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CHAPTER 12

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

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

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

We choose to investigate characteristics of ACPA in individuals from this cohort because North American Natives have previously been reported to have a younger age at disease onset and an increased prevalence and severity of RA. Genetic studies have also revealed a higher prevalence of HLA shared epitope (SE) alleles in North American Natives and increased frequency of RF positivity in patients with RA in several North American native populations.\textsuperscript{11} The data so far present about ACPA in healthy individuals originates from retrospective studies, collecting serum samples from blood donors with RA before disease onset.\textsuperscript{7, 8} These studies, how-
ever did not investigate the characteristics of the ACPA response in health versus disease. The most striking finding in our study, the interaction between RF and ACPA, implies that these autoimmune responses may converge to precipitate disease. Although the biological mechanism underlying this observation is unknown, this association could be explained by a model proposed in a study from Wipke et al, suggesting that that autoantibody-mediated articular inflammation in mice may be facilitated by soluble immune complexes that enable the access of pathogenic antibodies into the joint. Because RF antibodies recognize IgG molecules, they can form soluble immune complexes, which could facilitate access of ACPA joints. An alternative explanation is that RF could amplify the effector mechanisms induced by ACPA in the joint, thereby exacerbating joint inflammation.

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

As described above, the transition from asymptomatic autoimmunity to clinically detectable arthritis is not well understood. In chapter 3 we therefore studied an individual patient from a multi-case RA family from a North American Native
population. This young female had initially asymptomatic autoimmunity and subsequently developed clinical features suggestive of early RA. These data suggest that onset of clinical synovitis was heralded by an expansion in ACPA isotype usage, along with epitope spreading to citrullinated fibrinogen-associated antigens. Interestingly, the autoantibody levels continued to rise despite disappearance of the clinical symptoms with pregnancy. This observation implies that the clinical improvement typically seen during RA pregnancy may not necessarily reflect an attenuation of the underlying autoimmune mechanisms, at least at this early stage of the process. At the time of the study, it was not possible to determine with certainty whether this patient’s synovitis represents the earliest clinical evidence of RA, since she had yet to meet the American College of Rheumatology criteria set. On the other hand, it is possible that the very early use of antimalarials may indeed serve to prevent the full expression of the clinical RA syndrome. This has been shown with the use of methotrexate in anti-cyclic citrullinated peptide-positive patients with undifferentiated arthritis.\textsuperscript{15} The serological evolution and synovial features are consistent with the hypothesis that isotype expansion and epitope spreading of ACPA responses to synovial autoantigens are associated with the onset of synovitis in an individual who is genetically susceptible to RA.

ACPA positive and ACPA negative disease have been shown to be associated with different genetic and environmental risk factors, fuelling the hypothesis that different pathways mechanisms underlie these two separate disease subsets.\textsuperscript{16-18} For example, ACPA negative RA associated with HLA-DR3\textsuperscript{19, 20}, whereas the HLA SE alleles predispose to ACPA positive disease.\textsuperscript{21} Likewise, the contribution of smoking to disease risk is mainly confined to the ACPA positive HLA SE positive group.\textsuperscript{18} The HLA SE alleles are also involved in shaping the fine specificity response, as these alleles predispose to the development of antibodies against citrullinated vimentin but not against citrullinated fibrinogen.\textsuperscript{22} This would indicate that HLA SE alleles influence the magnitude and the specificity of the ACPA response. It has even been suggested that a specific interaction between environment (that is, smoking) and genetic background (HLA expression profile) might explain the reactivity against a specific citrullinated antigen.\textsuperscript{23} Since specific interaction between these two risk factors had been postulated in association with autoimmunity to citrullinated a-enolase peptide (CEP-1)\textsuperscript{23}, it has been investigated whether the interaction was truly specific for this antigen or if it might also exist for other peptides. Indeed, a more recent paper from our group revealed that this interaction was not specific for CEP-1, but rather extended to other citrullinated antigens as well, for example with antibodies directed against citrullinated vimentin.\textsuperscript{24} These data indicated that the interaction between HLA SE alleles and smoking extended to several citrullinated autoantigens, which might even be explained by a general predisposition to ACPA development.
In chapter 4 we wished to explore whether this effect of interaction would still be present in ACPA positive patients. These findings could be of relevance, since interactions between genotype, smoking, and autoimmunity to certain citrullinated antigens could further expand our understanding of RA pathogenesis. However, no stratification for ACPA status was performed in previous studies, allowing the possibility that the interaction effects were not explained by their influence on the formation of autoimmune reactions against specific citrullinated antigens, but rather by their influence on the formation of ACPA. Therefore, we reasoned that if such interaction effects only shaped the response to certain specific epitopes and not to other citrullinated epitopes, these effects should still be observed among the subset of ACPA-positive patients. However, after stratification for ACPA, there was no gene-environment interaction present in the shaping the reactivity against specific citrullinated antigens. This indicates that the association found is caused by the presence of ACPA rather than by the presence of an autoimmune reaction to specific citrullinated epitopes.

