proteins in the central nervous system or citrullinated forms of keratin and filaggrin in the skin[8,9].

When compared to ACPA, anti-carbamylated protein antibodies (anti-CarP antibodies) were discovered more recently[10]. Anti-CarP antibodies are increased in RA patients[10-14] and they associate with a more severe disease course as well[10,12]. However, anti-CarP antibodies are a different autoantibody system, with a limited cross-reactivity towards citrullinated respectively carbamylated proteins[15,16]. In human serum samples of RA patients, often both ACPA and anti-CarP antibodies are present. On the other hand, animal models, such as collagen-induced arthritis in mice or primates are characterized by the absence of ACPA, while anti-CarP antibodies can readily be detected[17] (MKV, unpublished observation). Furthermore, anti-CarP antibodies have been detected in non-RA patients as well, although at lower percentages than in RA patients[13,18,19]. Anti-CarP antibodies target proteins that contain homocitrullines, also called carbamylated proteins. Carbamylation is a chemical reaction that is carried out in the presence of cyanate. Both PAD enzymes and cyanate can be increased upon chronic inflammation, which might therefore increase the formation of citrulline and homocitrulline residues. The increase in cyanate during inflammation can be due to the conversion of thiocyanate into cyanate by myeloperoxidase[20]. Furthermore, cyanate can be increased in renal failure, due to its equilibrium with urea[21-23]. Smoking is also thought to increase the presence of cyanate and therefore carbamylation[24].

Although there are similarities between the two autoantibody systems (ACPA and anti-CarP antibodies), they do target two different post-translational modifications. As the name of both residues already implies, the difference between a citrulline and a homocitrulline is small. We therefore often observe situations in which the distinction between a citrulline and a homocitrulline is difficult. However, it is important to distinguish between the two post-translational modifications in order to further investigate the role of ACPA and anti-CarP antibodies separately. Although both antibodies can be detected years before disease onset[2,12], it is at the moment not clear whether the RA autoantibody response is first initiated against a citrulline or a homocitrulline residue. In the future, it may therefore be important to gain a deeper understanding of these modifications, especially when further investigating the possible pathogenesis of RA.

Here, we describe the most specific methods to determine whether a modification is a citrulline or a homocitrulline. We show the that use of polyclonal and monoclonal antibodies for ELISA or western blot, are not always sufficiently specific to make formal conclusions on the nature of the modified proteins. Furthermore, we discuss the use of the chemical “Senshu” modification in different settings and conclude with the pitfalls in detecting citrulline and homocitrulline using mass spectrometry (MS). In the end, we conclude that antibodies or other (chemical) detection methods are suitable to determine whether an *in vitro* modification was successful, but that MS is required to distinguish between the two amino acids in more complex settings.

Description of different methods to measure carbamylation and citrullinaton

Antigens and modifications

For our analyses we have used citrullinated and carbamylated versions of three antigens, namely fetal calf serum (FCS, Bodinco), human serum albumin (HSA, Sigma) and human fibrinogen (Fib, Sigma). The non-modified counterparts served as controls. Carbamylation of proteins was carried out by incubating the protein in a final concentration of 1M potassium cyanate (KOCN, Sigma) diluted in PBS. The protein concentration was between 2mg/ml and 5mg/ml, depending on the protein involved. This mixture was incubated overnight at 37°C, after which the solution was dialyzed against PBS for 48 hours at 4°C. During this time, the PBS was refreshed at least 5 times. Citrullination of HSA, Fib and FCS was carried out by diluting the protein in 0.1M Tris-HCL, 5mM DTT and 10mM CaCl2. For each mg of protein 10 units of PAD (Sigma) were added and the solution was incubated at 53°C for six hours. The presence of citrullination and or carbamylation was confirmed by both ELISA and MS.

Commercially available Antibodies

Four commercially available anti-citrulline antibodies and two anti-homocitrulline antibodies were used in these experiments. The anti-citrulline antibodies were mouse monoclonal anti-citrulline antibody, clone F95 (Millipore, MABN328), rabbit polyclonal anti-citrulline antibody (Upstate, 07-377), rabbit polyclonal anti-citrulline antibody (Abcam, ab100932) and rabbit polyclonal anti-citrulline antibody (Abcam, ab6464). The anti-homocitrulline antibodies were goat polyclonal anti-homocitrulline antibody (Abcam, ab175576) and rabbit polyclonal anti-homocitrulline antibody (Cell biolabs, STA-078). As secondary antibodies, to detect binding of the antibodies described above, the following antibodies were used: goat anti-rabbit Ig – HRP (DAKO, P0214), goat anti-mouse Ig – HRP (DAKO, P0447) and rabbit anti-goat Ig – HRP (DAKO, P0449). Monoclonal antibody binding to modified proteins was detected with goat anti-mouse IgG2a – HRP (Southern biotech, 1080-05) or rabbit anti-human IgG - HRP (DAKO, P0214).

Monoclonal antibodies

The monoclonal antibody targeting anti-CarP antibodies was made previously[16]. Antibody-producing hybridomas were made by fusing spleen cells of Ca-OVA immunized mice with SP2/0 myeloma cells using PEG1500 (Roche).

The monoclonal anti-citrulline antibody was made previously by culturing single B cells from an ACPA-positive RA patient, enriched via FACS sorting using a citrullinated fibrinogen peptide tetramer staining, for 14 days followed by screening of supernatants for production of antibodies reactive to citrullinated fibrinogen[25].Variable domain sequences were obtained from RACE PCR products, and synthetic DNA constructs cloned into a pcDNA3.1 vector for protein expression in Freestyle HEK293 cells.

ELISA

Nunc Maxisorp plates (Thermo scientific) were coated with 10μg/ml of the non-modified or modified antigens and incubated overnight at 4°C. Plates were blocked with PBS with 1% bovine serum albumin (BSA, Sigma) for at least 6 hours at 4°C, followed by addition of the primary antibody in different concentrations, which were diluted in PBS, 1% BSA and 0.05% Tween20 (Sigma). The primary antibody was incubated overnight at 4°C. The secondary antibody was incubated for 3.5 hours at 4°C. The presence of antibody-bound HRP was detected with ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid). In between the different steps, plates were washed 3x with PBS 0.05% Tween20.

Western Blot

5μg of (modified) FCS and 2μg of (modified) HSA or fibrinogen was loaded on a 4%-15% gel (Bio-Rad) for gel electrophoresis. The Pageruler Plus Prestained protein ladder (Thermo Scientific, 26619) was used as a size comparison for the samples. Protein transfer was carried out using a Bio-Rad system and PVDF transfer packs (Bio-Rad, 170-4156) Membranes were blocked for 1 hour with PBS, 3% skim milk powder (Sigma) and 0.05% Tween 20 at room temperature (rt). Primary antibodies were incubated for 2 hours at rt and secondary antibodies were incubated for 1 hour at rt. Washing steps were carried out with PBS 0.05% Tween20. The HRP signal was developed using ECL (GE healthcare, RPN2109).

Senshu method

The so-called anti-modified citrulline-“Senshu”-method was carried out as described[15,26]. The initial steps are similar as for the western blotting procedure. However, after protein transfer, the membrane is blocked in 0.1% ovalbumin for 15 minutes at rt and the membrane is fixed with 4% paraformaldehyde for 15 minutes at rt. After each of these two steps, milliQ is used for washing. Solution A, consisting of 0.25% $FeCl_3$, 25% H_2SO_4 and 20% H_3PO_4 and solution B consisting of 0.5% butanedione monoxime, 0.25% antipyrine and 0.5M acetic acid are both diluted in milliQ. The solutions are mixed 1:1 and incubated overnight at 37°C with the membrane, while shaking. The blot is blocked with PBS, 5% skim milk powder and 0.05% Tween20 for 1 hour at rt. Human anti-modified citrulline antibody (Modiquest, MQR2.601) is incubated at 1μg/ml for 3 hours at rt. As a secondary antibody, rabbit-anti-human IgG-HRP was used (DAKO, P0214). The “blocking buffer” is also used for the antibody dilutions. Washing steps were carried out with PBS 0.05% Tween20, unless mentioned otherwise. The HRP signal was detected using ECL (enhanced chemiluminescence, GE Healthcare).

Results

<u>All tested commercial anti-citrulline antibodies also recognize carbamylated proteins in ELISA</u>

We tested the reactivity of 6 commercially available antibodies, of which 2 are reported to be specific for homocitrulline and 4 are reported to be specific for citrulline, against three carbamylated, citrullinated or non-modified proteins in ELISA. The antigens used were FCS, HSA and Fib. The antibodies targeting homocitrullines display a binding pattern rather specific for carbamylated proteins, although low reactivity against citrullinated proteins is also observed for one of the antibodies (Figure 1A).
Out of the 4 anti-citrulline antibodies, all 4 show, next to reactivity against citrullinated proteins, also extensive reactivity against carbamylated proteins (Figure 1B). Often, the reactivity observed towards the carbamylated version of the protein is even higher than the reactivity towards the citrullinated version of the same protein. Reactivity to the non-modified protein was hardly present in all cases.

<u>Antibodies can be specific for citrulline or homocitrulline in ELISA</u>

Although we observed that many of the commercial antibodies are not specific for citrulline or homocitrulline, this does not directly implicate that antibodies in general cannot be specific for these post-translational modifications. Previously, we have shown for human antibodies in a polyclonal setting that antibody specificity for either carbamylation or citrullination does exist [15]. Here we also show this specificity for a monoclonal antibody response. We have previously acquired a human ACPA monoclonal antibody and generated a mouse anti-CarP monoclonal antibody, which we also tested in a similar ELISA setting as the commercial antibodies (Figure 2). The results indicate that the ACPA monoclonal is very specific for citrullinated proteins, while the anti-CarP monoclonal also shows some reactivity towards the citrullinated proteins. This indicates that specificity towards citrulline or homocitrulline does exist, but is dependent on the antibodies that are used.

<u>Detection of carbamylated and citrullinated proteins in western blot shows similar specificity profiles as in ELISA</u>

Besides ELISA, there are also other methods to detect carbamylated or citrullinated proteins. The anti-citrulline and anti-homocitrulline antibody that seemed to be most specific were selected and used for further experiments in western blot (figure 3a-b), using the same antigens as in ELISA. These data confirm that the anti-citrulline antibody is not specific for citrulline alone, while the anti-homocitrulline antibody does not seem to react with the citrullinated proteins.
Combined, these data therefore indicate that the antibodies that are thought to specifically target citrullinated proteins should be used with caution.

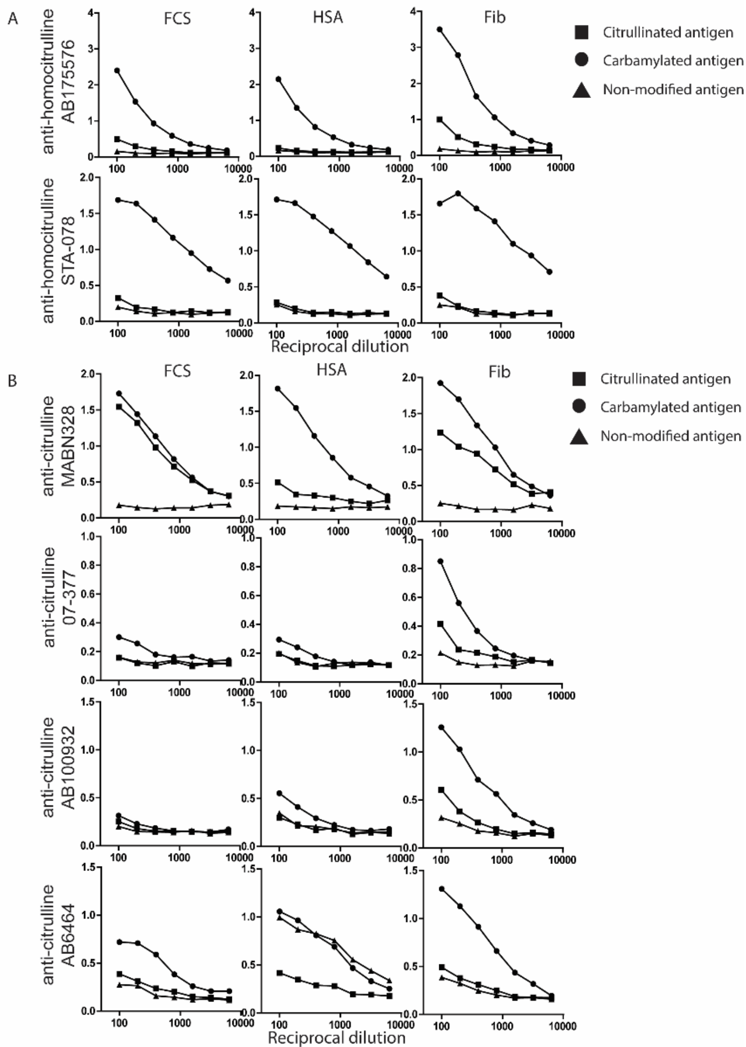

Figure 1 - Reactivity of commercial antibodies against three carbamylated and citrullinated proteins**.** Antibody reactivity of 2 anti-homocitrulline antibodies (A) and 4 anti-citrulline antibodies (B) against carbamylated (circle), citrullinated (square) and non-modified (triangle) proteins was determined by ELISA. The numbers on the Y-axis represent the absorbance values measured at 415 nm, while the x-axis shows reciprocal dilution. FCS: fetal calf serum, HSA: human serum albumin, Fib: fibrinogen.

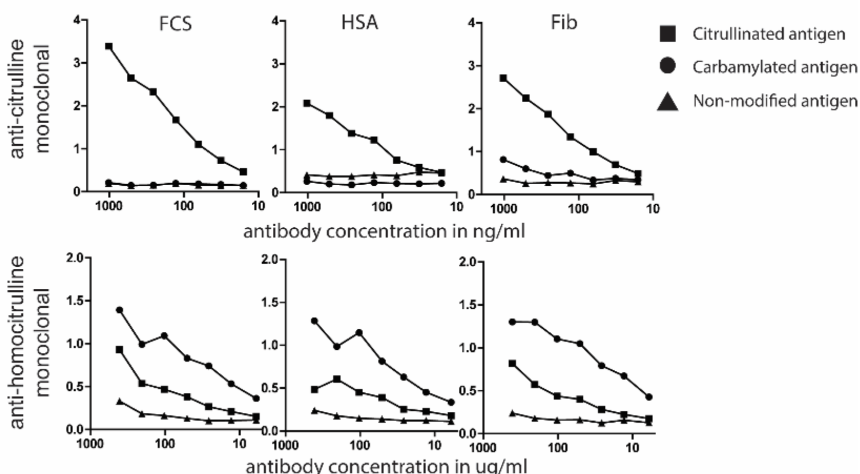

Figure 2 – Reactivity of two monoclonal antibodies against three carbamylated and citrullinated proteins. Antibody reactivity of one human anti-citrulline monoclonal and one mouse anti-homocitrulline monoclonal against carbamylated (circle), citrullinated (square) and non-modified (triangle) proteins was determined by ELISA. The numbers on the x-axis represent the concentration of the monoclonal antibody. The numbers on the Y-axis represent the absorbance values measured at 415 nm. FCS: fetal calf serum, HSA: human serum albumin, Fib: fibrinogen.

<u>The Senshu method is also not able to distinguish between citrulline and homocitrulline residues</u>

Another method to detect either citrulline or homocitrulline is the anti-modified citrulline (AMC)-Senshu method[26]. This method was initially used for the detection of citrullinated proteins. But more recent studies have indicated that next to citrulline also homocitrulline is modified in exactly the same way[15,27]. This method is based on the chemical modification of citrulline and homocitrulline residues, after which this additional chemical modification can be recognized by antibodies. This is also the case for the proteins that we modified (Figure 3c), indicating that this method is indeed not specific. The chemical modification used for the Senshu method can also be used for 96 well-plate assays or immunohistochemistry staining (data not shown)[28]

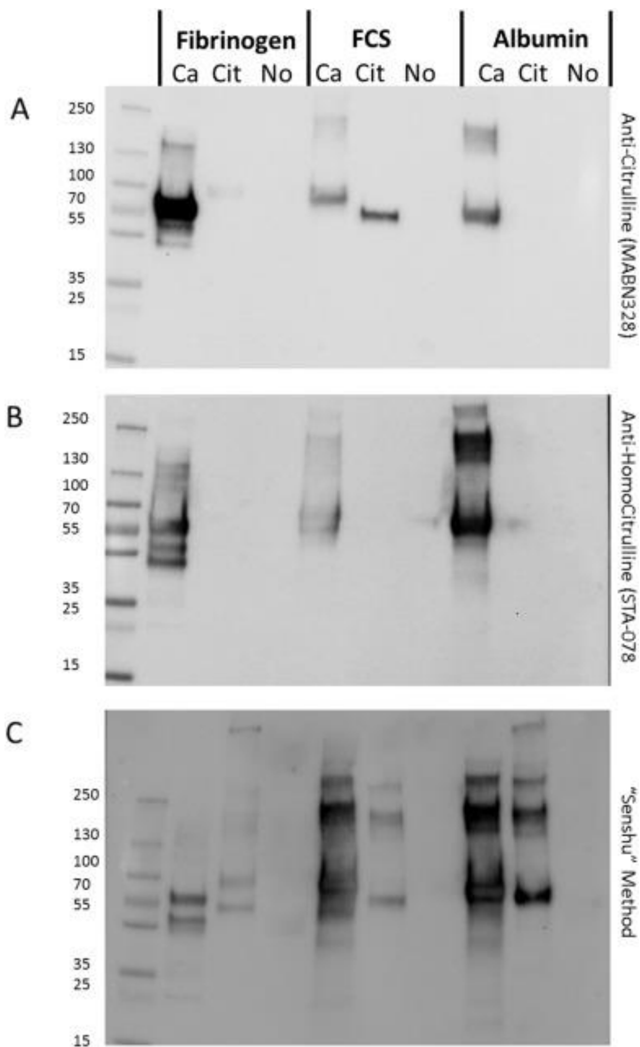

Figure 3 – Reactivity of two commercially available antibodies in western blot and a senshu on the same proteins. Antibody reactivity of anti-citrulline antibody (Millipore MABN328) (A) and anti-homocitrulline antibody (Cell biolabs, STA-078) (B) against carbamylated, citrullinated and non-modified proteins was determined by western blot. The same proteins were also used to carry out a Senshu, a chemical detection method for carbamylation and citrullination.(C).

Mass Spectrometry

Methods to detect post-translational modifications with MS were described previously [16,29]. Distinguishing between a citrulline and a homocitrulline residue is straightforward because both residues are present at a different position within the protein (they are derived from either an arginine or a citrulline). Furthermore, homocitrulline differs from citrulline by 14 Da. We have recently used this approach for the identification of carbamylated alpha-1 antitrypsin and albumin in human samples[16,29]. However, confident determination of citrullination (ΔMCit-Arg=+1 Da), is prone to false positive assignments using automated standard proteomic workflows. A number of complicating factors (of which some may also apply for the identification of homocitrulline residues) are briefly discussed below.

Citrullination is a sub stoichiometric post-translational modification. Therefore, low abundant peptides have to be analyzed in a full proteome background. Because of their low abundance the spectral quality can be poor. In addition, the low abundance also leads to more interferences during precursor ion isolation, i.e. co-isolation of other species, which leads to mixed or contaminated MS/MS spectra that are more difficult to interpret (and more prone to wrong assignments). An extra complication is that the deamidation of an asparagine residue ($\Delta m=+1$ Da) is rather common and has the same mass change. Therefore, deamidation has to be included as a variable modification during the matching of the recorded tandem mass spectra with the protein database. If deamidation is not included the software can wrongly fit the tandem mass spectrum to include e.g. an citrulline residue at an arginine position. However, the inclusion of two variable modifications, both leading to a +1 Da mass shift, is also a further complication. A citrulline may also be assigned wrongly at an arginine position due to inadvertent calling and selection of the second isotope of the precursor isotope pattern as the precursor mass. Finally, conversion of an arginine into a citrulline makes that particular site insensitive to trypsin cleavage, resulting in longer peptides, which generally yield less complete fragment ion series as well. Of note, C-terminally citrullinated tryptic peptides (protein C-terminal residue excluded), regularly 'identified' by the database matching process, are a clear illustration of false positive assignments.

In spite of the above, citrulline residues can be confidently assigned if performed with great caution from the tandem mass spectra, e.g. as performed before[30] in which citrullinated fibrinogen peptides in SF were identified. Using a similar approach we identified the same citrullinated peptides (data not shown). The best solution to correct assignment of tandem mass spectra is the comparison of the tentatively identified sequences and their synthetic counterparts. It unequivocally establishes the correctness of the tentatively assigned peptide sequence. In full proteomics experiments, a high number of identifications might prevent a one-by-one comparison strategy because of costs involved. However, if investments are made in follow-up work, checking the correct identifications with synthetic peptides is strongly recommended.

Discussion

Here we describe that many of the commercially available antibodies are often not suitable for the distinction between carbamylation and citrullination in both ELISA and western blot. Especially the tested anti-citrulline antibodies, recognize both citrulline and homocitrulline. Changing experimental conditions, such as buffers and incubation times, might improve the specificity of the antibodies. However, most other protocols that we applied for some of the anti-citrulline antibodies resulted in a reduced specificity and/or sensitivity (data not shown). Changes in experimental set-up may however influence the observed specificity of these antibodies.

We also show that the use of a monoclonal antibody can result in increased specificity, indicating that antibodies can be more specific for citrulline. Furthermore, the commercial antibodies targeting carbamylation seemed to be more specific than the commercial anti-citrulline antibodies. For all of the tested polyclonal antibodies, a small molecule or peptide, sometimes coupled to KLH was used for animal immunization. Often, affinity purification was carried out (not for AB100932 and AB6464) as well, but cross-reactivity still seems to be present, especially for the anti-citrulline antibodies. Using stricter protocols and negative selection for the unwanted reactivity may result in improved specificity. However, it cannot be excluded that these antibodies recognize other, (non-)modified proteins when applied to a complex mixture of proteins. Therefore the use of these antibodies to identify the presence of one of these two modifications in complex biological samples is in our opinion very limited. Determination of the success of an *in vitro* modification, such as carbamylation or citrullination on the other hand, would be a very useful application for which these antibodies can be used, when the non-modified protein is taken along as a negative control.

The use of chemical modifications, such as the Senshu method was shown before to be insensitive for the distinction between citrulline and homocitrulline[15,27]. Here we confirm these data and show that although this method is very efficient in identification of the post-translational modifications, discrimination is not possible. Another method, using a rhodamine-phenylglyoxal probe also recognizes both citrulline and homocitrulline residues[31].

Finally, we have discussed that MS is a very effective method to identify the presence of a citrulline or a homocitrulline. However, identification of citrullination requires additional attention to avoid false-positive assignments.

Even though the detection of carbamylation or citrullination with antibodies or chemical methods may not be most optimal, these methods have often been used in the past, when the focus in the RA-field was more on ACPA and not yet on anti-CarP antibodies. The findings that most of the methods used here are also able to detect carbamylation do not completely invalidate these previous studies. However, it is important to retrospectively evaluate these studies which may result in a different view on the relative role of citrullinated versus carbamylated proteins in health and disease.

Conclusion

To summarize, the use of commercial antibodies or chemical modification at this point in time, is not optimal to distinguish between carbamylation and citrullination. MS on the other hand, is a very effective method for making this distinction, at least when all pitfalls are avoided successfully.

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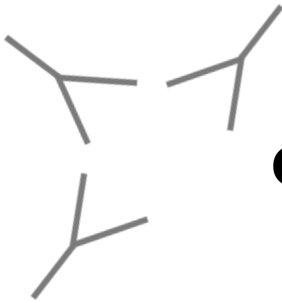

Chapter 9

The identification of carbamylated alpha 1 anti-trypsin (A1AT) as an antigenic target of anti-CarP antibodies in patients with rheumatoid arthritis

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In 2011 a novel autoantibody system, anti-carbamylated protein (anti-CarP) antibodies, was described in rheumatoid arthritis (RA) patients. Anti-CarP antibody positivity associates with a more severe disease course, is observed years before disease onset, and may predict the development of RA in arthralgia patients. Although many clinical observations have been carried out, information on the antigenic targets of anti-CarP antibodies is limited. Most studies on anti-CarP antibodies utilize an ELISA-based assay with carbamylated fetal calf serum (Ca-FCS) as antigen, a complex mixture of proteins. Therefore, we analysed the molecular identity of proteins within Ca-FCS that are recognized by anti-CarP antibodies.

Ca-FCS was fractionated using ion exchange chromatography, selecting one of the fractions for further investigation. Using mass-spectrometry, carbamylated alpha-1-antitrypsin (Ca-A1AT) was identified as a potential antigenic target of anti-CarP antibodies in RA patients. A1AT contains several lysines on the protein surface that can readily be carbamylated.

A large proportion of the RA patients harbour antibodies that bind human Ca-A1AT in ELISA, indicating that Ca-A1AT is indeed an autoantigen for anti-CarP antibodies. Next to the Ca-A1AT protein, several homocitrulline-containing peptides of A1AT were recognized by RA sera. Moreover, we identified a carbamylated peptide of A1AT in the synovial fluid of an RA patient using mass spectrometry.
We conclude that Ca-A1AT is not only a target of anti-CarP antibodies but is also present in the synovial compartment, suggesting that Ca-A1AT recognized by anti-CarP antibodies in the joint may contribute to synovial inflammation in anti-CarP-positive RA.

Introduction

Anti-citrullinated protein antibodies (ACPA) and rheumatoid factor (RF) are important serological markers in the diagnosis of rheumatoid arthritis (RA)[1,2] and are therefore part of the American College of Rheumatology (ACR) / European League Against Rheumatism (EULAR) disease classification criteria for RA[3]. ACPA are mostly detected using anti-cyclic citrullinated peptide (CCP) antibody assays. In 2011, we described antibodies to carbamylated proteins (anti-CarP antibodies) in RA patients using an ELISA, based on carbamylated fetal calf serum (Ca-FCS)[4-6]. In contrast to citrullination, which is mediated by enzymes, carbamylation is a chemical reaction mediated by cyanate, converting lysine into a homocitrulline[7] which is very similar to citrulline (only one CH2 longer side chain)[8].

Importantly, anti-CarP antibodies have been detected in both ACPA-positive and ACPA-negative RA patients, indicating a potential diagnostic value of anti-CarP antibody testing[9-11].

A recent meta-analysis estimated the sensitivity, specificity and odds ratio of anti-CarP antibodies as 42% (95% CI 38-45), 96% (95% CI 95-97) and 17 (95% CI 12-24), respectively when comparing RA patients to healthy controls[12]. Also, using a large cohort of longitudinal samples, it was demonstrated that anti-CarP antibodies predict joint damage as assessed by total Sharp van der Heijde Score[4]. Additionally, anti-CarP antibodies are present in individuals that developed RA already many years prior to disease onset[13-15]. The presence of anti-CarP antibodies has a prognostic value in arthralgia regarding the future development of RA[5]. Similar to ACPA and RF, anti-CarP antibodies have been reported in first degree relatives of RA patients and could help to identify individuals that might develop RA[16]. Even though anti-CarP antibodies are predominantly found in RA patients, they can also present in other inflammatory conditions at considerably lower frequencies[17-19].
Studies have also shown that carbamylation can occur *in-vivo*[20,21]. However, little is known about the antigens that can be recognized by anti-CarP antibodies. Although it is wel understood that Ca-FCS is recognized by anti-CarP antibodies, it is yet unknown which antigens within the complex mixture of Ca-FCS are targeted by anti-CarP antibodies. The identification of the molecular targets by anti-CarP antibodies in RA patients could aid in the etiological understanding of RA. Therefore, the goal of the current study was to characterize the protein(s) present in carbamylated FCS that are targeted by anti-CarP antibodies, where we, as a proof of concept, focus on one of the identified proteins.

Materials and methods

Patients and sera

We selected 80 ACPA-positive and 80 ACPA-negative RA patients from the Leiden Early Arthritis Clinic (EAC; Leiden, The Netherlands). Serum samples from disease controls were also derived from the EAC cohort; patients with gout (n=51), psoriatic arthritis (n=40), osteoarthritis (n=40), sarcoidosis (n=36) and spondylarthropaty (n=40).

In addition, serum samples were collected from 80 healthy controls from the Leiden area[4]. ACPA, RF and anti-CarP antibody status were acquired previously for the samples from RA patients and healthy controls[4]. Patient identity was not disclosed and the data were used anonymously in accordance with the Helsinki Declaration of human research ethics.

	RA patients	Healthy controls	Disease controls
n	160	80	207
age (average, stdev)	54,9 (14,7)	41,8 (12,7)	48,5 (16,5)
gender (% female)	66.4	51.3	44.0
DAS (average, stdev)	3 (1.3)		
ACPA (% positive)	50	1.3	5.1
RF (% positive)	56.9	5	8.3
anti-CarP antibodies (% positive)	37.5	5	10,8

Table 1 – Biographical data on RA patients and healthy controls. The table shows the available characteristics for RA patients and healthy controls. RA = rheumatoid arthritis, stdev = standard deviation, DAS = disease activity score, ACPA = anti-citrullinated protein antibodies, RF = rheumatoid factor, anti-CarP = anti-carbamylated protein, measured using FCS as antigen.

Carbamylation of antigens

Carbamylated proteins were produced by allowing the protein to react with potassium cyanate (KOCN, Sigma-Aldrich, St. Louis, MO) as previously described[4]. In brief, a 2M solution of KOCN was prepared in PBS. Fetal calf serum (FCS, Bodinco, Alkmaar, the Netherlands) or Alpha-1-antitrypsin (A1AT, Lee Biosolutions, Maryland heights, USA) were mixed with the 2M KOCN solution in a 1:1 volume-by-volume proportion. The mixed solution was incubated overnight at 37°C to produce Ca-FCS and Ca-A1AT. Following the incubation period, the resulting carbamylated solution was dialyzed against PBS (2L) for 48hrs, during which the PBS was refreshed at least 5 times. The *in-vitro* carbamylation of A1AT and FCS was confirmed by MS (data not shown).

Immunoassays

Anti-CarP antibodies were detected using Ca-FCS or Ca-A1AT as previously described[4]. In brief, unmodified FCS and Ca-FCS were coated overnight on NUNC MAXISORP® plates (Thermo Scientific, Waltham, MA). Following washing and blocking, the wells were incubated with serum samples obtained from human RA patients and healthy volunteers. Bound human IgG was detected either indirectly using rabbit anti-human IgG (Dako, Glostrup, Denmark), followed by HRP-labeled goat anti-rabbit IgG antibody (Dako), or directly using an HRP-labeled rabbit anti-human IgG (Dako). Following additional wash steps, HRP enzyme activity was measured using ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)). The cut-off for a positive response was chosen as the mean plus two times the standard deviation (SD) of the anti-CarP reactivity of healthy controls.
For epitope mapping studies using peptide fragments of A1AT, anti-biotinylated peptide ELISAs were carried out. NUNC MAXISORP® plates were coated overnight with streptavidin (Invitrogen) followed by incubation of 10ug/ml of biotinylated peptide diluted in PBS with 0.1% BSA for a period of 1 hour at room temperature.

Serum samples were diluted 50x in PBS with 1% BSA and 0.05% Tween and also incubated for 1 hour at 37 °C. Reactivity was detected using the HRP-labeled goat anti-rabbit IgG antibody for 1 hour at 37°C. Again, HRP enzyme reactivity was measured using ABTS.

Identification of A1AT by ion exchange fractionation and mass spectometry
Ca-FCS was fractionated by ion-exchange HPLC (high-performance liquid chromatography) using a MonoQ column. HPLC fractions were analyzed by measurement of absorption for their overall protein content using a plate reader as well as by ELISA used to test anti-CarP antibody reactivity (not normalized for protein content). Five fractions, covering the entire spectrum were selected for further ELISA experiments. Antibody reactivity against these fractions was determined in six serum samples, now coating equal concentrations. The samples included two serum samples with anti-CarP antibodies and no ACPA (CarP+/ACPA-), two serum samples with ACPA antibodies and no anti-CarP antibodies (CarP-/ACPA+), as well as two negative control serum samples from healthy volunteers. The fraction that showed highest reactivity in ELISA was run on an SDS-PAGE. Coomassie stained bands were excised and subjected to chymotryptic digestion and mass spectrometry (MS).

Synthetic overlapping peptides
Synthetic 21-mer peptides were synthesized based on the human A1AT sequence. Each lysine within the human A1AT sequence was identified and used as the basis for designing a peptide. On each side, the 10 flanking amino acids were added. If multiple lysines were present within 21 amino acids, multiple peptides were made. All peptides contained a biotin at the N-terminal end for ELISA testing. The peptides were synthesized as partially overlapping fragments, covering all lysines in the human A1AT sequence.

Modeling of A1AT
The known protein sequence of A1AT was entered into RasMol to generate a 3 dimensional model of the protein. RasMol (www.openrasmol.org) is a molecular graphics program intended for the visualisation of proteins, nucleic acids and small molecules. The program is aimed at display, teaching and generation of publication quality images. The program reads in a molecule coordinate file and interactively displays the molecule on the screen in a variety of colour schemes and molecule representations. All lysin residues were marked on the A1AT molecule and illustrations from two different perspectives were generated.

Mass spectrometric identification of carbamylated A1AT
MS analysis was used to identify the protein(s) present in the selected protein band in SDS-PAGE and to confirm full conversion to homocitrulline in the in vitro carbamylated A1AT preparations used in our studies. MS was also used to identify

the presence of carbamylated A1AT in the joint of an RA patient. The details of the MS procedures are described in the supplementary information.

Statistical evaluation

Data were statistically evaluated using Analyse-it software (Version 4.51; Analyse-it Software, Ltd., Leeds, UK). Comparative descriptive analyses were carried out to analyze the discrimination between different patient groups. Since values were not normally distributed according to Kolmogorov-Smirnov, non-parametric statistics (median and percentiles) have been used. The spearman rank test was used to analyze the correlation between biomarker results and disease activity as well as joint erosions. Mann Whitney tests were used to investigate the differences between two groups. Receiver operating characteristic (ROC) analyses were used to assess the discriminatory power of the different assays. P values < 0.05 were considered as significant.

Results

Ca-A1AT is one of the antigens in Ca-FCS targeted by antibodies in sera of RA patients

To identify possible antigens of anti-CarP antibodies within Ca-FCS, the protein mixture was first separated into fractions using ion exchange chromatography. Both protein content and antibody reactivity against each of these fractions were determined using ELISA measurements (Figure 1A). While the antibody reactivity with a healthy serum sample was minimal, reactivity with an anti-CarP-positive serum sample was evident in most of the fractions. Next, five fractions, namely 6, 19, 24, 29 and 35 were selected for further investigation. Each of these fractions was coated at an equal protein concentration and subsequently tested for antibody reactivity, using six different serum samples. All five fractions were able to bind to antibodies from two anti-CarP-positive serum samples while the signal for the four negative control serum samples was barely detectable (Figure 1B). The fraction with the highest ratio between the positive and the negative controls, fraction 19, was used for a Coomassie staining (Figure 1C) and protein identification using mass spectrometry (MS). MS analysis of the main protein band in fraction 19, resulted in the identification of 4 different carbamylated proteins. These proteins were carbamylated alpha-1-antitrypsin (A1AT), Alpha-2-HS-glycoprotein, Alpha-1-acid glycoprotein and Fetuin-B of which carbamylated alpha-1 antitrypsin (A1AT) was the most pronounced candidate, since most high-quality carbamylated peptides identified were derived from this protein (Supplementary figure 1).
Following the identification of bovine Ca-A1AT in Ca-FCS as a target of anti-CarP antibodies we analysed the protein for the presence of lysine residues and their surface exposure. In order to localize lysine residues exposed on the surface of A1AT a 3 dimensional theoretical model was generated. 32 lysine residues were identified in the bovine protein sequence (34 in the human sequence) of which a significant number is exposed on the surface and which therefore represent potential sites for carbamylation (Figure 2).

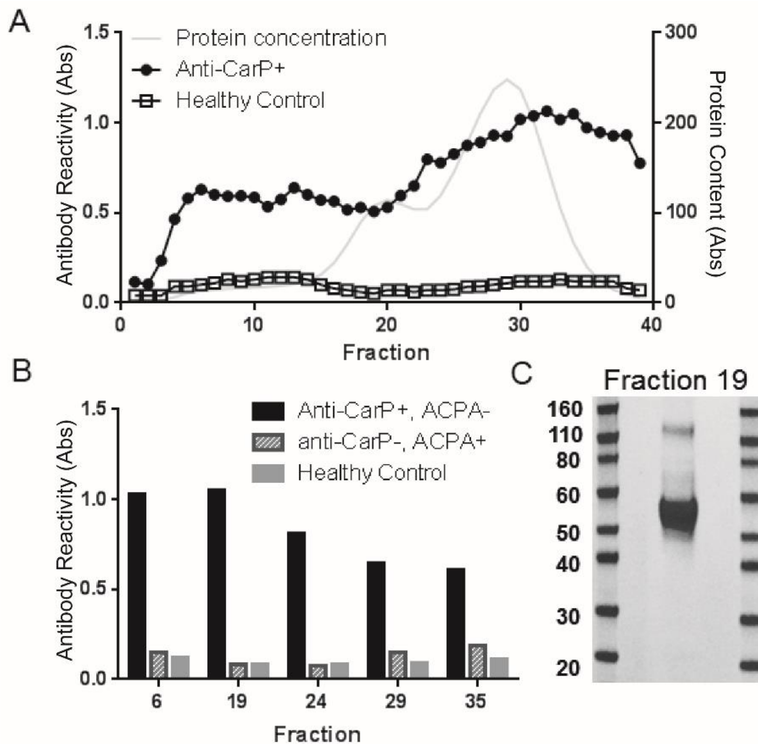

Figure 1 - Identification of alpha 1 anti-trypsin (A1AT) as a major carbamylated protein recognized by anti-CarP antibodies. In a.) the characteristics of the protein fractions obtained by ion exchange chromatograpy are shown. On the left Y-axis, antibody reactivity against each of the fractions is shown for a patient sample positive for anti-Carbamylated protein (anti-CarP) antibodies (solid squares) and for a healthy serum sample (solid circles). The protein content of the individual HPLC fractions as measured by ELISA is shown on the right Y-axis (grey line). Abs; absorbance In b.) the antibody reactivity of 5 HPLC fractions (all coated at the same concentration) with serum samples from rheumatoid arthritis patients healthy controls (two samples in each category) are displayed. For each serum sample group, the average of the two samples is shown. Abs; absorbance Furthermore, c.) illustrates the analysis of the 19th HPLC-fraction on a SDS-PAGE gel (4-12% SDS Page; simply blue safe stain). The main protein band was excised and subjected to chymotrypsin digests.