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

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

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

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

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

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

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

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

Furthermore, the recognition of different citrullinated peptides at baseline correlated with similar clinical characteristics, irrespective of differences in peptide-backbone structure, indicating that breaking of tolerance towards citrullinated proteins as such provides more information than the recognition of a particular peptide or set of peptides. As subgrouping patients based on their epitope recognition profile could be potentially useful to get more homogenous patient groups, we analysed different recognition profiles in detail. These data suggested that the recognition profile of patients with RA displays a large heterogeneity and that patients are not characterised by a unique and specific epitope recognition pattern. The lack of association between ACPA fine specificities and clinical characteristics might not be surprising, given the observation that even the baseline differences between ACPA-positive and ACPA-negative patients with RA are rather small. Nonetheless, these latter subgroups differ considerably with respect to disease course as measured by radiological progression. We feel that it is unlikely that ACPA fine specificity within ACPA-positive disease will have a similar impact as
found for ACPA status within RA. This notion is supported by our observation that a similar rate of joint destruction is observed between ACPA positive patients with high- and low-baseline anti-CCP2 levels as proxy for the extent of epitope recognition. Likewise, the data presented in chapter 6 and a recent observations by Fisher et al analysing the possible connection between reactivity against a specific citrullinated epitope provided similar indications.32

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

Although the ACPA fine specificity repertoire seems not to play a role in the rate of joint destruction in RA patients, the presence of ACPA does play an important role.31, 33-35 The observation that ACPA status is, to some extent, related to therapeutic outcome in early disease is intriguing. For a number of diseases, such as diabetes mellitus, it has been suggested that a critical period exists in which interventions might reverse the disease process.36 For RA, such a ‘window of opportunity’ might also exist, because symptom duration >12 weeks at treatment initiation is a strong and independent risk factor for a persistent disease course with more joint destruction.37-40 Although this observation could be explained by the assumption that acute-versus-insidious symptom onset characterizes the manifestation of different disease subsets, it could also be indicative of a window of opportunity.
The difference in outcome in relation to symptom duration raised the question of whether a difference in ACPA characteristics could be involved. However, in chapter 8, we show that ACPA-positive patients with symptoms of RA for <12 weeks display no difference in the specificity and isotype repertoire of their ACPA response compared with patients with longer symptom duration. These findings indicate that the ACPA-characteristics analyzed do not have an impact on the putative window of opportunity and emphasize further that maturation of the autoantibody response occurs at an early stage, before the first signs and symptoms of disease appear.

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

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

Moreover, ACPA IgG is cross-reactive to multiple citrullinated antigens15,16, and
therefore recognition of a citrullinated antigen by ACPA IgG does not indicate that this antigen is necessarily involved in the induction of B-cell responses.

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

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

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

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

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

Besides antibody responses directed against citrullinated proteins, also antibodies directed against carbamylated proteins (anti-CarP) have recently been shown to be present in RA.\textsuperscript{43} Interestingly, these anti-CarP antibodies are also present in around 20% of the ACPA negative RA patients and are associated with more severe joint damage in this group. ACPA recognize proteins only after the enzymatic conversion of the amino acid arginine by PAD enzymes to become the amino acid citrulline. Next to citrullination, also other post-translational modifications are known to occur. Therefore, it is likely that proteins that have undergone a differ-
ent type of post-translational modification are also recognized by autoantibodies. One of these other post-translational processes is the process of carbamylation. In this chemical reaction, mediated by cyanate, the amino acid lysine is changed to become the amino acid homocitrulline. Cyanate, necessary for such carbamylation is naturally present in the body and in equilibrium with urea.\textsuperscript{44} In the healthy situation the concentration of urea is rather low. It is likely that under such conditions especially long lived proteins, such as matrix molecules, become carbamylated. Renail failure, a condition with increased urea concentrations, is known to be associated with enhanced protein carbamylation.\textsuperscript{45} Also smoking, the most prominent environmental risk factor for RA, enhances carbamylation by increasing the cyanate concentration.\textsuperscript{46} Extensive carbamylation is especially thought to occur during (chronic) inflammation, when myeloperoxidase is released from neutrophils as this enzyme shifts the equilibrium of thiocyanate towards cyanate.\textsuperscript{46} As smoking and chronic inflammation are important in the context of RA, it is likely that in the inflamed synovium carbamylation is taking place. The post-translationally modified amino acids citrulline and homocitrulline are very similar structures (figure 1).

![Citrullination and Carbamylation](image)

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

In both cases a positively charged amino acid is replaced by a neutral one. The only structural difference is the difference in length; homocitrulline is one CH\textsubscript{3} group longer. As these structures are really similar, in chapter 11 we wished to determine to what extent human antibodies can differentiate between them. Unlike human antibodies, the anti-modified citrulline (AMC) antibody developed by Dr Senshu\textsuperscript{47} is able to recognize citrullinated epitopes irrespective of the neighbouring amino acids. Thus, we also aimed to verify whether the AMC assay could distinguish between those two amino acids.
Both the citrullinated and the carbamylated forms of the proteins tested were strongly recognized, whereas, the non-modified form did not reveal any staining. Staining similar western blots with selected human sera revealed that sera-positive for ACPA and anti-CarP stained both citrullinated and carbamylated forms of Fib, whereas, single positive sera stained only one form of modified Fib. These data indicate that although the ‘AMC-Senshu’ method does not discriminate between these two modifications, human sera of RA patients are able to. Furthermore, we also showed that part, but not all ACPA and anti-CarP antibodies are cross-reactive.

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

CONCLUSION

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