<ins>Serum samples from RA patients display autoreactivity against human Ca-A1AT</ins>

After the identification of bovine Ca-A1AT as a possible antigen for anti-CarP antibodies the autoantibody reactivity against human A1AT and human Ca-A1AT was investigated using sera from 160 RA patients and 80 controls. As a comparison and to show the anti-CarP antibody status of these patients and controls, Ca-FCS and FCS were also included as antigen in ELISA. Analysis of autoantibody binding using 240 sera revealed a strong binding to Ca-A1AT in RA patients and only minimal background binding to non-modified A1AT (Figure 3A), similar as to what was

observed for FCS (figure 3E). The levels of antibodies that target Ca-A1AT and Ca-FCS is higher in sera of RA patients when compared to sera of healthy controls (Mann-Whitney test, $p<0.001$) (Figure 3B and 3E).

We also observed a correlation between the levels of antibodies against Ca-FCS and Ca-A1AT (Spearman's rho = 0.58; $p <0.001$) (figure 3C). Correlations were also found between anti-Ca-A1AT antibodies and RF or ACPA (figure 3D). ROC analysis showed similar discrimination between RA and controls for Ca-FCS and Ca-A1AT as expressed by similar AUC values (0.73 for Ca-A1AT and 0.69 for Ca-FCS) (Figure 3F). A detailed comparison of the diagnostic properties of anti-Ca-FCS and anti-Ca-A1AT antibodies is shown in Table 1. The ROC curve for anti-Ca-A1AT antibodies discriminating between RA patients and disease controls is shown in figure 3G (AUC = 0,652). Combined these data indicate that Ca-A1AT, a single protein derived from Ca-FCS can be recognized in a similar manner as the complex protein mixture FCS. Also, these data show that a possible self-antigen, carbamylated human A1AT, can also be recognized by anti-CarP autoantibodies.

Alpha 1 antitrypsin as in vivo target of carbamylation

To investigate whether Ca-A1AT could indeed serve as an autoantigen in RA patients, an unbiased, proteome-wide MS analysis was carried out. As input material we have used a synovial fluid sample from an RA patient. Using MS we could identify a carbamylated peptide, derived from A1AT (supplementary figure 2). The peptide sequence is AVH**K**AVLTIDEK, where the bold k indicates the site of carbamylation. This indicates that A1AT can be found in carbamylated form at the site of inflammation in RA patients.

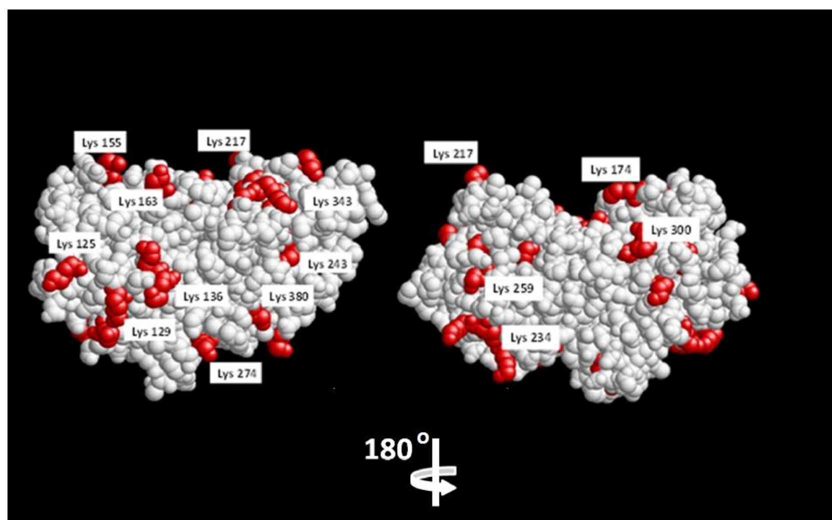

Figure 2 - Theoretical three dimensional structure model of human alpha 1 anti-trypsin (A1AT). The lysine residues of A1AT were mapped in a three dimensional structure, showing the samples mapped at the surface of the molecule.

Anti-CarP antibodies also recognize A1AT-derived carbamylated peptides

We have observed that Ca-A1AT as full-length protein can be recognized by antibodies from RA patients. However, the *in vitro* carbamylated A1AT might potentially involve other modifications besides the creation of a homocitrulline. To investigate the recognition of A1AT containing homocitrulline residues, synthetic 21-mer peptides, derived from A1AT, were designed. In these peptides, homocitrulline residues were incorporated at the location of the lysine residue in the original sequence. The peptides were tested in ELISA using samples from RA patients (n=24) [anti-Ca-A1AT positive (n=9) and negative (n=15)] and healthy controls (n=6).

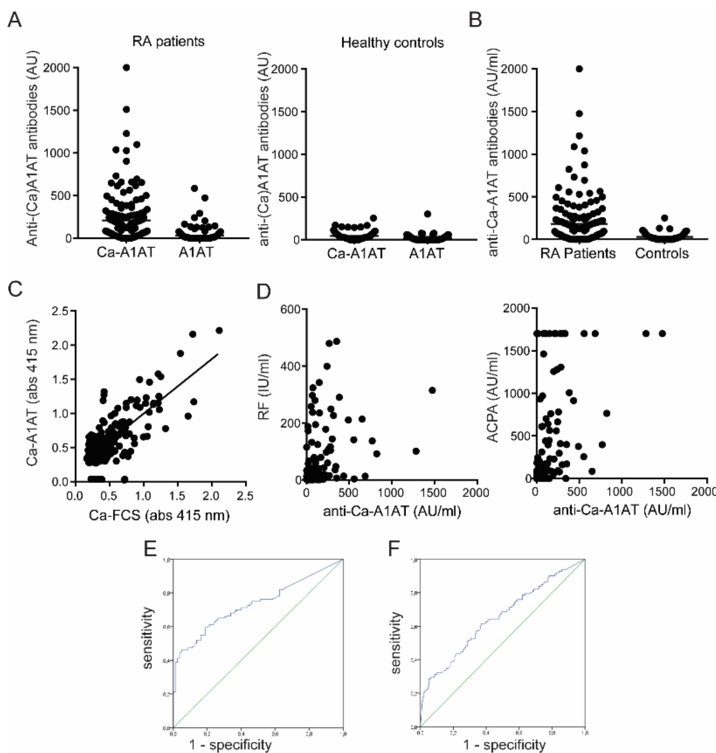

Figure 3 - Analysis of alpha 1 anti-trypsin (A1AT) as potential antigen by ELISA. In a.) antibody reactivity against Ca-A1AT and A1AT as measured by ELISA is shown for rheumatoid arthritis patients (RA, n = 160, left) and healthy controls (n = 80, right). b.) shows a complete overview of the anti-Ca-A1AT antibodies in which the A1AT signal has been subtracted from the Ca-A1AT signal for both RA patients and healthy controls. In c.) the correlation between anti-Ca-FCS and anti-Ca-A1AT antibodies is analysed and a clear correlation was observed (spearman correlation, rho=0.58; p<0.001). In d.) the correlation between anti-Ca-A1AT antibodies and RF or ACPA are shown (rho = 0.56 and 0.61 respectively, both p<0.001). e) shows the receiver operating characteristics (ROC) Ca-A1AT (green) as the antigen for discrimination between RA patients and healthy controls (AUC = 0,733). The diagonal reference line indicates no discrimination. In f) the ROC for Ca-A1AT as the antigen for discrimination between RA patients and disease controls (n=207) is shown (AUC = 0,652). The diagonal reference line indicates no discrimination.

The reactivity against the carbamylated peptides is virtually absent the serum samples from healthy controls, especially when compared to a non-carbamylated control peptide (Figure 4A and a cluster analysis in supplementary figure 3).In the sera of RA patients, an increased reactivity towards the carbamylated peptides can be observed. The sera of the RA patients that were positive in the anti-Ca-A1AT ELISA displayed a much more pronounced reactivity towards the peptides as compared to the RA samples that were negative in the anti-Ca-A1AT ELISA. We observed that many different peptides are recognized and that there is not one peptide that is recognized by the majority of the anti-CarP positive serum samples (Figure 4A). Out of these peptides, 6 peptides with a high reactivity in the RA patients were selected and a non-carbamylated control peptide was used in ELISA (figure 4B). Also in this situation, an increased reactivity was seen in anti-Ca-A1AT-positive RA patients when compared to anti-Ca-A1AT-negative RA patients or healthy controls.Furthermore, a trend is observed that the presence of multiple homocitrulline residues results in increased antibody reactivity in RA patients (supplementary figure 3B). Peptide 22-25 contain the homocitrulline residue that was found to be carbamylated *in vivo*. Interestingly, this region of the A1AT molecule is also recognized by anti-CarP antibodies.
Combined, these data indicate that A1AT peptides containing a homocitrulline can also be recognized by anti-CarP antibodies from RA patients.

	Ca-A1AT	**Ca-FCS**
Sensitivity at ~99% specificity	35.0 (28.0-42.7)	31.3 (24.2-38.8)
Likelihood ratio +	28.0 (5.1-159.7)	25.0 (4.6-142.8)
Likelihood ratio -	0.66 (0.58-0.73)	0.70 (0.62-0.77)
Odds ratio	42.5 (7.2-248.6)	35.9 (6.1-210.1)

Table 2 - Overview of the diagnostic performance of ELISAs using carbamylated Alpha-1 antitrypsin Ca-A1AT or carbamylated fetal calf serum (Ca-FCS) as antigen.

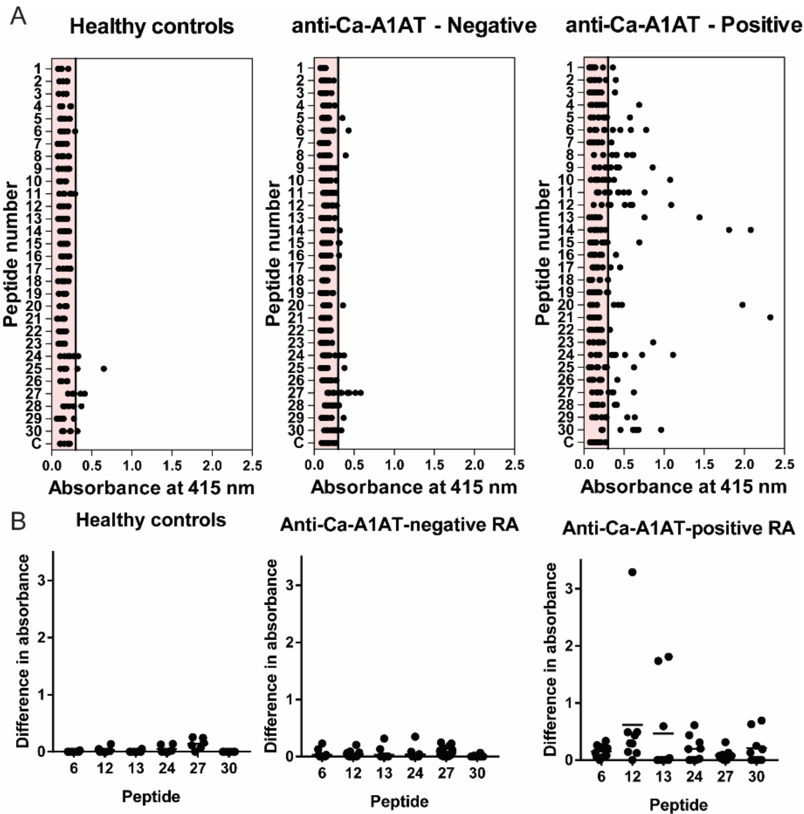

Figure 4 - Anti-CarP reactivity against alpha 1 anti-trypsin (A1AT) derived peptides. a.) A1AT peptides were generated synthetically and lysines were replaced by homocitrulline residues. The reactivity of anti-CarP antibodies against these peptides was tested by ELISA, using both samples from rheumatoid arthritis (RA, n=24) [anti-Ca-A1AT positive (n=9) and negative (n=15)] as well as from healthy controls (n=6). Significant reactivity was found with several of the A1AT peptides, mostly in the RA patient samples that were positive for anti-Ca-A1AT antibodies. C indicates a control peptide that does not contain a homocitrulline residue. b.) shows the difference in antibody reactivity between 6 carbamylated and the 6 matching non-carbamylated peptides for samples from RA patients (n=23) [anti-Ca-A1AT-positive (n=9) and negative (n=14)] as well as for healthy controls (n=6)

Discussion

Here we have described the identification of Ca-A1AT as one of the antigenic targets of anti-CarP antibodies present in Ca-FCS and showed that anti-CarP antibodies are able to recognize human Ca-A1AT as well. In addition, we have identified a carbamylated peptide derived from A1AT in the synovial fluid of an RA patient. Moreover, anti-CarP antibodies are able to recognize homocitrulline containing peptides that are derived from A1AT.

The peptide sequence as identified in synovial fluid is recognized by a substantial portion of the anti-CarP positive sera. Collectively this demonstrates that human Ca-A1AT serves as an autoantigen for anti-CarP antibodies from RA patients. Other protein antigens that have been described previously are mainly carbamylated fibrinogen[4,5,13,19,22,23] as well as enolase[24] and vimentin derived peptides[25]. However, to our knowledge these particular antigens have not yet been described in carbamylated form in RA patients. In other conditions, such as renal disease, many different carbamylated proteins have been identified[20,21], indicating that it is unlikely that A1AT is the only carbamylated protein in RA patients.

Our approach to identify linear B-cell epitopes on A1AT identified several epitopes that are recognized by antibodies targeting Ca-A1AT. However, none of the peptides were recognized by the majority of the patient samples containing anti-Ca-A1AT antibodies. Additionally, we did not find a correlation between the reactivity against the individual A1AT peptides and the complete protein (data not shown). Consequently, it is highly likely that conformational epitopes are the main target of the anti-CarP antibodies. However, it is possible that patients positive for antibodies to specific carbamylated A1AT derived peptides represent important subsets of RA patients with specific clinical characteristics[4,26-30]. Studies are needed to analyse if antibodies to these peptides show correlation to disease severity, disease activity and / or treatment response in certain RA patient subsets. Larger cohorts will have to be tested on Ca-A1AT to verify the clinical utility of the antibodies. A desirable outcome could be that anti-Ca-A1AT antibodies show similar characteristics as anti-Ca-FCS antibodies in terms of erosive disease, but yield higher disease specificity.

Furthermore, A1AT has interesting characteristics and the protein has been suggested to be relevant in RA as A1AT is a protease inhibitor belonging to the serpin superfamily, and is also known as serum trypsin inhibitor or alpha-1 proteinase inhibitor. In addition, A1AT protects tissues from enzymes of inflammatory cells, especially neutrophil elastase, and has a reference range in blood of 1.5 - 3.5 gram/liter, and the concentration can rise manifold upon acute inflammation. In its absence, neutrophil elastase is free to break down several molecules including elastin, which contributes to e.g. the elasticity of the lungs, resulting in respiratory complications such as emphysema, or chronic obstructive pulmonary disease (COPD) in adults and in cirrhosis in adults or children. Besides these actions, A1AT might also exert other anti-inflammatory or tissue-protecting effects via dendritic cells or regulatory T cells[31,32]. A1AT is a 46 kDa (394 amino acids) protein with 34 lysine residues, most of them exposed on the surface of the protein which makes the protein a suitable target for carbamylation. Compared to the average amino acid content of mammalian proteins, the content of lysine in A1AT is higher (11.9% vs. 7.2%).

Interestingly, anti-A1AT IgA complexes have been previously described in RA patients[33,34], but our study is the first to show that Ca-A1AT represents an immunogenic target of autoantibodies. It has also been suggested that there might be a genetic association between certain A1AT phenotypes or A1AT heterozygosity[35] with the presence of RA[31], although a smaller study was not able to replicate this finding[32].

On the basis of preclinical and clinical studies, A1AT therapy for non-deficient individuals may prevent disease progression in several inflammatory and immune-mediated diseases. In animal models for example, A1AT might be able to prevent arthritis[36,37]. In RA, patients with carbamylated and therefore possibly enzymatically inactive A1AT or with anti-Ca-A1AT antibodies might consequently lack the protective effect of this enzyme and would benefit from A1AT treatment. This approach is in line with recent concepts in precision medicine and companion diagnostics in RA[9,38].

We therefore conclude that Ca-A1AT might be one of the *in vivo* antigens for anti-CarP antibodies in RA patients. The presence of Ca-A1AT within the synovial fluid of an RA patient, suggests that carbamylation of A1AT might play a role in the development of synovial inflammation in anti-CarP positive RA patients.

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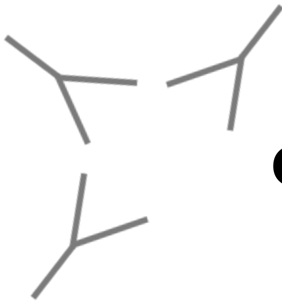

Chapter 10

Mass-spectrometric identification of carbamylated proteins present in the joints of RA patients and controls.

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Antibodies that target carbamylated proteins (anti-CarP antibodies) are present in sera of patients with rheumatoid arthritis(RA). Anti-CarP antibodies can be detected many years prior to clinical diagnosis of RA, associate with increased risk of RA development and with severity of joint destruction. Whether these antibodies contribute to RA is currently unknown, since it is not known which proteins are carbamylated in the RA joint. Therefore, we set out to determine the presence of carbamylated proteins in the human (inflamed) joint.

We obtained synovium, cartilage and synovial fluid from the RA joint and in addition acquired cartilage and synovium from controls. Samples were processed and used for immunohistochemistry or mass spectrometric analysis to investigate the presence of carbamylated proteins. Anti-CarP antibody reactivity towards identified carbamylated proteins was tested by ELISA.

Immunohistochemistry showed extensive staining of synovial tissue, derived from both RA and OA patients. Whole proteome analyses of joint tissues revealed the presence of a large number of carbamylated peptidyllysine residues. Comparing synovial tissue, cartilage and synovial fluid, we observed that carbamylated proteins are especially identified in cartilage. Interestingly, the presence of carbamylated peptides was not restricted to RA samples but also occurred the control tissues. A number of the carbamylated proteins identified were also confirmed to be recognized by anti-CarP antibodies in the sera of RA patients.

We conclude that numerous carbamylated proteins are present in the RA joint and can be recognized by anti-CarP antibodies, substantiating the notion that anti-CarP-directed autoimmunity may play a role in the pathogenesis of RA.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by the presence of joint inflammation leading to cartilage and bone damage. RA can be present in up to 1% of the western population and is often associated with the presence of autoantibodies. At present, several autoantibodies have been identified in both serum as well as the affected joints of RA patients. Well-known autoantibodies in RA are rheumatoid factor, anti-citrullinated protein antibodies (ACPA) and anti-Carbamylated protein antibodies (anti-CarP)[1,2]. Many clinical associations have been described for these autoantibodies, such as associations with joint damage or disease development, making them interesting potential players in the pathogenesis of RA[1,3,4]. However, for autoantibodies to mediate direct pathogenic effects, binding to their cognate antigen locally in the joint is likely required. Several studies have been carried out to investigate the presence of citrulline residues in the RA joint. Citrulline residing in a protein arises from the enzymatic conversion of an arginine by peptidylarginine deiminases (PADs).

Examples of citrullinated proteins present in the joints of RA patients are fibrin, vimentin and enolase,[5,6]. Also, full proteome analyses have been carried out to investigate the presence of citrullination in the RA joint, identifying the presence of a large number of modified proteins[7]. Many of these proteins are not exclusively present in the RA joint as they can also be identified in the non-RA joint[8].
Anti-CarP antibodies on the other hand target homocitrulline residues that arise from the post-translational modification of a lysine residue. This is a chemical reaction mediated by cyanate. Since anti-CarP antibodies were discovered more recently compared to ACPA and RF, less is known on the presence of carbamylated proteins in RA patients. However, carbamylation has been studied in renal disease, in which uremia (resulting in higher cyanate levels) may increase carbamylation[9]. Indeed, several proteins were identified in carbamylated form in renal disease, but many of the studies performed mainly focused on (a selected number of) serum proteins, such as haemoglobin and albumin[9,10]. Also in mice, carbamylation has been studied, showing for example that carbamylation seems to increase with age[11]. In RA, several proteins have been identified in the joint in carbamylated form, such as human serum albumin, alpha-1 antitrypsin and IgG[12-14]. Also, the amount of homocitrulline residues has been measured in several (inflamed) joints of 1 RA patient[15], showing that similar amounts of carbamylation could be detected in both the affected and non-affected joints of RA patients. However, it has not yet been described how many different proteins, and which proteins are present in carbamylated form in the RA joint. Therefore, we investigated the presence of carbamylated proteins in RA synovium, cartilage and synovial fluid, to provide a more complete overview of the "carbamylome".

Methods

Patients

Joint tissue samples (cartilage, synovium and synovial fluid) were acquired as anonymized leftover material from joint-replacement surgeries, at the departments of Orthopedic Surgery, either at the LUMC (Leiden) or at the Alrijne Hospital (Leiderdorp). This procedure was approved by the local ethical committee. Additional synovial fluid was acquired from patients visiting the outpatient clinic for the Rheumatology department at the LUMC. An overview of all the tissue samples used for mass spectrometry can be seen in table 1.
Serum samples from RA patients were selected[13], resulting in 80 ACPA-positive and 80 ACPA- patients. Also, serum samples from 80 healthy controls were collected in the Leiden area[1]. For all of the patient material, patient identity was not disclosed and the data were used anonymously in accordance with the Helsinki Declaration of human research ethics.

Immunohistochemistry

Paraffin embedded synovial tissue samples were used for immunohistochemistry. Paraffin on the synovial tissue slides was removed in xylene, followed by ethanol rehydration.

Antigen retrieval was carried out with EDTA pH9 (DAKO, S2367) at 96°C for 30 minutes. The presence of carbamylated proteins was visualised with a polyclonal rabbit anti-carbamyl-lysine antibody (aCBL, Cell Biolabs, STA-078) which was compared to a matching isotype control. For some conditions, the aCBL antibody was pre-incubated for 60 minutes at room temperature with 0.2mg/ml of carbamylated fetal calf serum or non-modified fetal calf serum (Ca-FCS, FCS). The primary antibody was allowed to bind to the tissue for 2 hours. As a secondary antibody, polyclonal goat anti-rabbit Ig/HRP (DAKO, P0448) was incubated for 1 hour. The staining was finalized with DAB peroxidase (Vector Labs, SK4100) and combined with haematoxylin (Klinipath, 4085-9001) staining. Pictures of the stained slides were made on a Zeiss Axio ScopeA1 microscope.

ID	Group	Tissue	Gender	Age	Location
1	RA	Cartilage	Female	76	Knee
2	RA	Cartilage	Male	56	Hip
3	RA	Synovium	Female	76	Knee
4	RA	Synovium	Male	56	Hip
5	RA	Synovial Fluid	Male	49	Knee
6	RA	Synovial Fluid	Male	67	Knee
7	OA	Cartilage	Male	77	Knee, affected
8	OA	Cartilage	Male	75	
9	OA	Synovium	Male	75	
10	OA	Synovium	Female	70	
11	OA	Synovial Fluid	Female	75	Knee
12	OA	Synovial Fluid	Male	75	Knee
13	OA	Cartilage	Male	77	Knee, preserved
14	Sports Injury	Cartilage	Male	33	Shoulder

Table 1: Characteristics of samples used for mass spectrometric analysis. Multiple samples were derived from the same person (1 and 3, 2 and 4, 8 and 9, 7 and 13). Missing data cannot be acquired, since patient identity was not disclosed. ID; sample identification number, RA; rheumatoid arthritis, OA; osteoarthritis.

Mass spectrometry

Mass spectrometry was carried out as described previously[12,13,16]. Synovial fluid (SF) was centrifuged at 2000 rpm for 5 minutes, the supernatant collected and stored in aliquots at -80C until further use. SF samples (500 µg protein) were first depleted for the top-12 most abundant serum proteins (Pierce/Thermo) according to the instructions of the supplier. The depleted sample (50 µg) was subjected to filter-aided sample preparation (FASP II)[17] using ^{13}C-urea instead of regular ^{12}C urea to distinguish artificial *in vitro* ^{13}C carbamylation during the FASP procedure from genuine *in vivo* ^{12}C carbamylation events. In vitro carbamylation events were not observed after FASP II procedure.

Cartilage and synovium samples (20 mg) were washed with PBS to remove adherent body fluids like SF and blood. Samples were incubated in ST lysis buffer (4% SDS in 0.1 M Tris-Cl pH 7.6) for 15 min at 70°C. Initially, SDS lysates were subjected to FASP II as described above, but yielding low numbers of carbamylated peptide hits. In contrast, subsequent trypsinization of the cartilage samples yielded much more hits. Therefore, the cartilage and synovium samples (after their extraction with hot SDS to remove adherent and easily soluble protein) were digested with trypsin using the following procedure: Samples were incubated in 100 µl 100 mM DTT in 25 mM NH_4HCO_3 for 20 min at 54°C. After a 5-min centrifugation at max speed, the supernatant was saved and the pellet incubated in 150 µl 15 mM iodoacetamide in 25 mM NH_4HCO_3 for 30 min at room temperature. After a 5-min centrifugation at max speed, the supernatant was saved and the pellet incubated in 200 µl 25 mM NH_4HCO_3 containing 10 µg trypsin for 4 hours at 37°C. The combined supernatants from DTT and iodoacetamide incubation were concentrated on a 30 kDa filter (Microcon, Millipore), washed 3 times with 100 µl 25 mM NH_4HCO_3 and also incubated with 1 µg trypsin for 4 hours at 37°C. Next the supernatant containing digested protein from the pellet was added to the digest on the filter. The filter was washed once with 100 µl 0.5 M NaCl. Peptides were recovered from the filtrate and subjected to solid phase extraction on C18 cartridges (Oasis HLB Waters).

Proteome analysis and mass spectrometric identification of carbamylation

Peptides were analysed via on-line C18-nano-HPLC-MS with a system consisting of an Easy nLC 1000 gradient HPLC system (Thermo, Bremen, Germany), and a Q-Exactive mass spectrometer (Thermo). Fractions were injected onto a homemade precolumn (100 µm × 15 mm; Reprosil-Pur C18-AQ 3 µm, Dr. Maisch, Ammerbuch, Germany) and eluted via a homemade analytical nano-HPLC column (15 cm × 50 µm; Reprosil-Pur C18-AQ 3 um). The gradient was run from 0% to 50% solvent B (100/0.1 water/formic acid (FA) v/v) in 120 min. The nano-HPLC column was drawn to a tip of 5 µm and acted as the electrospray needle of the MS source. The Q-Exactive mass spectrometer was operated in top10-mode. Parameters were resolution 70,000 at an AGC target value of 3 million maximum fill time of 100 ms (full scan), and resolution 17,500 at an AGC target value of 100,000/maximum fill time of 60 ms for MS/MS at an intensity threshold of 17,000. Apex trigger was set to 1 to 5 seconds, and allowed charges were 2-5. For peptide identification, MS/MS spectra were submitted to the uniprot Homo Sapiens database (UP000005640; Jan 2015; 67911 entries) using Mascot Version 2.2.04 (Matrix Science) with the following settings: 10 ppm and 20 millimass units deviation for precursor and fragment masses, respectively; trypsin was set as enzyme. Fixed modifications was carbamidomethyl on Cys. Variable modifications were carbamylation on K and protein N-terminus, oxidation on M and acetylation on the protein N-terminus. A decoy database was used during the search and only peptides above the false discovery rate (FDR) threshold of 1% are reported.

Protein carbamylation

For the investigated proteins, carbamylation was carried out directly in the ELISA plate (Nunc Maxisorp, Thermo Scientific). First, proteins were coated overnight at a concentration of 10ug/ml, incubating the plate at 4 degrees. This was followed by an overnight incubation with 1M of KOCN or PBS at 37 degrees. The KOCN solution was removed by washing with PBS 0.05% Tween20. Using the 1M of KOCN on empty wells did not result in recognition by anti-CarP antibodies (data not shown).The following proteins were used for in-plate carbamylation: human C3 (Quidel, A401), Fibronectin from human plasma (Sigma, F2006), Fibrinogen from human plasma (Sigma, F3879), human serum albumin (HSA, Sigma, A9511), human transferrin (Sigma, T8158).

Anti-CarP antibody ELISAs

ELISAs to measure the presence of antibodies towards carbamylated proteins were carried out as described previously[1], with some minor adjustments. Here we coated non-modified proteins and used in-plate carbamylation, followed by a blocking step with PBS 1% BSA. As a secondary antibody, rabbit anti-human IgG was used (DAKO, P0214). Finally, HRP enzyme activity was detected using ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)).

Results

Immunohistochemistry staining shows carbamylation in RA and OA synovium

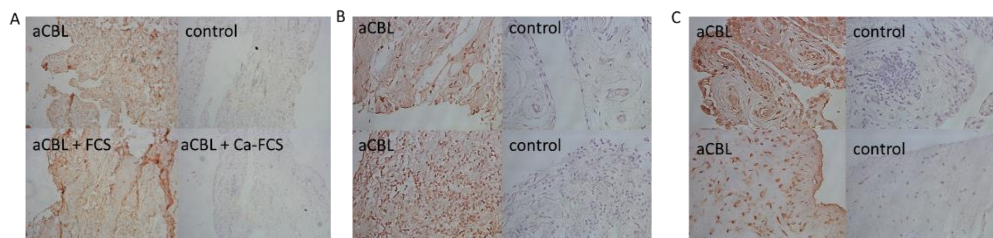


Figure 1 – Immunohistochemistry shows the presence of carbamylation in synovial tissue. Synovial tissue was stained with an aCBL antibody, targeting carbamylated proteins and is compared to an isotype control. (A) Additonally, the staining with the aCBL antibody is pre-treated with either FCS or Ca-FCS, to investigate specificity. Both RA synovial tissue (B) and OA synovial tissue (C) were used for further immunohistochemistry stainings with the aCBL and control antibody. aCBL; anti-carbamyl-lysine, FCS; fetal calf serum, Ca-FCS; carbamylated fetal calf serum, RA; rheumatoid arthritis, OA; osteoarthritis.

To investigate whether carbamylation is detectable in synovium, immunohistochemistry was carried out. We have previously tested the used antibody extensively in ELISA and confirmed its specificity for carbamylated proteins[16]. Here, the specificity of the antibody used for detection of homocitrulline in synovium was verified by blocking the antibody staining with either Ca-FCS or FCS (Figure 1A). A clear staining of the synovium was observed when using the aCBL antibody alone or the aCBL antibody pre-incubated with FCS. Pre-incubation of the aCBL antibody with Ca-FCS however, completely reduced the signal.

The fact that the aCBL antibody can be blocked by Ca-FCS, but not by unmodified FCS indicates that the antibody detects carbamylated proteins present in the synovium. Next we compared staining of carbamylated proteins by this antibody using synovium of 2 RA (figure 1B) and 2 OA (figure 1C) patients. We did not observe major differences in the staining intensity or pattern between these two patient groups with regards to aCBL binding. Combined, these data indicate that carbamylation, as measured by immunohistochemistry, is present in synovial tissue of RA patients as well as of OA patients.

<u>Carbamylated proteins are present in the joints of RA patients, OA patients and healthy controls</u>

To further detail the nature of the carbamylated proteins present in the joint, mass spectrometry was carried out, investigating synovium (n = 4), cartilage (n = 6) and synovial fluid (n = 4) from RA patients, OA patients and other controls. An overview of these 14 samples can be seen in table 1. We were able to identify a large number of unique peptides in the different joint tissues (Figure 2A). Furthermore many carbamylated peptides could be identified. (Figure 2B), confirming the results obtained by antibody staining. To make sure that the peptides were correctly assigned by the software to be carbamylated[16] a total of 48 of the identified peptides were synthesized as well. The synthetic peptides with exactly the same sequence as identified in the tissue by MS were ordered and analysed with the same methods. Out of these peptides, the spectra from 45 matched the initial spectra acquired from the complex mixture. Table 2 shows an overview of the identified peptides and their corresponding proteins. A complete overview of the observed spectra from the 45 confirmed peptides are depicted in supplementary data 1. To obtain an estimation on the relative abundance of carbamylated peptides present in the joint we next divided the number of uniquely carbamylated peptides by the total number of unique peptides detected in a particular sample (Figure 2C).

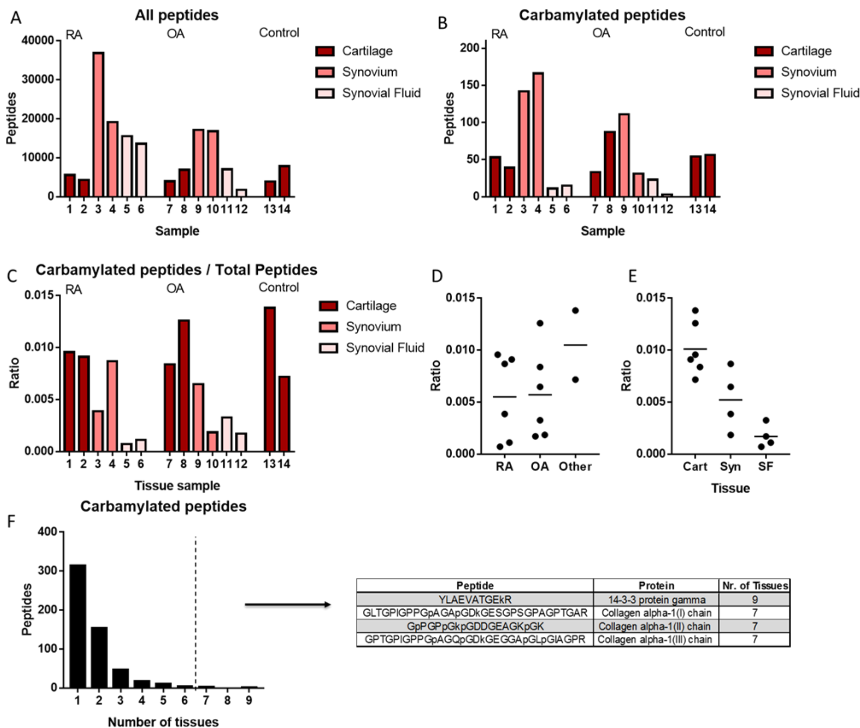
When comparing the results obtained with MS between RA patients to OA patients, we observed no differences with regards to the calculated ratio (Figure 2D), further substantiating that the presence of carbamylated proteins does not differ substantially in these two conditions. Upon further examination of the data obtained we noted that the ratio of carbamylated peptides over total peptides was higher in cartilage tissue as compared to synovium or synovial fluid (Figure 2E). These data indicate that carbamylated proteins are relatively abundant in cartilage, a site in which long-lived matrix proteins are present.

Table 2: Identified carbamylated peptides that were confirmed with synthetic peptides

Peptide	Protein
<u>AEFAEVSkLVTDLTK</u>	Serum Albumin
<u>AGEkGLPGAPGLR</u>	Collagen alpha 1(II)
<u>AVAEPGIQLkAVK</u>	Thrombospondin 4 / Cartilage oligomeric matrix protein
<u>cDckPGYR</u>	Fibrillin-1

DGPkGARGDSGPpGR	Collagen alpha 1(II)
EGcYGDkDEFPGVR	Aggrecan Core Protein
EIQcSGYTLPTkVAK	Cartilage intermediate layer protein 1
eIVLTQSPGTLSLSPGER	Immunoglobulin kappa light chain
EkEVVLLVATEGR	Aggrecan Core Protein
EkMAEPEKLTAR	Sushi-repeat containing protein X-linked 2 (precursor)
ESkPLTAQQTTK	Fibronectin
eVQLVESGGGLVQPGGSLR	Immunoglobulin heavy chain variable region
eVQLVESGGGLVQPGR	Immunoglobulin heavy chain variable region
GADGSPGkDGVR	Collagen alpha 1(I)
GDVGEkGPEGApGKDGGR	Collagen alpha 1(II)
GFpGADGVAGPkGPAGER	Collagen alpha 1(I)
GLEPGQEYNVLLTAEkGR	Tenascin
GLLGpkGPpGIpGPpGVR	Collagen alpha 2(XI)
GLpGTAGLpGmkGHR	Collagen alpha 1(I)
GPAGPNGIpGEkGPAGER	Collagen alpha 1(III)
GPAGPSGPAGkDGR	Collagen alpha2(I)
GpPGPpGkpGDDGEAGKpGK	Collagen alpha 1(II)
GPQGALGEpGkQGSR	Collagen alpha 2(VI)
HkVYAcEVTHQGLSSPVTK	Ig Kappa chain
HLGVkVFSVAITPDHLEPR	Collagen alpha1(VI)
IGDQWDkQHDMGHMmR	Fibronectin
IGILITDGkSQDDIIPPSR	Collagen alpha1(XIV)
ISEAkLTGIPK	Biglycan
KVESLQEEIAFLkK	Vimentin
kVPQVSTPTLVEVSR	Serum Albumin
LAkEIAIGR	Cartilage intermediate layer protein 2
LFAVAPNQNLkEQGLR	Collagen alpha 2(VI)
NGDkGHAGLAGAR	Collagen alpha 2(I)
NIDSEEVGkIASNSATAFR	Collagen alpha 3(VI)
NLkYLPFVPSR	Fibromodulin
NTDEDkWGDAcDNcR	Cartilage oligomeric matrix protein
QYNVGPSVSkYPLR	Fibronectin
TLLIkTVETR	Vimentin
TLVkVIPQGScR	Cartilage intermediate layer protein 1
VFDEFkPLVEEPQNLIK	Serum Albumin
VGVVQFSNDVFPEFYLkTYR	Collagen alpha 3(VI)
VkIVGPLEVNVR	Cartilage intermediate layer protein 1
VSKEkEVVLLVATEGR	Aggrecan Core Protein
VVQcSDLGLkSVPK	Biglycan

The carbamylated lysine is indicated with a lower case “k”. The lowercase “p” represents a hydroxyproline. The lowercase “c” represents a carbamidomethylated cysteine



Peptide	Protein	Nr. of Tissues
YLAEVATGEkR	14-3-3 protein gamma	9
GLTGPIGPPGpAGApGDkGESGPSGPAGPTGAR	Collagen alpha-1(I) chain	7
GpPGPpGkpGDDGEAGKpGK	Collagen alpha-1(II) chain	7
GPTGPIGPPGpAGQpGDkGEGGApGLpGIAGPR	Collagen alpha-1(III) chain	7

Figure 2 – Carbamylated peptides in the joint. Joint tissues samples, as described in detail in table 1, were subjected to mass spectrometry. (A) The total number of peptides identified in each of the tissues is shown. (B) only shows the number of carbamylated peptides and (C) depicts a ratio between the carbamylated and the total number of peptides, separated for each of the samples. In (D), the ratio as in C is shown while organising the samples based on original disease state(RA, OA or control). In (E) the ratio as in C is shown by organising the samples based on tissue (cartilage, synovium or synovial fluid). (F) shows the number of tissues that carbamylated peptides were identified in, with an additional table containing the peptides that were identified in carbamylated form in seven or more tissues. No peptide was identified in more than 9 out of the 14 samples. RA; rheumatoid arthritis, OA; osteoarthritis

Furthermore, upon more detailed analysis of the individual tissues, many of the identified modified peptides were unique for a particular tissue. However, some of the unique carbamylated peptides could be identified in up to 9 out of the 14 investigated samples (Figure 2F). A complete overview for each of the individual peptides and samples can be found in supplementary data 2.

These data combined indicate that carbamylation is present in the joint of RA patients. However, carbamylation does not seem to be specific for RA and can be detected in OA and control samples as well.

Together, these data match the immunohistochemistry observations and indicate that carbamylation is not confined to RA synovial compartments despite the presence of an inflammatory environment which could drive carbamylation through the presence of myeloperoxidase that can also generate cyanate. Likewise the number of carbamylated peptides identified in cartilage was increased when compared to other compartments of the RA joint.

Carbamylated proteins in the RA joint can be recognized by anti-CarP antibodies in sera of RA patients

After the identification of carbamylated proteins in the joint, we next wished to investigate whether these proteins can be recognized in carbamylated form by antibodies that are present in the sera of RA patients. To this end we selected a set of 4 proteins (C3, fibrinogen, fibronectin and transferrin), for these experiments. These proteins, were selected mainly on their commercial availability in purified format. As depicted in figure 3, the RA sera showed increased reactivity towards all the tested carbamylated proteins when compared to healthy controls. These data indicate that proteins present in carbamylated form in the joint of RA patients can act as target for anti-CarP antibodies in RA patients.

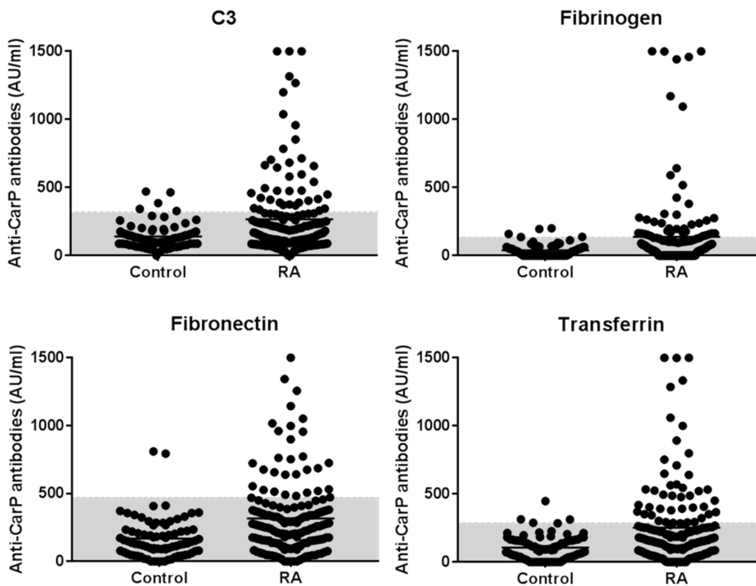


Figure 3 – Antibody reactivity towards carbamylated proteins in sera of RA patients compared to healthy controls. Reactivity of 160 RA serum samples (80 ACPA-positive, 80 ACPA-negative) and 80 healthy control serum samples against C3, fibrinogen, fibronectin and transferrin are shown. The grey area indicates the samples that are below the cut-off. Anti-CarP; anti-carbamylated protein, AU/ml; arbitrary units per millilitre.

Discussion

Immunohistochemistry stainings with an aCBL antibody showed that carbamylated antigens are present in all areas of the synovium. To our knowledge, this is the first time that this particular antibody has been used to stain synovial tissue and we were somewhat surprised to see such an extensive staining. Previously, we have tested this antibody in ELISA to detect carbamylated proteins, as proteins harbouring other post-translational modifications were not recognized[16]. The use of Ca-FCS or FCS to block the IHC staining further supports the specificity of the currently used antibody.

Our observations in immunohistochemistry were supported by mass spectrometry, also showing that a large variety of carbamylated “neo-epitopes” can be present in the joint. Importantly, proteins that were identified previously as carbamylated in the joint, such as alpha-1 antitrypsin, could also be identified in our dataset[13]. However, this feature was not specific for RA as similar findings were obtained upon analyses of synovial tissue from OA-patients and healthy controls. These data were in line with a previous study analysing material from one patient as it was shown that the amount of homocitrulline present in an affected joint was similar to the amount of homocitrulline present in an unaffected joint[15]. It has been suggested that the presence of inflammation increases carbamylation, through conversion of thiocyanate into cyanate by myeloperoxidase[18]. However our data suggest that carbamylation is not substantially increased at an inflammatory site.

Interestingly, we observed that the number of identified carbamylated peptides compared to the total number of identified peptides in each of the tissues was increased in cartilage when compared to synovium and synovial fluid. These data are interesting as they point to the notion that especially cartilage will be targeted by anti-CarP-antibodies and/or ant-CarP-antibody expressing B-cells. The reason why carbamylation of cartilage is more prominent as compared to synovial tissue or fluid is not clear, but is tempting to speculate that this relates to the longer half-life of proteins in cartilage. The longer life span of such proteins may allow accumulation of carbamylated sites over time, thereby increasing the total carbamylation in this tissue.

Besides the presence of carbamylated proteins in the RA joint, other proteins with posttranslational modification can also be identified in the joint. One of these is citrullination, but also acetylation and malondialdhyde adducts and others might be present in the joints of RA patients[19,20]. This is the first study to investigate and compare the carbamylation in different joint tissues of RA patients. We identified a large number of carbamylated residues in proteins especially in cartilage. In RA patients that are positive for anti-CarP antibodies, these antibodies recognize the carbamylated proteins that have been identified to be present in the joint. Therefore it is conceivable that anti-CarP antibodies bind to carbamylated proteins in the joint contributing to the disease process of RA.

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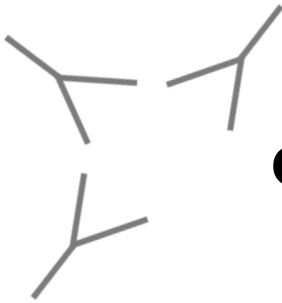

Chapter 11

Breach of autoreactive B-cell tolerance by post-translationally modified proteins

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Over 50% of rheumatoid arthritis (RA) patients harbor a variety of Anti-Modified Protein Antibodies (AMPA) against different post-translationally modified (PTM) proteins, including anti-carbamylated protein (anti-CarP) antibodies. At present it is unknown how AMPA are generated and how autoreactive B-cell responses against PTM proteins are induced. Here we studied whether PTM foreign antigens can breach B cell tolerance towards PTM self-proteins.

Serum reactivity towards five carbamylated proteins was determined for 160 RA-patients and 40 healthy individuals. Antibody cross-reactivity was studied by inhibition experiments. Mass spectrometry was performed to identify carbamylated self-proteins in human rheumatic joint tissue. Mice were immunized with carbamylated- or non-modified (auto)antigens and analyzed for autoantibody responses.

We show that anti-CarP antibodies in RA are highly cross-reactive towards multiple carbamylated proteins, including modified self- as well as modified non-self proteins. Studies in mice show that anti-CarP antibody responses recognizing carbamylated self-proteins are not only induced by immunization with carbamylated self-proteins but also by immunization with carbamylated proteins of non-self origin. Similar to the data observed with sera from RA patients, the murine anti-CarP antibody response was, both at the monoclonal- and polyclonal level, highly cross-reactive towards multiple carbamylated proteins, including carbamylated self-proteins.

Self-reactive AMPA-responses can be induced by exposure to foreign proteins containing PTM. These data show how autoreactive B-cell responses against PTM self-proteins can be induced by exposure to PTM foreign proteins and provide new insights on the breach of autoreactive B-cell tolerance.

Introduction

Autoimmunity in Rheumatoid arthritis (RA) patients is characterized by a spectrum of anti-modified protein antibodies (AMPA) directed against post-translationally modified (PTM) proteins. The best-known AMPA in RA are autoantibodies directed against citrullinated proteins. Anti-citrullinated protein antibodies (ACPA) target proteins that have undergone a post-translational modification of arginine into citrulline by an enzymatic process mediated by peptidylarginine deiminases (PAD)[1,2]. The identification of ACPA as specific serological marker have had a major impact on the understanding of RA and disease prognosis as their presence predicts a more destructive disease process[3-7]. Much less is known about the occurrence and aetiology of other AMPA responses in RA such as autoantibodies directed to malondialdehyde-acetaldehyde (MAA) adducts, acetylated antigens, and carbamylated proteins[8-11]. Anti-carbamylated protein (anti-CarP) autoantibodies recognize carbamylated proteins containing a homocitrulline, a PTM structurally similar to citrulline[8,12].

Like ACPA and rheumatoid factor, also anti-CarP antibodies can be detected in serum many years before RA manifestation [13-18] and similar to these autoantibodies, the presence of anti-CarP antibodies is predictive for increased radiological damage[13]. In contrast to deimination (citrullination), carbamylation occurs through an enzyme-independent reaction in which a lysine is converted into a homocitrulline through a reaction with cyanate[19], (Figure 1A).

With the presence of various AMPA responses in RA, PTM proteins have been implicated in the breach of autoreactive B-cell tolerance leading to the formation of these autoantibodies[20,21]. Following activation by PTM proteins, AMPA-producing B-cells are thought to undergo the process of class switching and somatic hypermutation. So far, attempts to provide more insight into how autoreactive B-cell responses against citrullinated proteins are induced have been hampered by the fact that ACPA do not occur in murine models of arthritis[22].

Interestingly, anti-CarP antibodies, do occur in mice with collagen-induced arthritis (CIA)[23]. The kinetics of anti-CarP antibodies in CIA mice display similarity to RA as these antibodies can be detected before disease onset. In addition, humoral responses to carbamylated proteins are only present in arthritis models that require active involvement of the adaptive immune system[24]. Given the observation that anti-CarP antibodies, in contrast to ACPA, do occur in both humans and mice, we here investigated for the first time how PTM proteins could contribute to a breach of B-cell tolerance.

Our findings show that autoreactive B-cell responses against PTM proteins can be induced by exposure to PTM foreign proteins and provide new insights on the breach of autoreactive B-cell tolerance by foreign proteins.

Methods

Human serum samples

Serum samples from 160 RA patients of the Leiden Early Arthritis Cohort (EAC)[25] and 40 healthy controls were used to study anti-CarP antibody cross-reactivity. All RA patients fulfilled the 1987 RA classification criteria. All subjects provided informed consent prior to inclusion and ethical permission was provided by the institutional review board.

Carbamylation and citrullination

Fetal calf serum (FCS, Bodinco), myelin basic protein (MBP, Sigma), human serum albumin (HSA, Sigma), H1 Histone (H1 Merck Millipore), Prothrombin (ProT, provided by Prof. Blom Malmö, Sweden), ovalbumin (OVA, Sigma-Aldrich) and mouse albumin (mAlb, EMD Millipore) were incubated with 1 M potassium cyanate (Sigma-Aldrich) during 12 hours at 37 °C, followed by dialysis. Fibrinogen, mouse (Cell Sciences) and human (Sigma-Aldrich), was incubated with 0.5 M potassium cyanate during 7 days.

For citrullination; 10 mg FCS or 2 mg fibrinogen in 1 ml containing 0.1 M Tris-HCl pH 7.6 and 0.15 M CaCl2 was incubated with 40U PAD4 (Sigma-Aldrich) for 3 hours at 37 °C. Protein carbamylation and citrullination was determined afterwards by in-house developed and standardized ELISAs confirmed by mass spectrometry.

Antibody detection and inhibition assays

Human anti-CarP antibodies were detected as described previously[8]. Briefly, 10μg/ml of each antigen (carbamylated and non-modified) was coated on plates and after blocking with PBS-1%BSA, serum samples (50 times diluted) were incubated overnight at 4°C. Antibody binding was detected with HRP-conjugated rabbit-anti-human IgG (DAKO, P0214). The cut-off for positivity was set as the mean plus two times standard deviation of healthy controls.
For inhibition assays, sera were incubated with 0 or 0.2 mg/ml carbamylated or non-modified versions of one from five antigens before addition of serum samples to the ELISA plate. Mixtures were pre-incubated for 1 hour at room temperature.
Mouse anti-CarP antibodies were detected as described previously[23]. Serial dilutions of a pooled serum from mice with CIA were used as a standard.

Mass Spectrometric-analysis of joint tissue

RA joint tissue samples were obtained from knee-replacement surgery leftover material (Department of Orthopedic surgery, LUMC). This procedure was approved by the local ethical committee. Sample preparation for mass spectrometric-analysis and peptide identification is described in detail in the supplementary text.

Mice and immunizations

Animal experiments were performed conform national guidelines following approval by the local Ethical Committee for Animal Experimentation. DBA/1 mice were obtained from our breeding colony (originally Harlan) and C57BL/6 mice were purchased from Charles River (8-10 week old).
For alum immunizations mice received two i.p. injections containing a 1:1 mixture of antigen (100 μg) and alhydrogel (Invivogen). Complete Freund's adjuvant (CFA) immunizations were given at the tail base, 100 μg antigen in CFA (Difco). Three weeks later a subcutaneous boost was given, 100 μg antigen in incomplete Freund's adjuvant (IFA; Sigma-Aldrich). *For* For immunizations in the absence of adjuvant, mice received two injections with 100μg antigen diluted in PBS.

Production of monoclonal antibodies

Generation of anti-CarP monoclonal antibodies, variable region cloning and antibody production is described in detail in the supplementary text.

Statistics

Prism 7 (GraphPad) or IBM SPSS Statistics 23 was used for statistical testing. Statistical differences in inhibition experiments were determined by the Wilcoxon signed-rank test.

Differences in antibody levels between subjects and controls were determined by the Mann-Whitney U test. Differences in antibody positivity were determined by Pearson's chi-squared test. Spearman's rank test was performed to evaluate correlations.

Results

Anti-CarP antibodies of RA patients recognize multiple carbamylated antigens.

To characterize the antigen recognition profile of human anti-CarP antibodies, we studied antibody reactivity against a set of five different carbamylated proteins; fetal calf serum (FCS), human serum albumin (HSA), human prothrombin (ProT), bovine histone H1 (H1) and bovine myelin basic protein (MBP). As depicted in Figure 1B and supplementary Figure 1A, serum samples from 160 RA patients showed increased recognition of multiple carbamylated antigens as compared to serum from healthy individuals (n=40) (Mann-Whitney U test, $p<0.001$ for each test). Although the overall number of RA patients displaying antibody reactivity towards these five different antigens is similar (ranging from 39% till 58%) (Figure 1C), the antigen recognition profile among individuals differs. In sera of healthy individuals, anti-CarP antibody reactivity, when present, is limited to only one or two carbamylated antigens, while up to 5 out of 5 carbamylated protein antigens are recognized by 24% of RA patients (Figure 1D). Furthermore, antibody levels correlate with the total number of proteins recognized (Figure 1E and supplementary Figure S1B). A strong correlation is observed between the mutual recognition of several carbamylated antigens (Figure 1F). Together, these data show that anti-CarP antibodies have a broad antigen recognition profile that correlates with antibody levels. Within this broad antibody response, both self- and foreign carbamylated antigens can be recognized.

Human anti-CarP antibodies are highly cross-reactive.

Since anti-CarP antibodies recognize a variety of carbamylated antigens and a high correlation was observed between reactivities, we next investigated whether antibody cross-reactivity could explain these features. Cross-reactivity was determined by inhibition assays using carbamylated proteins or their unmodified counterparts. Titrations and control experiments were performed to ensure non-saturating conditions for sera and inhibitors (supplementary Figure S2A-B). Inhibition assays with the exact same protein used as both coating antigen and inhibitor was used as a positive control. Successful signal inhibition is observed for each of the five antigens (FCS, HSA, ProT, H1 and MBP) using carbamylated inhibitors, while no inhibition is observed for their non-carbamylated counterparts (S2C). Next ten serum samples both reactive to Ca-MBP and Ca-ProT were selected and used for subsequent inhibition experiments. Binding of anti-Ca-MBP antibodies could be inhibited by incubation with Ca-ProT (Figure 2A and SD2), unlike incubation with unmodified ProT or control.

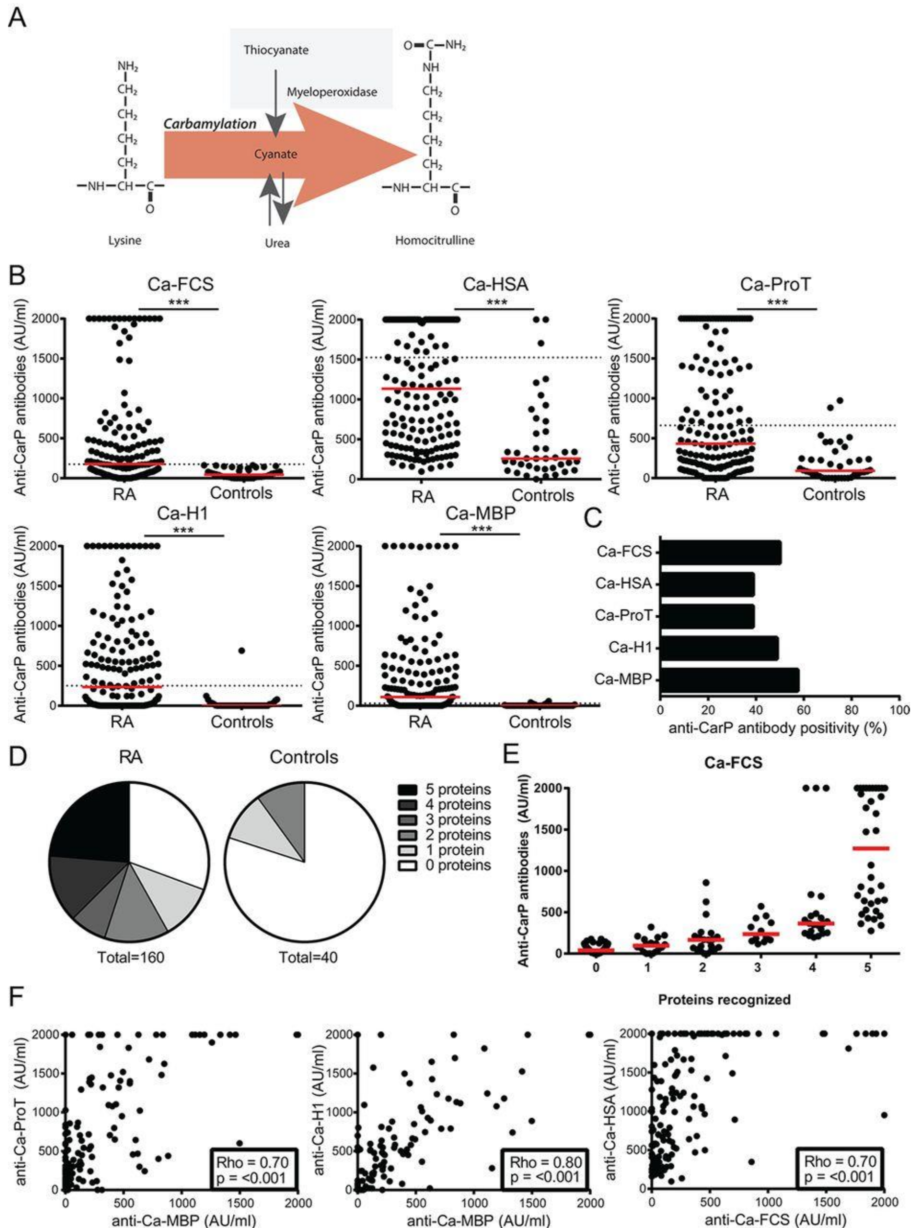

Figure 1 - Characterisation of anti-CarP antibody reactivities in patients with RA. (A) During carbamylation, a lysine residue is converted into a homocitrulline residue through a chemical reaction with cyanate. Levels of cyanate are in equilibrium with urea and can be increased, for example, during kidney disease. Cyanate levels can also be elevated during inflammation by the action of myeloperoxidase. (B) Anti-CarP antibody reactivities against five carbamylated and non-modified counterparts were measured by ELISA in 160 patients with RA and 40 healthy controls. A standard serum pool was used to calculate the arbitrary units.

The represented value was calculated by subtracting the non-modified antigen reactivity from the carbamylated antigen reactivity. The dotted line represents the cut-off while the continuous line represents the median. Statistical differences were determined by the Mann-Whitney U test (***$p<0.001$). (C) Percentages of anti-CarP antibody positivity for all five antigens in patients with RA. (D) Fractions of patients that display antibody reactivity towards multiple carbamylated antigens are shown for patients with RA and controls. (E) Correlation between the amount of antigens recognised by individual serum samples and the anti-Ca-FCS antibody levels. The small continuous line represents the median. Reactivity towards Ca-FCS is shown as an example, Spearman rank test, $p<0.001$ for all carbamylated antigens. (F) The correlation between two anti-CarP antibody reactivities is shown for different carbamylated antigens. The Spearman rank test was carried out to determine the degree of statistical correlation. anti-CarP, anti-carbamylated protein; AU/ml, arbitrary units per millilitre; Ca, carbamylated; FCS, fetal calf serum; H1, H1 histones; HSA, human serum albumin; MBP, myelin basic protein; ProT, prothrombin; RA, rheumatoid arthritis.

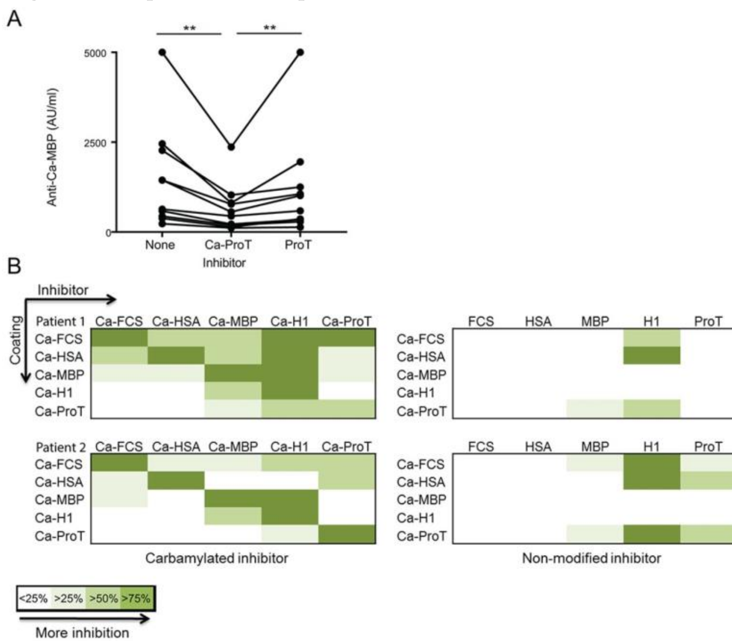

Figure 2 - Anti-CarP antibodies are highly cross-reactive. (A) Ten serum samples containing both anti-Ca-MBP and anti-Ca-ProT antibodies were used for inhibition experiments. Anti-Ca-MBP antibody binding was measured with or without the presence of Ca-ProT (0.2 mg/ml) or ProT (0.2 mg/ml) as inhibitor. Statistical differences were determined by a Wilcoxon signed-rank test (**$p<0.01$). (B) An overview of the cross-reactivity of anti-CarP antibodies is depicted. Inhibition assays by ELISA were carried out to determine the degree of cross-reactivity using two representative serum samples containing antibodies against five carbamylated antigens. Plates were coated with one protein (indicated vertically) and binding of the antibodies towards this coating was inhibited with another, or the same protein (indicated horizontally). Inhibition was calculated as a percentage of the antibody binding without inhibition. The blank was subtracted before calculating. The darkest colour indicates an inhibition of more than 75%, followed by an inhibition of more than 50%, and the light colour indicates an inhibition of more than 25%. The white blocks indicate that the inhibition is less than 25%. anti-CarP, anti-carbamylated protein; Ca, carbamylated; FCS, fetal calf serum; H1, H1 histones; HSA, human serum albumin; MBP, myelin basic protein; ProT, prothrombin.

To characterize the anti-CarP antibody cross-reactivity profile in more detail, two representative serum samples containing antibodies reactive towards all five antigens were studied. In both samples, each carbamylated and non-carbamylated antigen was used to inhibit all five carbamylated antigens (Figure 2B). Most of the carbamylated inhibitors can interfere with antibody binding, although differences between samples and inhibitors exist.
Non-carbamylated counterparts do not display an extensive inhibition profile although some inhibition was observed for H1, possibly due to the presence of other PTM on histones. Together, we observed that carbamylated proteins are able to interfere with antibody binding to other unrelated carbamylated proteins, indicating that anti-CarP antibody cross-reactivity is present in RA patients.

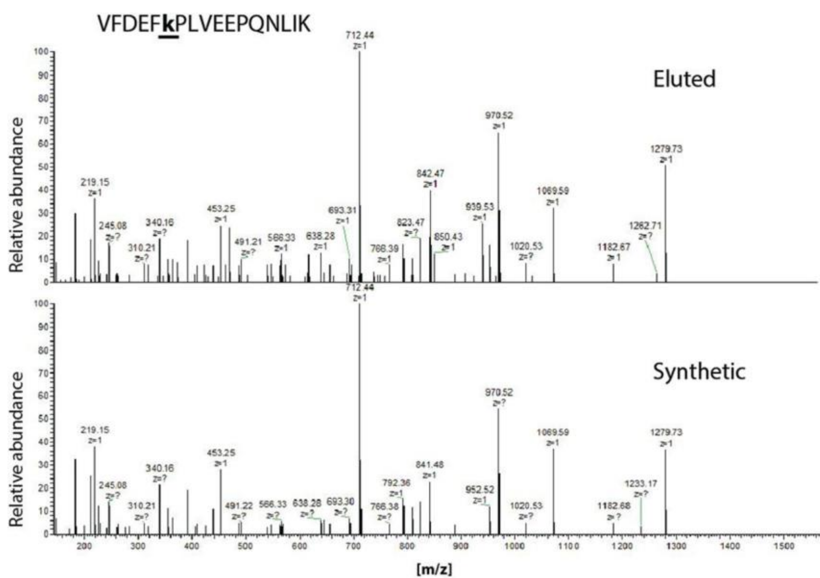

Figure 3 - Identification of carbamylated albumin in RA synovial tissue. MS2 spectrum from eluted VFDEF k PLVEEPQNLIK peptide (upper panel) derived from carbamylated albumin identified in RA synovial tissue. The synthetic VFDEF k PLVEEPQNLIK peptide (lower panel) was submitted to MS2 on the same instrument. The bold, underlined non-capital k indicates the position of homocitrulline residue. MS2, tandem mass spectrometry; RA, rheumatoid arthritis.

Carbamylated human albumin is present in RA synovial tissue.

The cross-reactive nature of anti-CarP antibodies suggests that these antibodies might react to a variety of carbamylated proteins present in target tissue. However, little is known about the presence of carbamylated proteins within affected tissue of RA patients. Therefore, we aimed to identify carbamylated self-proteins in synovial tissue of RA patients by mass spectrometry. From a list of potential hits, four peptides from carbamylated human albumin were selected for further analysis:

VFDEF**k**PLVEEPQNLIK, **k**LVAASQAALGL, **k**VPQVSTPTLVEVSR, and ADDKETcFAEEG**k**K. The bold, non-capital k, indicates the homocitrulline residue. Three of these four carbamylation sites could be identified in both patients. A representative MS-spectrum of carbamylated albumin-derived peptide VFDEF**k**PLVEEPQNLIK is depicted in Figure 3. Importantly, synthetic peptides with the same sequence displayed highly similar MS-spectra confirming the correct identification of these peptides. MS-spectra of the other albumin-derived peptides are depicted in supplementary Figure S3A-C. Other verified proteins in which carbamylation was detected in RA patients include common proteins, such as several collagens, fibronectin, fibromodulin, albumin, and more unknown proteins including Sushi-repeat containing protein SRPX2. Although, the extent of carbamylation was not quantified, these results indicate that a carbamylated self-protein, human albumin, is present locally in the synovial compartment of RA patients.

Immunization with carbamylated foreign proteins induces anti-CarP antibodies recognizing carbamylated foreign and self-antigens.

As anti-CarP antibodies from RA patients can recognize both carbamylated self- and non-self proteins, we next investigated whether a carbamylated foreign antigen can facilitate a breach of B-cell tolerance towards carbamylated self. Therefore, we immunized mice with Ca-OVA or native OVA in aluminum hydroxide (alum) as a protein free adjuvant. As depicted in Figure 4A and supplementary Figure 4A, immunization with both OVA and Ca-OVA results in the induction of a strong antibody response recognizing both modified and non-modified OVA. We subsequently analyzed whether murine anti-CarP-antibodies were cross-reactive by determining antibody reactivity to another carbamylated foreign protein, Ca-FCS (Figure 4B). In this setting, antibody reactivity to the OVA backbone will not be detected. Sera from OVA-immunized mice do not react to Ca-FCS, whereas sera from Ca-OVA immunized mice do contain antibodies reactive to Ca-FCS.

To determine whether AMPAs induced by PTM foreign proteins can cross-react with self-proteins, we examined whether serum from Ca-OVA immunized mice contained antibodies recognizing carbamylated mouse Albumin (mAlb) and fibrinogen (mFib). As depicted in Figure 4C, also Ca-mAlb or Ca-mFib are recognized by sera from Ca-OVA-immunized mice but not by sera from OVA-immunized control animals. Importantly, unmodified mAlb or mFib are not recognized by sera from Ca-OVA-immunized mice. These data show that auto-reactive AMPA-responses can be induced by exposure to carbamylated foreign proteins. These findings were not confined to foreign antigens, as also immunization with carbamylated self–proteins (mAlb and mFib) induced, a cross-reactive anti-CarP antibody response (Figure 4D-E and supplementary Figure S4B-D). Nonetheless, these data are important as they show that even in the context of a highly immunogenic 'foreign' antigen the immune response also specifically recognizes small PTMs as evidenced by the presence of anti-CarP antibody responses.

Because of the high structural homology between citrulline and homocitrulline we next determined whether murine anti-CarP antibodies could recognize citrullinated antigens as well. However, despite minor difference in chemical structure, no binding to Cit-Fib or Cit-FCS was detectable using anti-CarP antibody containing sera from Ca-OVA immunized mice (Figure 4F). In contrast, ACPA-containing sera from RA patients do recognize these citrullinated antigens.

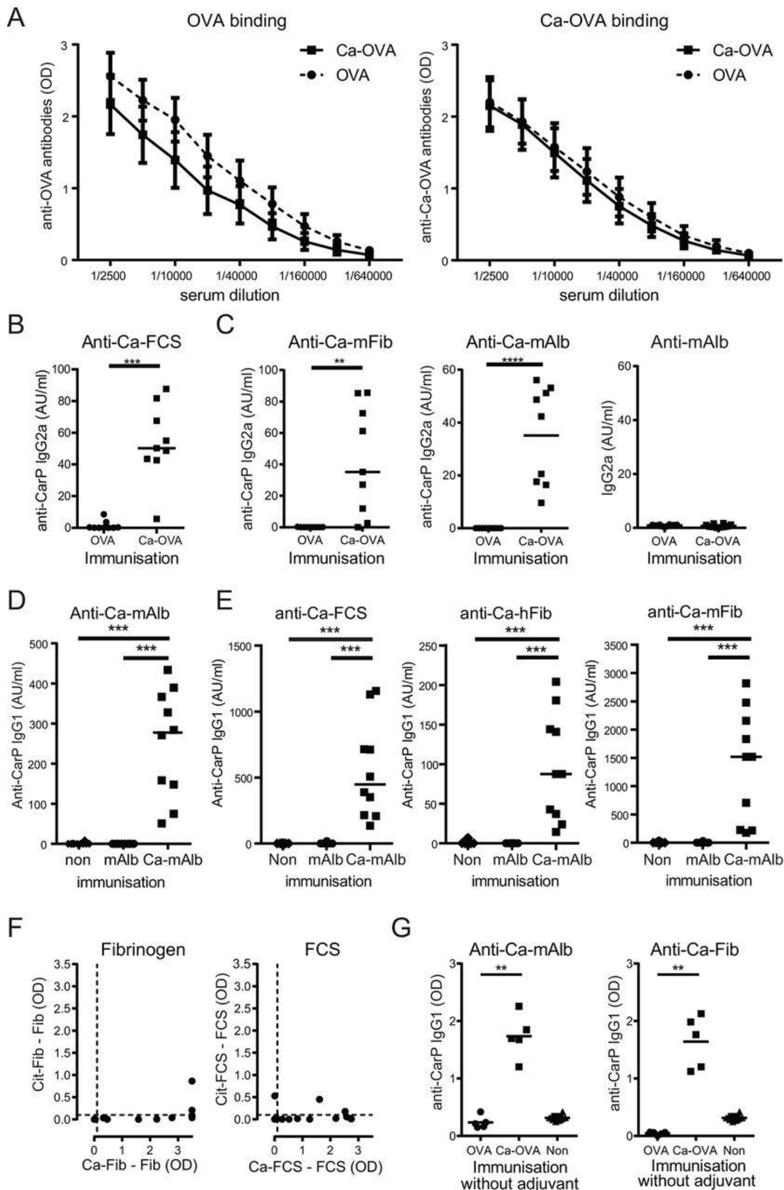

Figure 4 - Carbamylated foreign proteins can induce cross-reactive anti-CarP antibodies. (A) Mice were immunised with foreign antigens OVA or Ca-OVA. Sera from immunised mice, OVA (circles) and Ca-OVA (squares), were analysed for binding towards OVA (left panel) and anti-Ca-OVA (right panel) by ELISA (n=10). (B) Antibody reactivity towards Ca-FCS from mice immunised with OVA (circles) and Ca-OVA (squares) was determined by ELISA. Representative data from three experiments are shown. Each dot represents data from one mouse (n= 10, ***p<0.001 and **p<0.01, Mann-Whitney U test). (C) Antibody reactivity towards Ca-mFib (left panel), Ca-mAlb (middle panel) and native mAlb (right panel) from immunised mice, OVA (circles) and Ca-OVA (squares). Representative data from three experiments are shown (n= 10, Mann-Whitney U test, ***p<0.001). (D) Mice were immunised with a carbamylated self-antigen, mouse albumin (Ca-mAlb) or native albumin (mAlb) in aluminium hydroxide. Sera from immunised mice (Ca-mAlb) (depicted as squares), mAlb (depicted as circles) and non-immunised mice (depicted as triangles) were analysed for reactivity towards Ca-mAlb. Representative data from three experiments are shown. Each dot represents data from one mouse. Statistical difference was determined by the Mann-Whitney U test (n= 10, ***p<0.001). (E) Sera of (Ca-)mAlb immunised mice were analysed for reactivity towards Ca-FCS (left panel), Ca human fibrinogen (Ca-hFib) (middle panel) and Ca-mouse fibrinogen (Ca-mFib) (right panel). Sera from Ca-mAlb (depicted as squares), mAlb (depicted as circles) and non-immunised mice (depicted as triangles) were analysed by ELISA. Representative data from three experiments are shown. Each dot represents data from one mouse (n= 10, ***p<0.001, Mann-Whitney U test). (F) Correlation between antibody reactivity of Ca-OVA-immunised mice towards carbamylated human fibrinogen (Ca-Fib) and citrullinated human fibrinogen (Cit-Fib) (left panel) and correlation between antibody reactivity towards Ca-FCS versus citrullinated FCS (Cit-FCS) (right panel) (n=10, Spearman rank test). (G) Mice were immunised with foreign antigens OVA or Ca-OVA in the absence of adjuvant. Sera from immunised mice OVA (circles), Ca-OVA (squares) and non-immunised mice (triangles) were analysed for binding towards carbamylated self-proteins, Ca-mAlb (left panel) and Ca-mFib (right panel) (n= 5, Mann-Whitney U test, **p<0.01). anti-CarP, anti-carbamylated protein; Ca, carbamylated; FCS, fetal calf serum; IgG, immunoglobulin G; OD, optical density; OVA, ovalbumin.To examine whether carbamylated foreign proteins can break tolerance in absence of adjuvants, we next immunized mice with Ca-OVA in PBS. Interestingly, significant antibody responses against carbamylated self-proteins were induced (Figure 4G), showing that immunization with carbamylated foreign proteins in absence of adjuvant also results in a cross-reactive B-cell response against modified self-proteins.

<u>Monoclonal anti-CarP antibodies show a similar pattern of cross-reactivity towards carbamylated foreign and self-proteins.</u>

To confirm the cross-reactive nature of anti-CarP antibody responses, we generated a murine anti-CarP monoclonal from a mouse immunized with Ca-OVA. As depicted in Figure 5A, this monoclonal antibody binds both carbamylated foreign- and self-proteins. We observe a significant correlation between the monoclonal antibody binding to Ca-FCS and Ca-OVA and between Ca-Fib and Ca-FCS (Figure 5B) confirming its cross-reactive nature. Thus, as observed for polyclonal anti-CarP antibodies from mice immunized with a foreign antigen, strong cross-reactivity is observed towards different carbamylated foreign and self-proteins at the monoclonal antibody level, confirming that self-reactive AMPAs can be induced by exposure to foreign PTM proteins.

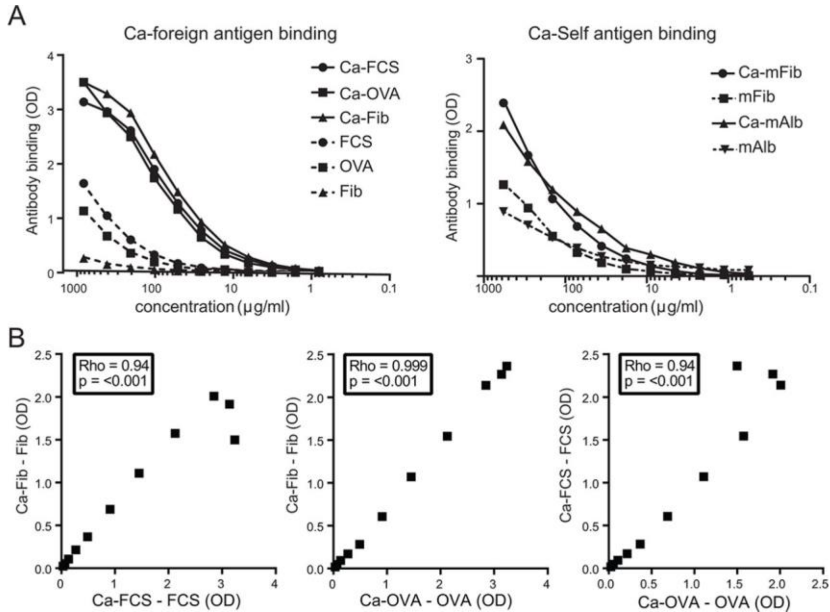

Figure 5 - Monoclonal antibodies are highly specific and cross-reactive towards carbamylated foreign and self-antigens. (A) Binding of a murine anti-CarP monoclonal antibody towards carbamylated foreign proteins; Ca-FCS, Ca-OVA, Ca-Fib (human fibrinogen) and non-modified counterparts were tested using ELISA (left panel). Reactivity of the anti-CarP monoclonal antibody towards carbamylated self-proteins; Ca-mFib (mouse fibrinogen), Ca-mAlb (mouse albumin) and non-modified counterparts were measured by ELISA (right panel). (B) The correlation between anti-CarP antibody reactivity towards Ca-Fib and Ca-FCS is shown in the left panel (rho=0.999) and for Ca-Fib compared with Ca-OVA is depicted in the middle panel (rho=0.936). Correlation between anti-CarP antibody reactivity towards Ca-OVA and Ca-FCS is depicted in the right panel (rho=0.936). The Spearman rank test was carried out to determine the degree of statistical correlation (n=11). anti-CarP, anti-carbamylated protein; Ca, carbamylated; FCS, fetal calf serum; OD, optical density; OVA, ovalbumin.

Discussion

A key characteristic of RA is the occurrence of autoantibodies against PTM proteins[2,8-10]. Here, we report that post-translational modification of foreign proteins, in particular carbamylation, represents one way in which immune tolerance at the B-cell level towards self can be broken. In RA patients we found that anti-CarP antibodies present within one serum sample are cross-reactive towards different carbamylated proteins, including foreign and self-proteins. To study how autoreactive B-cell responses against PTM self-proteins can be induced, we used carbamylated model antigens (OVA and mAlb) in mice. Our observations reveal that not only carbamylation of self- but also of foreign proteins is sufficient for a breach of immunological tolerance and the formation of autoreactive anti-CarP antibodies.

Although cross-reactive AMPA responses have been described [9,10,26], it has not been demonstrated that AMPA-producing B-cells recognizing a particular modified self-protein can be induced by other -unrelated- modified proteins. Previous animal studies showed that immunogenicity of proteins is enhanced upon citrullination, but did not show whether immunization with a modified (structurally unrelated) foreign protein leads to the induction of a cross-reactive AMPA-response against self[27-29]. Clearly, human studies demonstrating this principle are challenging as the autoantibody-inciting events are unknown and difficult to control[30].
Recently, we showed that mice are able to mount an antibody response against carbamylated proteins [23]. Therefore, we could now address the question whether exposure of a host to a carbamylated foreign protein can lead to the formation of an autoreactive B-cells response. Our data show that anti-CarP autoantibodies can, indeed, be induced by carbamylated foreign antigens. These autoantibodies react, both at the polyclonal- as well as the monoclonal level, to different carbamylated proteins, confirming that anti-CarP antibodies are cross-reactive. This high-level cross-reactivity is likely explaining why carbamylated foreign proteins can induce an autoreactive B-cell response, and indicate that the epitope recognized by responding B-cells can be present on a variety of proteins, either of self- or non-self origin.
In RA, we have shown that anti-CarP antibodies are able to recognize different carbamylated (auto)antigens. Similar findings have been reported for other AMPA responses[9,10,26]. For example, previous human studies have shown that also ACPA exhibit cross-reactive properties towards different citrullinated self- and foreign antigens[26,31-35]. Interestingly, although citrulline greatly resembles homocitrulline in structure, we were unable to detect an antibody response against citrullinated proteins in mice. Also vaccination with citrullinated proteins did not induce an ACPA response (data not shown). Therefore, we were not able to analyze whether autoreactive ACPA could also be induced by (citrullinated) foreign proteins. Nonetheless, given the cross-reactive properties of ACPA [36-39], it is highly conceivable that similar principles as identified for anti-CarP-antibody responses apply to other classes of AMPA as well.

Although it is unknown how autoantibodies against PTM proteins are generated in humans, it is often speculated that an autoreactive T-cell response recognizing such self-proteins is crucial for their appearance. Clearly, our results are not incompatible with this notion and do not indicate that such T-cell help would not contribute the induction of B-cell mediated autoimmunity against PTM proteins. However, our results provide first evidence that also T-cells recognizing “conventional” foreign antigens could be involved in the induction of AMPA-producing B-cell responses that recognize modified self-proteins. So far it is unclear to what extent T-cell tolerance is lost in RA as identification of PTM epitopes recognized by autoreactive T-cells has been proven difficult. Although T-cell responses against PTM self-proteins have been described [40-42], frequencies of citrulline specific T-cells are only about 1 in 100,000 CD4 cells compared to 1:10,000 CD4 cells for tetanus toxoid specific T-cells[42,43].

Data from animal studies suggest that PTM proteins can generate antigen-specific T-cell responses in mice [28], [19]. However, present it is still unclear to what extent these T-cells provide help to autoreactive B-cells in human RA. Our data indicate that T-cell help required for the generation of isotype-switched AMPA-responses can be provided by T-cells directed against foreign antigens (supplementary Figure S5), we consider it likely that the initiating event leading to the formation of autoantibodies against carbamylated proteins is not found in the induction of a (T-cell) response against carbamylated self-proteins but rather in the induction of immune responses against foreign antigens. The only requirement would be that the foreign antigen recognized by the T-cells contains PTMs seen by B-cells. Such requirement could be met during infection as the conditions to post-translationally modify microbe-derived proteins readily occur during infection. This could, for example, be mediated through release of PAD by neutrophils during netosis (citrullination), the release of myeloid peroxidase leading to enhanced carbamylation or the presence of bacterial-derived acetylated proteins[1,12,44]. In all these cases, microbe-derived proteins express or can acquire a PTM that can be targeted by responding B-cells. These B-cells are likely to obtain help from microbe-directed T-cells required for further somatic hypermutation. Since self-proteins can also undergo similar PTM, some B-cells will conceivably be selected on modified self-proteins leading to the development of a self-reactive B-cell response. In this scenario, autoimmunity can emerge without the presence of autoreactive T-cells.

These considerations are important for the development of tolerizing protocols aiming to dampen or inactivate putative autoreactive T-cells in an antigen-specific fashion. Likewise, they are also of relevance to define the autoimmune inciting antigen as the recognition of a particular antigen by autoreactive B-cells or antibodies might not relate to the antigen that was required to induce the B-cell response.

In conclusion, our results clearly indicate that carbamylated foreign proteins are able to induce a breach of tolerance at the B-cell level leading to the formation of cross-reactive anti-CarP antibodies recognizing modified self-proteins. We consider it likely that anti-CarP B-cell responses can result from inflammatory conditions induced for example by infection, as it is conceivable that in such conditions carbamylated foreign proteins are recognized by the responding immune system. The evoking anti-CarP immune response might subsequently cross-react to carbamylated self-proteins that are also expressed in the joints of RA patients thereby possibly contributing to the local inflammatory reaction present in RA.

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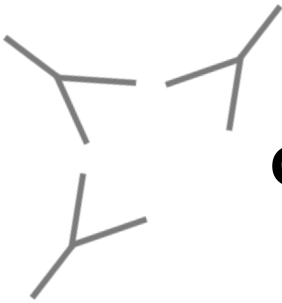

Chapter 12

Discussion

Rheumatoid arthritis (RA) is a disease that results from a complex interaction between genetic factors and the environment, resulting in chronic inflammation. There are many different immunological processes that may play a role in the development and continuation of RA. One of these immunological aspects is represented by autoantibodies[1] (Chapter 2). A subgroup of these antibodies, anti-carbamylated protein antibodies, were the main focus of this thesis, with the first aim focusing on the clinical relevance of anti-CarP antibodies.

The clinical role of autoantibodies in rheumatoid arthritis
To investigate the role of anti-CarP antibodies, their presence has been measured in a large variety of cohorts, providing information on their role in RA and other (related) diseases[2-11]. Many studies (including chapters 4 and 5) have confirmed that anti-CarP antibodies are present in RA patients and that their levels are increased when compared to control groups[9,12]. In chapter 5 further confirmation was provided for the presence of anti-CarP antibodies before disease onset and for the association between anti-CarP antibodies and joint damage. However, to determine the clinical value of anti-CarP antibodies, it is important to take into account the antibodies that are already in use in the clinic. Therefore a meta-analysis incorporating ACPA and RF as well was carried out in chapter 7 . Combining all data available indicated that using anti-CarP antibodies for the diagnosis of RA, in individuals already presenting clinical symptoms, may not add significant value since ACPA and RF combined perform as well on their own as with the addition of anti-CarP antibodies. Adding another measurement may therefore not be very cost-effective. However, when it comes to the prediction of RA, the addition of anti-CarP antibodies increases the odds at having / developing RA, with a very high specificity and at the cost of a lower sensitivity. This suggests that a in group of people positive for the combination of 3 autoantibodies contains a large proportion of potential future RA patients, which could be treated early to prevent disease development[13]. However, the studies investigated in the meta-analysis were all retrospective. In the future a large population-wide prospective study would be required to confirm the current observation that these 3 autoantibodies may be used to predict RA development. Besides these observations, it is also important to investigate whether anti-CarP antibodies may provide additional help to predict the treatment response in RA patients. Although a small study has been carried out into this direction, at this point no clear evidence is available to indicate that anti-CarP antibodies may contribute to these predictions[7].

Another aspect with regards to anti-CarP antibodies and associations that may warrant further investigations are genetics. One study, focusing on particular SNPs known to associate with RA, did not provide clear evidence for a correlation, at least not independently from ACPA[9]. Further information on the genetic components that relate to anti-CarP antibody positive disease may help to provide more insights into the mechanism and clinical consequences of anti-CarP antibodies.

Furthermore, it is important to note that many of the clinical associations that have been identified for anti-CarP antibodies are similar for ACPA, even though these autoantibodies are not the same subset and can be found independently in RA patients[12,14](Chapter 11). Besides these two more well-investigated autoantibodies, other autoantibodies have been identified in RA patients as well. Interestingly, attention is moving towards antibodies that target other post-translationally modified proteins[1]. Two of these are malondialdehyde modifications and acetylation[15-17]. Also for autoantibodies targeting these PTMs, there are several indications that they associate with joint damage and that they may behave in similar manner as ACPA and anti-CarP antibodies with regards to clinical associations. It should be noted that quite some overlap has been observed in RA patients in regards to autoantibody positivity towards PTMs. It is conceivable that more anti-PTM antibodies will be discovered in the future and it will become increasingly difficult to distinguish between the individual autoantibody subsets. Moreover, when combining all of these autoantibodies in RA patients, there remains a substantial group of patients that is negative for all autoantibodies. It therefore seems that – as has been argued by other as well[18,19] – RA may always exist of at least two distinct subsets: autoantibody-positive and autoantibody-negative. The clinical associations discussed above and the further speculations are therefore mainly relevant for the autoantibody-positive subset of RA patients.

The development of autoantibodies in RA

The fact that so many anti-PTM antibodies are able to develop in RA patients, raises the question how these autoantibodies arise. For ACPA, some theories have been formed based on genetic associations, for example with the HLA alleles[20]. However, these theories are difficult to prove, since most *in vivo* animal models lack ACPAs[21]. Anti-CarP antibodies on the other hand are common in animal models[21,22](Chapter 3) and are therefore quite suitable for further investigation into the development of autoantibodies in RA. Initial studies involving mouse models show that anti-CarP antibodies can, surprisingly be induces in a simple collagen-induced arthritis model, in which anti-CarP antibodies actually arise before disease onset[23]. Also, several other models of RA that involve the adaptive immune system show spontaneous development of anti-CarP antibodies[21]. These data seem to indicate that anti-CarP antibodies develop relatively easily when the adaptive immune system is activated.

In these systems, it is unclear which type of antigen is involved or how these arise. One possibility for the creation of carbamylated proteins is the combination of myeloperoxidase and thiocyanate, resulting in carbamylation in inflammatory environments[24]. Possibly, proteins that are normally inaccessible may become carbamylated under such inflammatory conditions and result in the development of anti-CarP antibodies. However, during life, many inflammatory situations are present and most people do not seem to develop these autoantibodies, as evidenced by the absence of anti-CarP antibodies in most healthy control populations[12,25].

Also, chapter 6 describes that a large increase in carbamylation due to inflammation or due to increase in urea does not explain the large amount of anti-CarP antibody-positive individuals in the group of RA patients, indicating that increased carbamylation alone is not sufficient for the development of anti-CarP antibodies in the human situation.

An interesting aspect is that chapter 11 describes that once tolerance is broken towards carbamylated proteins, many other carbamylated proteins can be recognized as well. The inducing protein can be a self-protein or a foreign protein, but both can result in recognition of carbamylated self-proteins and may eventually result in autoimmunity. Anti-CarP antibodies in human sera also show high cross-reactivity towards multiple carbamylated proteins, although to a lesser extent when compared to immunized mice. However, a break of tolerance towards carbamylated proteins due to the in vivo carbamylation of foreign pathogenic proteins seems to be an interesting working hypothesis.

A final aspect to take into consideration with regards to the development of anti-CarP antibodies is the low affinity of these antibodies when compared to immunization responses (unpublished data, Myrthe van Delft). The low affinity may suggest that we are at this moment not looking at the "original" antigen that is recognized by these antibodies or that the process to mature B cells producing anti-CarP antibodies differs from the classical immune response. Whether this is the case and which of these hypothesis may be true remains an interesting, but difficult topic for future investigations.

The possible functions of anti-CarP antibodies

Although it is unknown how anti-CarP antibodies develop in human RA, we do know that they exist. Therefore, the function of anti-CarP antibodies is interesting to investigate, especially in light of the clinical associations. At first sight, there is no reason to assume that the function of anti-CarP antibodies differs from other autoantibodies. A large amount of antibody-dependent functions has been described in autoimmune diseases[26,27]. This includes examples were antibodies can activate receptors such observed in Graves' disease or occasions where antibodies act as an antagonist, blocking receptor signaling such as in myasthenia gravis. Although it would be interesting if anti-CarP antibodies would have such actions, no evidence is available to support this. It is possible that the main mode of action of these antibodies in autoimmunity is related to their respective antigens, as investigated in chapter 10. These data indicated that a large amount of different proteins can function as antigen for anti-CarP antibodies. Also, it seems that carbamylation is present in many different tissues and may even be present throughout the entire human body. However, the longevity of cartilage (and other matrix proteins) may provide more time and opportunity to accumulate such modifications, as our observations indicated more carbamylation in cartilage tissue. Therefore, such tissues are the most likely targets of these autoantibodies.

Upon binding a (highly) carbamylated tissue, several actions could be mediated by anti-CarP antibodies. One of the first such mechanisms is through the complement system, via C1q binding[28]. This can result in the attraction of other immune cells due to the creation of chemoattractants C3a and C5a during the activation of the complement cascade. Whether the amount of carbamylation in cartilage, combined with the low avidity of anti-CarP antibodies is sufficient for complement activation is unknown but would be important for future investigations. However, low avidity ACPA seem to be better at complement activation than high avidity ACPA[29], which could also be the case for anti-CarP antibodies. A second mechanism through which anti-CarP antibodies may have an effect is by binding of Fcγ receptors on the surface of several cell types. If bound to activating receptors (all but FcγRIIb) this may result in local activation of immune cells, dependent on the type of Fc-receptor bound and on which cell this receptor is present[30]. Eventually, this could lead to a local inflammatory reaction, for example in the joint.

Another effect related to anti-CarP antibodies that should be considered is that these antibodies do not only function as fluid-phase antibodies, but also as a B cell receptor. Binding of a B cell receptor to a carbamylated antigen may also result in B cell activation and production of inflammatory cytokines such as TNF-α and IL-6, which may contribute to an inflammatory response as well[31].

Combined, it seems that there are many ways in which anti-CarP antibodies may exert their function. How much each of these pathways contributes to the development or prolongation of the immune response would be important to understand, especially with regards to possible targeted therapies to stop this particular process in RA patients.

Anti-CarP antibodies in the pathogenesis of RA

Although there are many functions by which anti-CarP antibodies could potentially contribute to the pathogenesis of RA, it is at this point unclear what their contribution is towards the development or chronicity of RA. A first conclusion that can be made is that anti-CarP antibodies are not essential for the development of RA in general, since there is a percentage of patients without anti-CarP antibodies or antibodies in general, as discussed in chapters 4, 5, 6 and 7. However, autoantibody-negative RA as such may have to be considered as different disease subset.
A second conclusion that can be made is that anti-CarP antibodies do not directly lead to RA as anti-CarP can also be found in small subsets of individuals suffering from other diseases or in the healthy population. This does not exclude that anti-CarP antibodies could play a role in the development of RA, through many different disease pathways. It is therefore interesting to investigate the potential role of anti-CarP antibodies in the autoantibody-positive subgroup of RA patients.

The fact that anti-CarP antibodies are present in animal models might make it easier to investigate their role in RA[21-23]. In chapter 11, mice have been immunized with different carbamylated proteins, upon which they start to produce high levels of anti-CarP antibodies (when compared to the more natural production of anti-CarP antibodies in CIA mice). These antibodies are able to recognize a large amount of carbamylated proteins, but the mice do not show any obvious signs of arthritis, indicating that high levels of anti-CarP antibodies alone are not sufficient for the induction of disease in this model. It should be noted though that the mice used for these experiment are relatively young, while carbamylation is thought to increase with ageing[32]. Older mice may therefore be more suitable for pathogenic experiments, with regards to anti-CarP antibodies. This situation may also be equal for humans, in which RA is developed at a later age as well[33].

Another model in which anti-CarP antibodies are present is shown in chapter 3, where anti-CarP antibodies are investigated in rhesus monkeys after collagen injections aimed to induce CIA. Only part of the animals developed disease and also only part of the animals developed anti-CarP antibodies during the observation period. However, these two groups did not match: some monkeys did develop anti-CarP antibodies but showed no signs of disease, or the other way around. This indicates again that anti-CarP antibodies do not necessarily induce disease.

The current data on animal models in general do not show an obvious effect of anti-CarP antibodies on the development of arthritic symptoms[21]. This may indicate that this is also the case in human RA, but translation of data found in animal models to the human situation should be carried out with caution. However, the presence of anti-CarP antibodies years before disease onset, already indicates that anti-CarP antibodies alone are not directly pathogenic in humans as well[34,35](chapter 5). However, given their possible functions, it seems probably that anti-CarP antibodies play a role in disease propagation and that they may contribute to the inflammatory cascade in the joint, once initiated by other mechanisms. Also, one could speculate that the disease process in general is quite slow and that it takes quite some time to develop a disease with clinical presentation after initiation of the disease process.

Conclusion and future perspective

At this point, we know that anti-CarP antibodies are present in RA patients in many different cohorts around the world. These autoantibodies may arise during an infection or other immune response, due to changes in carbamylation or due to the carbamylation of specific proteins. Once created, anti-CarP antibodies may function through complement activation, Fcy-Receptor binding or by functioning as a B cell receptor activating B-cells. Due to these functions anti-CarP antibodies may contribute to chronic inflammation in RA and increase disease severity.

From a clinical perspective, anti-CarP antibody measurements may not add to disease diagnosis once clinical symptoms occur, but may provide additive value as a prognostic factor in combination with ACPA and RF. Future clinical research should therefore focus on these three autoantibodies in large population-wide prospective studies to determine the value of anti-CarP antibodies, ACPA and RF in predicting the development of RA.

With regards to the development and role of anti-CarP antibodies, many aspects are yet unclear and unravelling these processes will be difficult. Potentially, the key to finding the solutions for these problems is found in (autoantibodies in RA that target) other post-translational modifications (PTMs). For example, not all PTMs result in autoantibody development in RA. Studying differences between these modifications may help understand why autoantibodies do develop towards some of these modifications. Also, more detailed studies into different autoantibody subsets based on clinical associations, subclasses, isotypes and sequences may provide more insight into the function of these autoantibodies, especially when focusing on the differences between the subsets.

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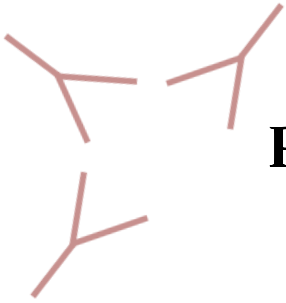

Part III

Addendum

Summary

Chapter 1 introduces rheumatoid arthritis (RA) as a complex, chronic autoimmune disease. It is unknown how this disease develops but both environmental (smoking) and genetic (HLA) risk factors have been discovered to play a role. Many treatment options have been developed that make RA more bearable, but no cure is available at the moment. However, very early treatment of the disease provides good results and may help to cure RA. This would however require early diagnosis or even prediction of disease development. Currently, rheumatoid arthritis is diagnosed based on joint inflammation and serum biomarkers such as autoantibodies. The autoantibodies currently most often used for diagnosis are rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA).
Chapter 2 describes the multitude of autoantibodies present in RA and psoriatic arthritis patients. Most of the antibodies described are only present in one of the two diseases and could be used to distinguish between RA and psoriatic arthritis. With regards to RA, one of the most studied antibody group that is not yet in use in the clinic are antibodies that target the post-translational modification carbamylation (anti-CarP antibodies). Therefore, the first part of this thesis focuses on the clinical role of anti-CarP antibodies and how these antibodies compare to ACPA and RF which are currently more commonly used in the clinic.

Chapter 3 investigates whether some of the RA-specific autoantibodies (RF, ACPA and anti-CarP antibodies) are also present in an animal model of RA. Surprisingly, anti-CarP antibodies could be detected in a rhesus macaque model of collagen-induced arthritis, while ACPA and RF were undetectable. Also, these anti-CarP antibodies were often present before the onset of clinical symptoms. Importantly, some animals without clinical symptoms did develop anti-CarP antibodies and some animals that were ill showed no detectable anti-CarP antibody levels.
Chapter 4 on the other hand analyses the presence of anti-CarP antibodies in the human situation. Previous measurement of these autoantibodies were restricted to Caucasian populations. This chapter provides the first evidence that anti-CarP antibodies are also present in an Asian population of RA patients. The distribution of anti-CarP antibodies in this cohort of Japanese RA patients is rather similar as observed in the Caucasian population, indicating that the measurement of anti-CarP antibodies may be relevant in a worldwide setting.
Chapter 5 investigates the presence of anti-CarP antibodies and several ACPA fine-specificities in a large cohort of RA patients, in which serum samples before and after the diagnosis of RA was available. It is shown that also in the human situation, anti-CarP antibodies can be detected many years prior to diagnosis. Anti-Carp levels and positivity are higher in pre-symptomatic individuals when compared to controls. A further increase in levels and frequency of anti-CarP antibodies could be observed after diagnosis of RA. Furthermore, it was demonstrated that anti-CarP antibodies associate with disease development and joint damage (also in ACPA-negative individuals).

Besides an increased presence of anti-CarP antibodies in RA patients, these antibodies may also be present in other diseases.
Therefore chapter 6 describes the presence of anti-CarP antibodies in conditions with increased carbamylation, namely smoking, renal disease and chronic inflammation. When compared to healthy controls, a slightly increased prevalence of anti-CarP antibodies could be observed for renal disease, but the percentage of antibody-positive RA patients was much higher than for any of the other conditions.
Since the presence of anti-CarP antibodies has been measured in a large number of cohorts, a meta-analysis based on these data was carried out in Chapter 7. The general aim of this study was to investigate whether anti-CarP antibodies could add to the diagnosis or prediction of RA, especially when taking into account that ACPA and RF are already in use in the clinic. For the diagnosis of RA, cohorts with RA patients, disease controls, healthy first-degree relatives and healthy controls were analysed. The results indicated that ACPA and RF may be sufficient to diagnose RA patients. To investigate the prediction of RA development in healthy individuals, we analysed three independent cohorts including the pre-symptomatic individuals, as in chapter 5. In this case, the measurement of ACPA, RF and anti-CarP antibodies provides an increase in the odds of developing RA, indicating that the combination of 3 autoantibodies may assist in prediction and early diagnosis of RA.

Although much information is available on the relationship between the presence of anti-CarP antibodies in relation to clinical presentation of RA, less is known on the antigens and the development of anti-CarP antibodies. These are the main subjects of the second part of this thesis.
Chapter 8 focuses on the detection of carbamylated and citrullinated proteins. Since the amino acids homocitrulline and citrulline, resulting from these reactions show similarities, it may be difficult to distinguish between the two. For example, most commercially available antibodies marketed to recognize citrulline also recognize homocitrulline in both ELISA and western blot. The tested homocitrulline-targeted antibodies on the other hand preferentially recognize carbamylated proteins. Therefore, mass spectrometric analysis – if carried out carefully – seem essential for the identification of citrullination and carbamylation in complex protein mixtures.
In Chapter 9 further research into potential carbamylated antigens is carried out. The mixture of carbamylated fetal calf serum (Ca-FCS), currently used in ELISA for the detection of anti-CarP antibodies, is investigated in more detail. One of the proteins that is well-recognized by anti-CarP antibodies in the sera of RA patients is carbamylated alpha-1-antitrypsin (Ca-A1AT). When investigated in further detail, Ca-FCS and Ca-A1AT both perform similar when it comes to the identification of RA patients. Also, several Ca-A1AT peptides can be recognized as well, although this is less universal that Ca-A1AT protein recognition. The data from this chapter may be used for further optimization of diagnostic tests to measure anti-CarP antibodies.
Further investigation into antigens for anti-CarP antibodies in RA patients is carried out in chapter 10, where carbamylation is determined in joint tissue of RA patients and controls, using mass spectrometry.

Even though it was expected that carbamylation in RA joint tissue would be increased, the number of unique carbamylated peptides identified was similar between RA and controls. However, when comparing cartilage, synovium and synovial fluid, more peptides were identified in cartilage tissue. Importantly, the identified carbamylated proteins could be recognized by anti-CarP antibodies. Combined, these data indicate that anti-CarP antibodies can potentially contribute to disease progression by binding to carbamylated proteins present in the joint of RA patients.
Finally chapter 11 describes studies on further characteristics of anti-CarP antibodies and their antigens. It is demonstrated that antibodies in the sera of RA patients are able to recognize a large variety of carbamylated proteins. Furthermore, quite some cross-reactivity towards different carbamylated proteins is observed, although the actual cross-reactivity pattern is different for each patient. Interestingly, mice, when immunized with a carbamylated self-protein also show high cross-reactivity towards other carbamylated proteins. Moreover, immunization with a foreign carbamylated protein results in a break of tolerance towards carbamylated self-proteins, indicating that an immune reaction towards a foreign carbamylated protein may result in a general autoimmune response targeting both foreign- and self-proteins in caramylated form.

In the discussion, Chapter 12, several possibilities in which anti-CarP antibodies may contribute to RA and to acquiring further knowledge on this chronic disease are described. From a clinical perspective, future research should focus on the use of anti-CarP antibodies in the prediction of RA, using large prospective studies. Furthermore, many aspects with regards to the development, function and role of anti-CarP antibodies remain unclear. Unraveling these aspects may prove difficult but may also provide further insight into the development of RA and possible treatment options for RA patients.

Nederlandse Samenvatting

Reumatoïde artritis(RA) is een gecompliceerde, chronische auto-immuunziekte. In Hoofdstuk 1 wordt het huidige inzicht over het ontstaan en beloop van RA uiteengezet. Op dit moment is het niet bekend waardoor deze ziekte ontstaat, maar enkele omgevings- en genetische risicofactoren zijn wel bekend; bijvoorbeeld roken en HLA. Er zijn veel verschillende behandelmethodes ontwikkeld voor RA, maar deze zorgen nog niet voor volledige genezing. Om RA werkelijk te genezen, lijkt een zo vroeg mogelijke behandeling nodig. Het is daardoor van belang om de ziekte in een vroeg stadium te ontdekken. De huidige diagnose word gebaseerd op gewrichtsontsteking en biomarkers in serum, zoals autoantilichamen. Autoantilichamen die nu vooral worden gebruikt voor diagnose zijn reumafactor (RF) en antilichamen tegen gecitrullineerde eiwitten (ACPA). Naast deze autoantilichamen zijn er ook andere antilichamen aanwezig in RA en gerelateerde ziektes. In Hoofdstuk 2 worden meerdere antilichamen beschreven die geschikt zouden kunnen zijn als biomarker voor RA. Eén van de meest bestudeerde antilichamen die op dit moment nog niet in de kliniek in gebruik is, is de groep antilichamen die carbamylatie herkent (anti-CarP antilichamen). In het eerste deel van dit proefschrift wordt daarom vooral aandacht besteed aan anti-CarP antilichamen, met een speciale focus op klinische relevantie, ook in vergelijking met ACPA en RF.

In Hoofdstuk 3 word onderzocht of verschillende RA-specifieke antilichamen (RF, ACPA en anti-CarP antilichamen) ook aanwezig zijn in RA diermodellen. Verrassend genoeg waren anti-CarP antilichamen wel detecteerbaar in het collageen geinduceerde arthitis model in rhesusapen, terwijl in ACPA en RF afwezig zijn in dit model. De anti-CarP antilichamen waren al aanwezig voor het begin van klinische symptomen. Het is hierbij wel belangrijk om te vermelden dat er apen waren met anti-CarP antilichamen maar zonder symptomen. Daarnaast waren niet alle zieke dieren positief voor anti-CarP antilichamen.

In Hoofdstuk 4 wordt juist gekeken naar anti-CarP antilichamen in de mens. Alle eerdere metingen van anti-CarP antilichamen zijn uitgevoerd in de Kaukasische populatie. Dit hoofdstuk is echter het bewijs dat anti-CarP antilichamen ook in de Aziatische populatie van RA patiënten aanwezig zijn. De hoeveelheid patiënten positief voor anti-CarP antilichamen is vergelijkbaar in de Japanse en Kaukasische RA patiënten.

In Hoofdstuk 5 worden studies beschreven naar de aanwezigheid van anti-CarP antilichamen en ACPA fijn-specificiteiten in RA patiënten. Van deze patiënten waren monsters van zowel voor als na de diagnose aanwezig. In de monsters voor diagnose is een hoger percentage personen positief voor de verschillende antilichamen, als dit wordt vergeleken met gezonde controles. Na de diagnose was een nog groter percentage van de monsters positief voor de verschillende antilichamen. Daarnaast was er een duidelijke associatie tussen de aanwezigheid van anti-CarP antilichamen en gewrichtsschade in dit cohort.

Autoantilichamen zoals anti-CarP antilichamen komen vaak in meerdere ziektes voor. Daarom wordt er in hoofdstuk 6 gekeken naar anti-CarP antilichamen in condities met verhoogde carbamylatie, namelijk roken, nierfalen en chronische ontsteking. Het is duidelijk dat het percentage anti-CarP-antilichaam positieve patiënten hoger is bij RA dan in de andere bestudeerde groepen.De hoeveelheid patienten positief voor anti-CarP antilichamen is wel licht verhoogd in nierfalen, vergeleken met gezonde controles.
Omdat anti-CarP antilichamen inmiddels in veel cohorten zijn gemeten werd in hoofdstuk 7 een meta-analyse uitgevoerd. Het doel van deze studie was om te onderzoeken of anti-CarP antilichamen gebruikt kunnen worden voor de diagnose van RA of om te voorspellen wie er in de gezonde populatie RA zal ontwikkelen. Hierbij hebben we rekening gehouden met het gegeven dat ACPA en RF al in gebruik zijn in de kliniek. Om het effect van anti-CarP antilichamen op de diagnose van RA te bestuderen werden RA patiënten, ziektecontroles, eerstegraads familieleden en gezonde controles geïncludeerd. In de meta-analyse wordt duidelijk dat ACPA en RF voldoende zijn om RA te diagnosticeren als patienten zich bij de kliniek melden met klachten. De toevoeging van anti-CarP antilichamen lijkt in dit geval overbodig. Om de voorspelling van het ontwikkelen van RA te onderzoeken, werden pre-symptomatische personen geïncludeerd, zoals in hoofdstuk 5. In dit geval is de combinatie van ACPA, RF en anti-CarP antilichamen vele malen beter dan de andere antilichaamcombinaties. Deze combinatie is daarom zeer interessant voor het voorspellen van RA ontwikkeling en mogelijk voor een zeer vroege diagnose van RA.

Op dit moment is er veel bekend over de klinische associaties van anti-CarP antilichamen, terwijl er veel minder bekend is over de antigenen waar deze antilichamen aan binden of over de antigenen die een anti-CarP antilichaam respons uitlokken. Dit is de focus van het tweede deel van dit proefschrift.
Hoofdstuk 8 beschrijft studies die zich richten op de methodes voor detectie van gecarbamyleerde en gecitrullineerde eiwitten. Omdat de aminozuren behorende bij deze reacties erg op elkaar lijken kan het moeilijk zijn deze te onderscheiden. Veel van de commercieel verkrijgbare antilichamen tegen citrulline waren bijvoorbeeld ook in staat om gecarbamyleerde eiwitten te herkennen in zowel ELISA als western blot. De geteste anti-homocitrulline antilichamen hadden wel een voorkeur voor gecarbamyleerde eiwitten. Voor het meten van deze modificaties in een complexe eiwitmix lijkt massa spectrometrie echter essentieel voor het identificeren van gecarbamyleerde of gecitrullineerde eiwitten.
Hoofdstuk 9 gaat verder in op mogelijke antigenen voor anti-CarP antilichamen. Op dit moment wordt gecarbamyleerd foetaal kalf serum (Ca-FCS) gebruikt in ELISA om anti-CarP antilichamen te detecteren. Deze eiwitmix wordt hier verder onderzocht. Eén van de eiwitten in deze mix die goed wordt herkend door anti-CarP antilichamen is gecarbamyleerd alpha-1-antitrypsine (Ca-A1AT). Het blijkt dat assays gebaseerd op Ca-FCS en Ca-A1AT beide gelijkwaardig zijn wat betreft de detectie van anti-CarP antistoffen en de identificatie van RA patiënten.

Daarnaast worden ook sommige van de Ca-A1AT peptides goed herkend door antilichamen, alhoewel dit minder universeel is dan de eiwitherkenning. Hoofdstuk 10 beschrijft studies die dieper in gaan op verschillende humane gecarbamyleerde eiwitten die als antigen herkend zouden kunnen worden door anti-CarP antilichamen. Massa spectrometrie wordt gebruikt om deze te identificeren in de gewrichten van RA patiënten en controles. Er werd verwacht dat carbamylatie verhoogd zou zijn in de gewrichten van RA patiënten, maar dat was niet het geval. Het aantal gecarbamyleerde peptides in RA en controles was vergelijkbaar. Opvallend genoeg was het aantal verschillende gecarbamyleerde peptides wel verhoogd in kraakbeen, vergeleken met synoviaal vocht en synovium. Een groot gedeelte van de geïdentificeerde gecarbamyleerde eiwitten konden ook worden herkend door anti-CarP antilichamen. Dit is een indicatie dat gecarbamyleerde eiwitten in de gewrichten van RA patiënten, vooral in kraakbeen, een mogelijk doelwit vormen voor anti-CarP antilichamen.
In Hoofdstuk 11 wordt uiteindelijk gekeken naar verdere eigenschappen van anti-CarP antilichamen. De antilichamen kunnen veel verschillende gecarbamyleerde eiwitten herkennen en blijken zeer kruisreactief te zijn. De mate van kruisreactiviteit voor gecarbamyleerde eiwitten verschilt per patiënt. Als muizen geïmmuniseerd worden met een gecarbamyleerd eiwit zijn de resulterende antilichamen ook kruisreactief tegen andere gecarbamyleerde eiwitten. Bovendien zorgt immunisatie met een vreemd gecarbamyleerd eiwit voor het doorbreken van de immunologische tolerantie richting eigen gecarbamyleerde eiwitten. Hierdoor zou een immunologische reactie tegen een vreemd gecarbamyleerd eiwit kunnen zorgen voor een gevaarlijke autoimmuunreactie.

De mogelijke rol van anti-CarP antilichamen in de pathogenese van RA wordt bediscusseerd in Hoofdstuk 12. Hierbij kan gedacht worden aan complement-activatie door antilichaambinding in het gewricht of aan een rol voor de anti-CarP positieve B cellen. Daarnaast worden suggesties gedaan voor het verdere onderzoek naar anti-CarP antilichamen. Vanuit een klinisch perpectief is het belangrijk om meer onderzoek te doen naar anti-CarP antilichamen in relatie tot het voorspellen van RA, bijvoorbeeld met grote prospectieve studies. Daarnaast zijn er nog vele onbekende factoren met betrekking tot de ontwikkeling, functie en rol van anti-CarP antilichamen. Het zal erg ingewikkeld zijn om dit verder uit te zoeken, maar dit kan wel belangrijke informatie opleveren over de ontwikkeling en mogelijke behandeling van RA.

List of Publications

Anti-carbamylated protein antibodies in the pre-symptomatic phase of rheumatoid arthritis, their relationship with multiple anti-citrulline peptide antibodies and association with radiological damage
Brink M*, Verheul MK*, Rönnelid J, Berglin E, Holmdahl R, Toes RE, Klareskog L, Trouw LA, Rantapää-Dahlqvist S.
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Curriculum Vitae

Marije Verheul werd geboren in 1990 te Gouda. In 2008 behaalde ze haar gymnasium diploma aan het Groene Hart Lyceum in Alphen aan den Rijn, waarna ze begon aan de studie biomedische wetenschappen in Leiden. Na het succesvol voltooien van de bachelor-fase van deze studie koos ze voor de research specialisatie in de master. Haar master rondde ze in 2013 af met een stage op de afdeling immunologie van het NKI-AVL onder begeleiding van Prof. Dr. Ton Schumacher, waar ze onderzoek deed naar intratumorale T cellen. Hierna begon Marije aan een PhD aan de afdeling reumatologie in het LUMC, Leiden. Hierbij werd ze begeleid door Prof. Dr. Rene Toes, Prof. Dr. Tom Huizinga en Dr. Leendert Trouw. Haar onderzoek richtte zich op autoantilichamen gericht tegen posttranslationele eiwitten in reumatoïde artritis. Momenteel is Marije werkzaam als postdoc bij de Oxford Vaccine Group, Afdeling kindergeneeskunde van de universiteit van Oxford, waar ze kijkt naar Salmonella Typhi infectie.

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